A Thesis Submitted for the fulfilment of Doctor of Philosophy (Ph.D.)

The Effects of Insulin and the Insulin-like Growth Factors (IGF-I and IGF-II) On the Proliferation and Differentiation of Cells Isolated from Placentae of Non-Diabetic and Diabetic Subjects.

Assoc. Prof. M.K. Towstoless (Principal supervisor)

Dr M. Bhave (Co-supervisor)

Ann Wilson

Centre for Bioprocessing and Food Technology

Victoria University of Technology

Dedication

This thesis represents my heart and soul, sweat and tears
for the last 6 years of my life.
I dedicate it as a gift to the ones I love, my family:
Mum, Dad, Mausi, Nani, Anita and Alfred.

Declaration

I hereby declare that all work carried out in this thesis was performed solely by me while I was enrolled as a student for the degree of Doctor of Philosophy at the Centre for Bioprocessing and Food Technology, Victoria University of Technology. To the best of my knowledge, this work has not been submitted in whole or part for any other degree or diploma in any University and no material contained in this thesis has been previously written or published by another person, except where due reference is made in the text.

Ann Wilson
March, 2001

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AGA	Appropriate for Gestational Age
CRH	Corticotropin Releasing Hormone
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EVT	Extravillous Trophoblast
FCS	Fetal Calf Serum
FFA	Free Fatty Acid
GDM	Gestational diabetes (diet controlled)
GnRH	Gonadotropin Releasing Hormone
hCG	Human Chorionic Gonadotropin
HLA	Human Leucocyte Antigen
hPL	Human Placental Lactogen
IDDM	Insulin Dependent Diabetes Mellitus
IGFBPs	Insulin-like Growth Factor Binding Proteins
IGF-I	Insulin-like Growth Factor I
IGF-II	Insulin-like Growth Factor II
IGFs	Refers to both IGF-I and IGF-II
LGA	Large for Gestational Age
LM	Light Microscope
mRNA	Messenger Ribonucleic Acid
N	Normal
NIDDM	Non-Insulin Dependent Diabetes Mellitus
PCNA	Proliferating Cell Nuclear Antigen
RNA	Ribonucleic Acid
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope
TGF	Transforming Growth Factor

Paper Presentations and Publications

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Wilson A., Bhave M. & Towstoless M. 1997. Effect of Insulin and Insulin-like Growth Factor 1 on Proliferation of Trophoblasts Isolated from Placental Tissue obtained from Insulin Dependent Diabetic Mothers (IDDM). (poster presentation) The 7th Meeting of the European Placenta Group, Vigso, Denmark. (Published in Placenta 18:A60.)

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Abstract

Maternal peripheral hyperinsulinemia and fetal hyperinsulinism that occurs as a consequence of the treatment of diabetic pregnancy have been associated with both gross and microscopic, cellular changes. These include an apparent hyperplacentation and fetal macrosomia and trophoblastic morphofunctional pathology, which may contribute to increased morbidity and mortality rates. Reports have indicated that fetal and placental macrosomia does not always result from maternal hyperglycemia and that insulin may stimulate cellular proliferation *in vitro*. A family of growth factors known as the Insulin-like Growth Factors (IGFs) have been identified and shown to share insulin's biological activities. These, too, are thought to play an important role in the growth and development of the fetus. Due to the structural similarities, insulin and the IGFs may bind with each other's receptors to elicit a response. The aim of this study was to investigate and compare the effects of insulin and the IGFs on the proliferation and differentiation of cells isolated from the human placenta.

The effects of insulin, IGF-I and IGF-II on growth rates were examined and compared in cultures of cells isolated from placental tissue obtained from 'normal' (n=30) and diabetic (n=22) pregnancies. Trophoblasts were purified and cell identity was determined using immunocytochemical markers. Insulin binding sites on trophoblast membranes were also studied and apoptotic activity was assessed.

Cell numbers were observed to increase, peaking at 24h, in 'normal' cultures. Significantly higher ($p<0.001$) numbers were observed in cultures of cells isolated from placentae of insulin-dependent diabetic (IDDM) mothers while diet controlled gestational diabetic (GDM) cell cultures were significantly lower ($p<0.001$) in cell numbers compared to 'normal'. This trend was found to be consistent for all culture treatments. In cells derived from 'normal' placentae, IGF (IGF-I or IGF-II) administration resulted in significantly higher ($p<0.001$) numbers compared to control and insulin treated cells with IGF-I treated cells being higher in number compared to that of IGF-II. However, no significant

differences were observed in cell numbers between insulin, IGF-I and IGF-II treated cultures of cells obtained from diabetic (IDDM or GDM) placentae. In cell cultures from 'normal' placentae the combination of the IGFs with insulin resulted in numbers being significantly lower ($p<0.01$) than that of treatment with either of the IGFs alone but significantly higher ($p<0.01$) than that of insulin treatment. These results were supported by thymidine uptake measurements and staining for PCNA.

Cell cultures in all treatments were observed to decrease in number after 24h. This coincided with the differentiation of cells to form syncytia. This was confirmed using immunocytochemical staining for desmoplakins I and II. Assays (TUNEL kit) for cell death revealed that very little to no apoptosis occurred during this stage of culture.

Receptor binding assays confirmed the presence of specific insulin binding sites on cytotrophoblast membranes with a significantly greater ($p<0.05$) number in cells of IDDM placentae compared to those of 'normal' and GDM placentae. There were no significant differences in the binding affinities between 'normal' and diabetic placentae. Experiments showed that some of the proliferative effects of insulin were mediated via the IGF-I receptor. Experiments using antibodies against the insulin receptor demonstrated the effect of insulin in enhancing the proliferation of the cells. Interestingly, IGF-II administration to IDDM cultures increased insulin binding, which was not reflected in the proliferation data.

Data, in this study, indicate that insulin affects the proliferation of trophoblasts *in vitro*. This study has demonstrated significant differences in the relative proliferative effects of insulin on trophoblast from placentae of 'normal' and diabetic pregnancies which may be explained by the alterations in the number of insulin binding sites observed on cells obtained from placental tissue of 'normal' and diabetic subjects. Insulin binding was shown to be reduced when cells were saturated with the IGFs. Thus, these studies have contributed into furthering the knowledge of the possible mechanisms involved in the macrosomic outcomes of fetus and placenta seen in pregnancies of diabetic mothers.

Statement of Relevance

This study is undertaken to better understand the physiological factors that may be involved in the growth and development of the placenta and that may contribute to fetal growth and development. In cases of placental insufficiency, where the placental mass and maturity are inadequate for optimal fetal growth and development, the fetus is subjected to chronic intrauterine hypoxia, hyperlactemia, acidosis and a reduced nutrient availability resulting in limited fetal growth. Conversely, pregnancies of women with pregestational diabetes are at greater risk than those with gestational diabetes of developing gigantic placentae and an abnormally high metabolic activity, resulting in fetal macrosomia and/or other growth anomalies. These conditions are closely associated with a range of pregnancy complications and an increased mortality rate.

The complex and diverse functions of the placenta required to meet the increasing demands of the developing fetus are dependent on the organisation of the placenta itself. Although the structure, growth and development of the placenta has been well described, very little is known about the factors and mechanisms involved in regulating the proliferation and differentiation of the constituent cells that comprise it. To better understand the function of the placenta there is a need to compare 'abnormal' or 'malfunctioning' placentae obtained from diseased conditions with those considered being 'normal'.

The purpose of this study is to gain further insight into the growth and functional development of the placenta, an organ essential for prenatal life. The role of a family of related factors, Insulin and the IGFs, in the regulation of proliferation and differentiation of placental cells will be investigated. It is believed that this information will be highly relevant in planning the future care of mother and child.

"The placenta is the most accurate record of the infant's prenatal experiences...

submitting this organ to a reasonably knowledgeable look and touch can provide much insight into prenatal life; the results are often helpful in caring for the neonate.....

and, most importantly, much of what can be learned cannot be put into the maternal prenatal history if the information is discarded with the organ."

Benirschke (1981)

CHAPTER ONE

THE GROWTH AND DEVELOPMENT OF THE HUMAN PLACENTA

The following chapter will review the literature on the structural and functional growth and development of the placenta from implantation through to maturity. This chapter will cover pivotal research in the structural architecture and developmental stages of the placental villous, the different cell types and the growth and function of the surrounding fetal membranes.

1.1. History of Knowledge of the Placenta

The term 'placenta', meaning circular cake in Latin, was first formulated in 1559 AD (as cited by Williams, 1985). However, knowledge of the afterbirth can be traced further into history where ancient texts and drawings, including the Old Testament, have referred to it as the seat of the "external soul". The word *chorion* was believed to be first used by Aristotle and it was not until the early 16th century, during the Renaissance, that a thirst for the anatomical knowledge of the human placenta developed.

This was followed by the discovery of the utero-placental circulation, a concept introduced by Harvey in 1628 (as cited by Moghissi & Hafez, 1974; Williams, 1985; Moore, 1989) who originally believed there was continuity between maternal and fetal vascular systems. An accurate concept of the structure and functional significance of the human placenta, put together by collective findings of many anatomists, along with the basic idea that there was a 'placental barrier', was clearly formulated by the late 17^{th} century. The endocrine function of the placenta was not recognised until much later.

With considerable improvement in light microscopy techniques, a time marked by the histological and cytological studies of the structure of the human placenta, came the clarification of many aspects of placental embryology and physiology (Moghissi & Hafez, 1974). Here the first accurate description of the decidua was made and a parietal lining was distinguished from a capsular one. Later, the *decidua basalis* was described (Panigel, 1974; Moore, 1989) and this was soon followed by accurate descriptions of what we now know as the intervillous space. Towards the end of the 19^{th} century the true nature of the chorionic villi was appreciated and the basic knowledge of the blood circulation in the intervillous space was established. During this period Langhans demonstrated clearly that two layers of cells covered the villi; the cells of the inner layer, the cytotrophoblasts, are thus often referred to as the Langhans' cells (Panigel, 1974; Williams, 1985). Hubrecht, meanwhile, introduced the term *trophoblast* to distinguish the portion of blastocyst that did not contribute to the cellular portion of the embryo. It was later demonstrated that the cells of the superficial layer of the chorionic villi were

syncytial in nature and is now generally referred to as syncytiotrophoblasts (Panigel, 1974; Williams, 1985).

Despite the rapid growth in knowledge of placental physiology, the placenta was, at one time, removed and discarded and only the most obvious physical abnormalities were diagnosed and recorded. Over the last few decades, light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) allowed two-dimensional features to be viewed as real three-dimensional structures. Such advancements in microscopic techniques allowed the accurate reconstruction of placental villous structures (Kaufmann *et al.,* 1987) enabling one to distinguish between levels of maturation (i.e, the immature versus the hypermature placenta) and to distinguish 'normal' growth and abnormal growth as observed in pre-eclampsia, diabetes mellitus and peripheral hypoxia.

The human placenta is described as discoid haemomonochorial (Bjorkman *et al.,* 1989). It is initially labyrinthine in structure, and then secondarily multivillous with a partial return to labyrinthine. Consequently, the structure and its relationships to function are varying and complex. The high metabolic activity at the surface of the feto-maternal exchange area is essential for embryonic differentiation and fetal growth and the transfer of numerous nutritive substances and essential metabolites from mother to fetus and waste products from fetus to mother. In addition, the placenta manufactures and modifies hormones necessary for maintaining pregnancy and provides protection for the developing fetus by serving as a physical barrier and supplying immunoglobulins.

There is limited information with regard to the mechanisms involved in regulating and effecting placental growth. However, studies have demonstrated the abundance of insulin-like growth factor receptors on placental membranes, and the synthesis of insulin-like growth factors (IGFs) in placenta and insulin-like growth factor binding proteins (IGFBPs) in the tissue surrounding the placenta. Collective evidence has shown the growth promoting effects of insulin and the insulin-like activity of the IGFs in different cell systems. Taken together, the evidence to date suggests that insulin and the family of growth factors known as the IGFs may play an important role in the regulation of cellular proliferation and

differentiation in placental tissue. However, the effects of insulin and the IGFs on such processes in the human placenta and, thus, fetal development have yet to be clearly understood.

But what do we know of placental growth and development and the factors regulating such processes? The following sections describe our knowledge to date of the processes involved in the growth, development, structure and function of the human placenta.

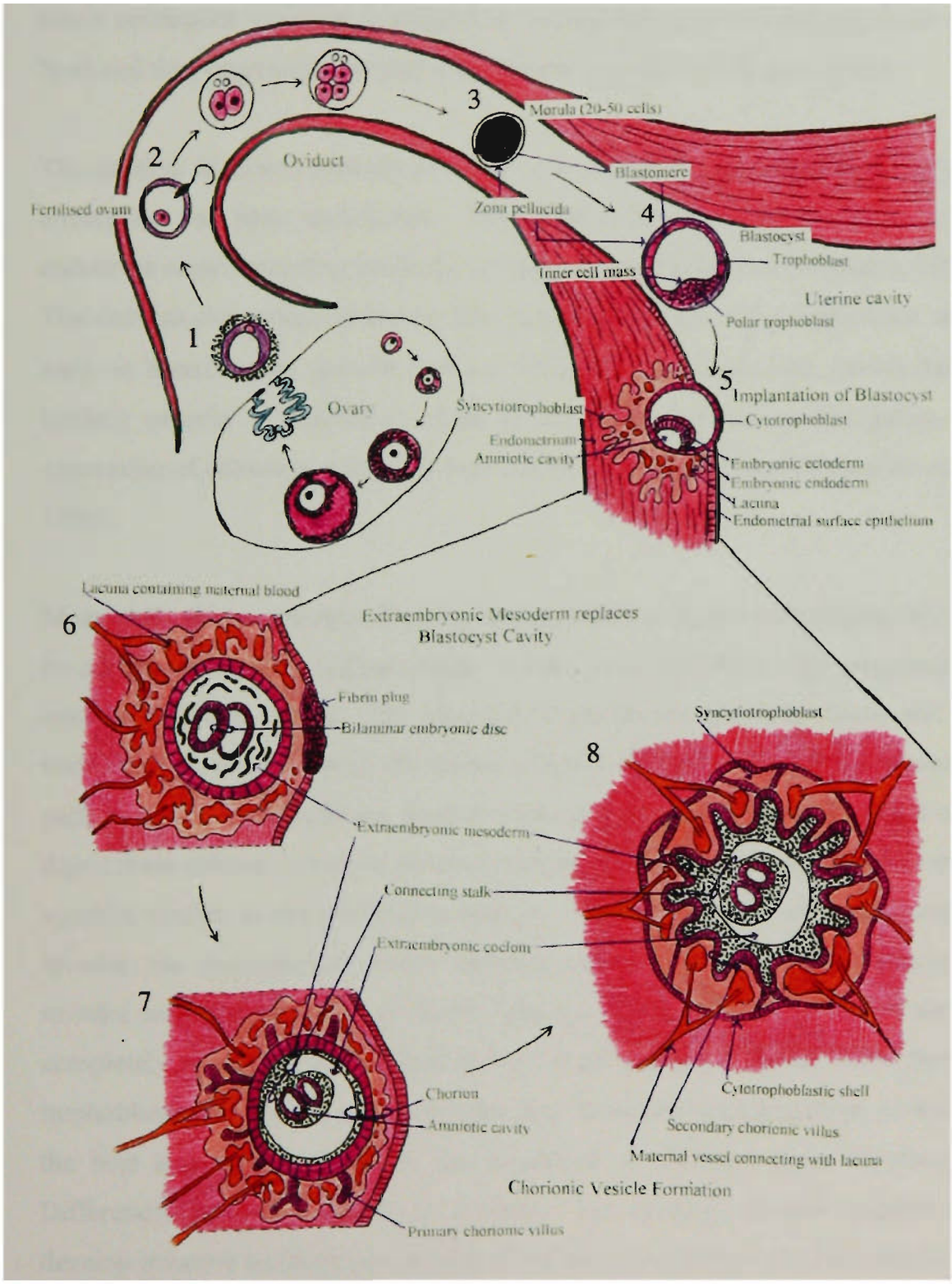
1.2. Implantation and the Decidua

The placenta develops from a complex system of embryonic membranes surrounding the fetus (Shanklin, 1986). The placenta encompasses a great diversity of morphological types, of which the human haemochorial disc and chorioallantoic organs are but specific variants. The formation and growth of the placenta begins with implantation (Figure 1.1).

Implantation, where the blastocyst makes contact with the endometrium usually on the posterior wall of the uterus, is a complex process that seems not to have been studied as a whole in any single species. Present concepts arise from fragments of information derived from several species. However, it must be noted that the definitive knowledge accumulated does not necessarily apply to all species. Various types of placentae are ultimately formed through different developmental pathways; eg. chorioallantoic (pig), choriovitelline (dog) and yolk sac (fish) placentae (Bjorkman *et al.*, 1989; Leiser and Kaufmann, 1994).

Figure 1.1. Diagrammatic representation of follicular growth, ovulation, fertilisation and embryonic development during the implantation process.

This figure depicts the following stages involved in implantation. (1) The egg is released from the ovary. (2) Fertilisation of the ova with sperm. (3) The morula (20-50 cell stage). (4) The blastocyst as seen in the early stage of implantation. (5) Implantation of the blastocyst. (6) Development has proceeded, extraembryonic mesoderm replaces the blastocyst cavity. (7 & 8) Chorionic vesicle formation and interstitial implantation. Drawings (1-5) adapted from Whittingham, D.G. (1979) Br Med Bull 35:105-11; and drawings (6-8) Wheater's Functional Histology, 3rd ed, (1993) Burkitt H.G., Young B., Heath J.W. (eds), Churchill Livingston, London.


1
2
3
4
5
6
7
8
Fertilised ovum
Oviduct
Morula (20-50 cells)
Blastomere
Zona pellucida
Blastocyst
Trophoblast
Inner cell mass
Uterine cavity
Polar trophoblast
Ovary
Implantation of Blastocyst
Syncytiotrophoblast
Cytotrophoblast
Endometrium
Amniotic cavity
Embryonic ectoderm
Embryonic endoderm
Lacuna
Endometrial surface epithelium
Lacuna containing maternal blood
Extraembryonic Mesoderm replaces Blastocyst Cavity
Fibrin plug
Bilaminar embryonic disc
Extraembryonic mesoderm
Connecting stalk
Extraembryonic coelom
Chorion
Amniotic cavity
Primary chorionic villus
Syncytiotrophoblast
Cytotrophoblastic shell
Secondary chorionic villus
Maternal vessel connecting with lacuna
Chorionic Vesicle Formation

Implantation has been shown to be under strict hormonal control (Williams, 1985; Moore, 1989). Progesterone and oestrogen, in specific sequence and quantities, are needed to induce the uterus to allow blastocyst implantation and uterine receptivity lasts for only a brief period. The decidua forms from the remodelling of the endometrium initiated with implantation. Here the underlying stromal tissue undergoes vascular modification, accumulation of cellular glycogen and lipid and the progressive atrophy of endometrial glands (Williams, 1985).

The decidua functions initially as a source of essential nutrients until the central circulation has been established. Progressively the decidua functions as an endocrine organ, secreting prolactin, relaxin and prostaglandins (Williams, 1985). The decidua also produces factors that regulate invasion of the trophoblast cells, such as transforming growth factor β (TGFβ) and insulin-like growth factor binding protein 1 (IGFBP-1) (Lala *et al.*, 1998) or that downregulate the expression of adhesion molecules on the surface of trophoblasts (Babawale *et al.*, 1996).

Meanwhile, the trophoblasts from the implanting blastocyst proliferate rapidly and invade the surrounding endometrium via pinocytotic and phagocytic processes (as cited in Williams, 1985). The invasion of the uterine glandular tissue and the underlying stroma results in the release of great amounts of metabolic substrates such as lipids, carbohydrates, nucleic acids and proteins. The trophoblast cells digest these substrates and the products are passed through the rapidly developing vascular system to the developing embryo. The extent to which the conceptus invades the endometrium varies between species; in humans the conceptus invades the maternal tissue so deeply that the uterine surface epithelium almost completely regrows over it. This is known as interstitial implantation. Not all trophoblasts are invasive and such properties of the implanting embryo, as well as the host endometrium, lead to the formation of different types of placenta. Differences between species are marked. For example, human zygotes that develop invasive trophoblasts produce the haemochorial-type placenta, whilst the diffuse epithelial chorial placenta (eg. pig) results from trophoblasts that remain cellular; i.e, do not invade the endometrium nor provoke a decidual reaction (Williams, 1985).

Of great theoretical and practical interest is the problem posed by the fact that the implanted embryo is in effect a homograft that is surprisingly well tolerated by the host. Reasons for tolerance by the mother of an antigenically foreign homograft are not fully understood, but appear to involve systemic hormonal factors as well as local tissue factors. It is the fetal trophoblast that forms the direct contact with the host tissue. The trophoblast has a unique organisation. Attachment to and invasion and metastasis of antigenically foreign tissue by the trophoblast have led some workers to surmise that trophoblasts and cancer are similar in some fundamental aspects. Furthermore, studies indicate that antigenic difference between the trophoblast and the host enhances rather than retards growth of transplanted mouse trophoblasts (Ringler & Strauss, 1990). Maternal immunoglobulin-G is transported across the placental syncytiotrophoblast and passively through the amniochorion during fetal development (Bright & Ockleford, 1995).

Meanwhile the primary yolk sac of blastocyst at day 10-11 partially fills with endodermal meshwork generated as a result of interstitial implantation of the embryo. A small secondary yolk sac develops by day 12-13 as a result of the pinching off of a portion of the larger primary yolk sac. The caudal margin of the primitive streak develops precociously in day 12-14 embryo and appears to be the source of all extraembryonic mesoderm of the chorion, chorionic villi and body stalk. The exocoelomic cavity forms inside the extraembryonic mesoderm alongside the placental chorionic plate and is believed to be an important transfer interface and reservoir of nutrients for the developing embryo. Maternal or placental proteins filtered in the extraembryonic coelom are probably absorbed by the secondary yolk sac, which is directly connected with the primitive digestive system throughout embryonic development (see Jauniaux *et al.*, 2000).

1.3 The Placental Villous Tree

The unique morphological features of the placenta include its dual composition and its extracorporeal location; functionally unique are its full growth and development within a limited life span and its anatomical adaptations to a number of diverse tasks. That a single tissue, the syncytiotrophoblast, may produce both

steroid and protein hormones is unusual and that the same tissue acts in both a restrictive and facilitative manner to protect and optimise fetal growth and differentiation is unique. Electron microscopy of the placenta has provided striking examples of the diversity and complexity of all placental components, dispelling the concept of the placental membrane as a semipermeable barrier. On the contrary, each component of the vital placental membrane has been shown to participate selectively in one or more metabolic functions.

Advanced microscopic techniques (LM, SEM and TEM) enabled the investigation of the three dimensional ultrastructural organisation of the human placental villous and observations into the relationships of the cells with other compartments of the placenta (Fox, 1978; Kaufmann *et al.,* 1979; Castellucci & Kaufmann, 1982; Kaufmann *et al.,* 1987). Different types of placental villi, appearing at different stages of placental maturity, have been characterised (Kaufmann, 1981; Castellucci & Kaufmann, 1982; Benirschke & Kaufmann, 1990). Initially, two types of placental villi are observed: the *free (floating) villi* and the *anchoring villi* (Figure 1.2).

Free villi are not in contact with the uterine wall and are bathed in maternal blood in the intervillous space. This allows for gas and nutrient exchange for the developing fetus. The anchoring villi attach the embryo to the uterine wall with cytotrophoblast columns. Extravillous cytotrophoblasts then invade the endometrium (interstitial invasion), myometrium and uterine blood vessels (endovascular invasion). As a result, oxygenated maternal blood flows to the intervillous space. *Mesenchymal villi* can be identified by day 14, after which total villous surface area increases (Baur, 1977). These villi are characterised by the presence of mesenchymal stroma (Castellucci & Kaufmann, 1982). Trophoblasts at the tips of the villi proliferate to produce cytotrophoblastic cell columns, which are not invaded by mesenchyme, anchor to the decidua at the basal plate. The floor of the intervillous space consists of cytotrophoblasts from cell columns, peripheral syncytium of the trophoblastic shell (trophectoderm), and decidua of the basal plate. On the other hand, the chorionic plate comprises cytotrophoblasts and syncytiotrophoblasts, externally, and fibrous mesoderm

internally, forming the roof of the intervillous space (Wislocki & Streeter, 1938 as cited in Williams, 1985).

Our understanding of the events that occur between the 18-19th days of development, when the embryo is in the primitive streak stage, are based on the pioneering work of Ramsey (1972). The trophoblastic shell is thick with villi formed of cytotrophoblastic projections, a central core of chorionic mesoderm (with developing blood vessels) and an external covering of syncytium (Figure 1.1). The blastocyst is buried within the decidua and the decidua basalis and decidua capsularis separate it from the myometrium and uterine epithelium, respectively. The embryo is trilaminar and the endoderm is continuous with the lining of the yolk sac. The extraembryonic mesoderm later form part of the walls of the amnion and yolk sac and connects the embryonic structures to the chorionic mesoderm by the body stalk, which develops into the umbilical cord. At this stage, the definitive yolk sac is completely lined by endoderm. In humans, the exocoelomic cavity, found externally to the yolk sac, fills with fluid and prevents the approximation of the yolk sac and trophoblast cells. This precludes the formation of a choriovitelline placenta.

About three weeks after fertilisation, the relations of chorion to decidua are clearly evident in the human embryo (Williams, 1985). The chorionic membrane consists of an inner connective tissue layer and an outer epithelium from which rudimentary villi project. The trophoblasts differentiate into cuboidal to round shaped cells of clear cytoplasm and vesicular nuclei (cytotrophoblasts or Langhans' cells) and an outer layer of syncytium that is multinucleated and has coarsely granulated cytoplasm. The villi in contact with the decidua basalis proliferate to form the leafy chorion which forms the fetal component of the placenta, whilst those in contact with the decidua capsularis begin to degenerate. This develops into the smooth chorion, chorion laeve, which is believed to be formed as a result of direct pressure and interference with the blood supply (Williams, 1985).

Much of the structural knowledge of the developing placenta is provided by the pivotal work performed by Castellucci and Kaufmann (1982). From weeks 7

through to 14 of pregnancy, the connective tissue core of the chorionic villi "assume a reticular aspect" to form *immature intermediate villi* (Castellucci *et al.*, 1982; 1984). Here, the villi are found arranged in compartments of different sized intercommunicating channels made of collagen fibrils and overlapping extensions of stromal cells. Numerous Hofbauer cells (macrophage-like cells) are found "communicating" through the stromal channels believed to serve as a substitute lymphatic system. Fetal capillaries are found located at the periphery of the villi near the trophoblastic covering with larger vessels located centrally within the villous core. The stroma contains many connective tissue cells known as reticulum cells that exhibit small cell bodies provided with highly branched cytoplasmic extensions, large nuclei, few cisternae of rough endoplasmic reticulum, distended Golgi apparatus and few polyribosomes and small vesicles.

After 14 weeks, a condensed network of collagen fibres starts to form in the proximal *immature intermediate villi*, resulting in compact fibrous stroma forming the *stem villi* (Castellucci & Kaufmann, 1982; Demir *et al.*, 1997). Large fetal vessels and capillaries are found running directly under the trophoblasts. The reticulum cells begin to resemble fibroblast cells. The Hofbauer cells either remain in the compartment-like surroundings or become enmeshed in the connective tissue fibres. This transformation proceeds gradually from the chorionic plate towards the peripheral ramifications of the immature intermediate villi (Castellucci & Kaufmann, 1982). By the beginning of the second half of pregnancy, *mature intermediate villi* are formed from the branching of the immature intermediate villi. A loose fibrillar network of connective tissue fibres, which fill the spaces between capillaries, replaces the reticular pattern characterising the immature intermediate villi (Figure 1.2).

Finally, *terminal villi* develop on the surface of the mature and immature intermediate villi and appear as small buds or outgrowths at around 30 weeks gestation. The stroma of the chorionic villus is almost completely occupied by fetal capillaries that are sinusoidally dilated and comprise both collagen fibres and small spindled-shaped (stromal) cells that have little cytoplasm, small Golgi apparatus and numerous polyribosomes (Castellucci & Kaufmann, 1982).

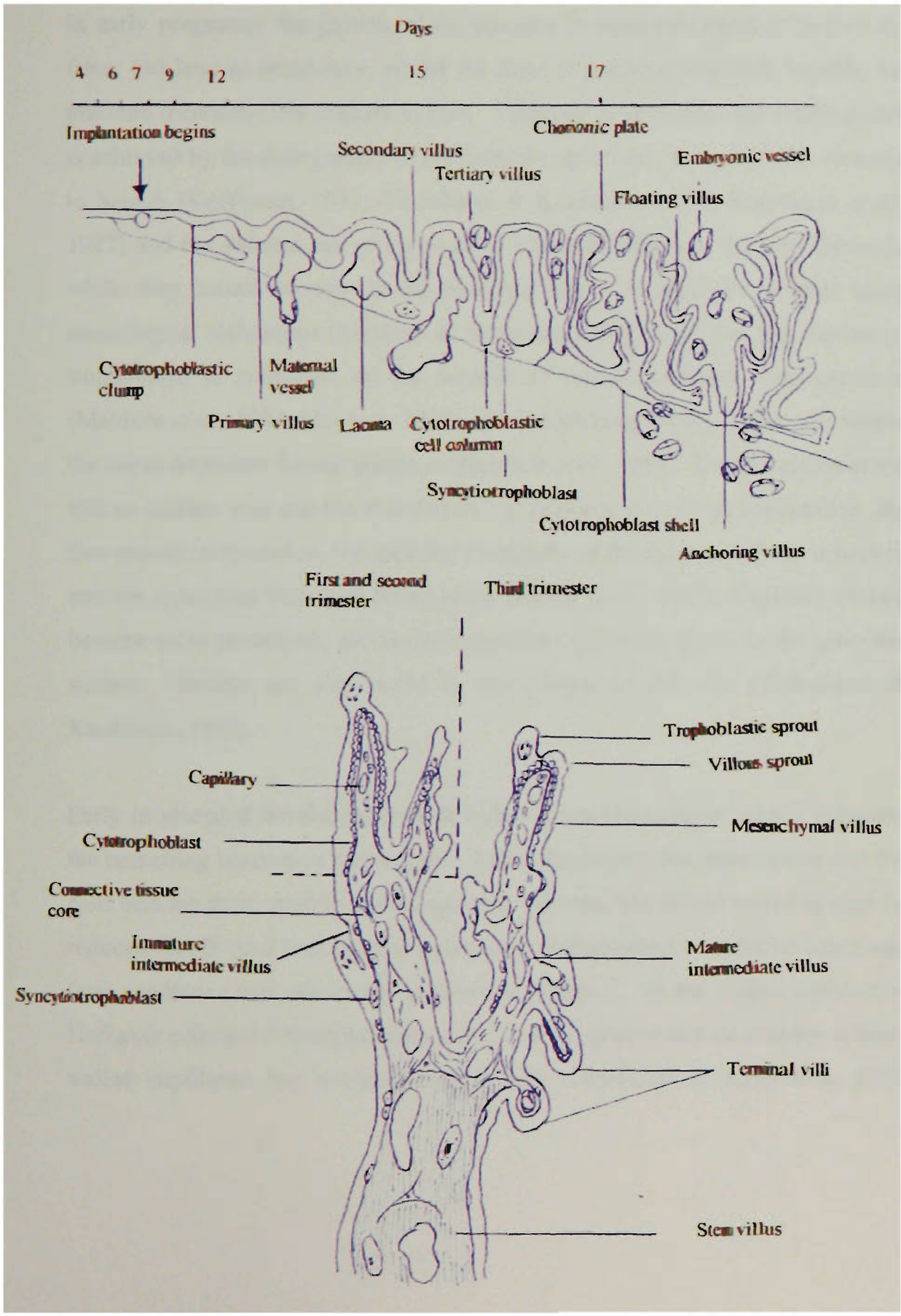
Figure 1.2. The developing villous tree of the human placenta

Diagram shows:

(a) The growth and development of the villous from first and second trimester through to third trimester.

(b) Villous comprises the villous sprout, the mesenchymal villous, the intermediate villous, the terminal villi and stem villi.

(Taken from (a) Carlson B.M. (1999) Human embryology and developmental biology. 2nd Edition, Mosby Inc, New York, p110; and (b) Castelluci *et al.,* (1990) Anat Embryol 181:117.)


Days
4 6 7 9 12 15 17
Implantation begins
Chorionic plate
Secondary villus
Tertiary villus
Embryonic vessel
Floating villus
Cytotrophoblastic clump
Maternal vessel
Primary villus
Lacuna
Cytotrophoblastic cell column
Syncytiotrophoblast
Cytotrophoblast shell
Anchoring villus
First and second trimester
Third trimester
Trophoblastic sprout
Villous sprout
Capillary
Mesenchymal villus
Cytotrophoblast
Connective tissue core
Immature intermediate villus
Mature intermediate villus
Syncytiotrophoblast
Terminal villi
Stem villus

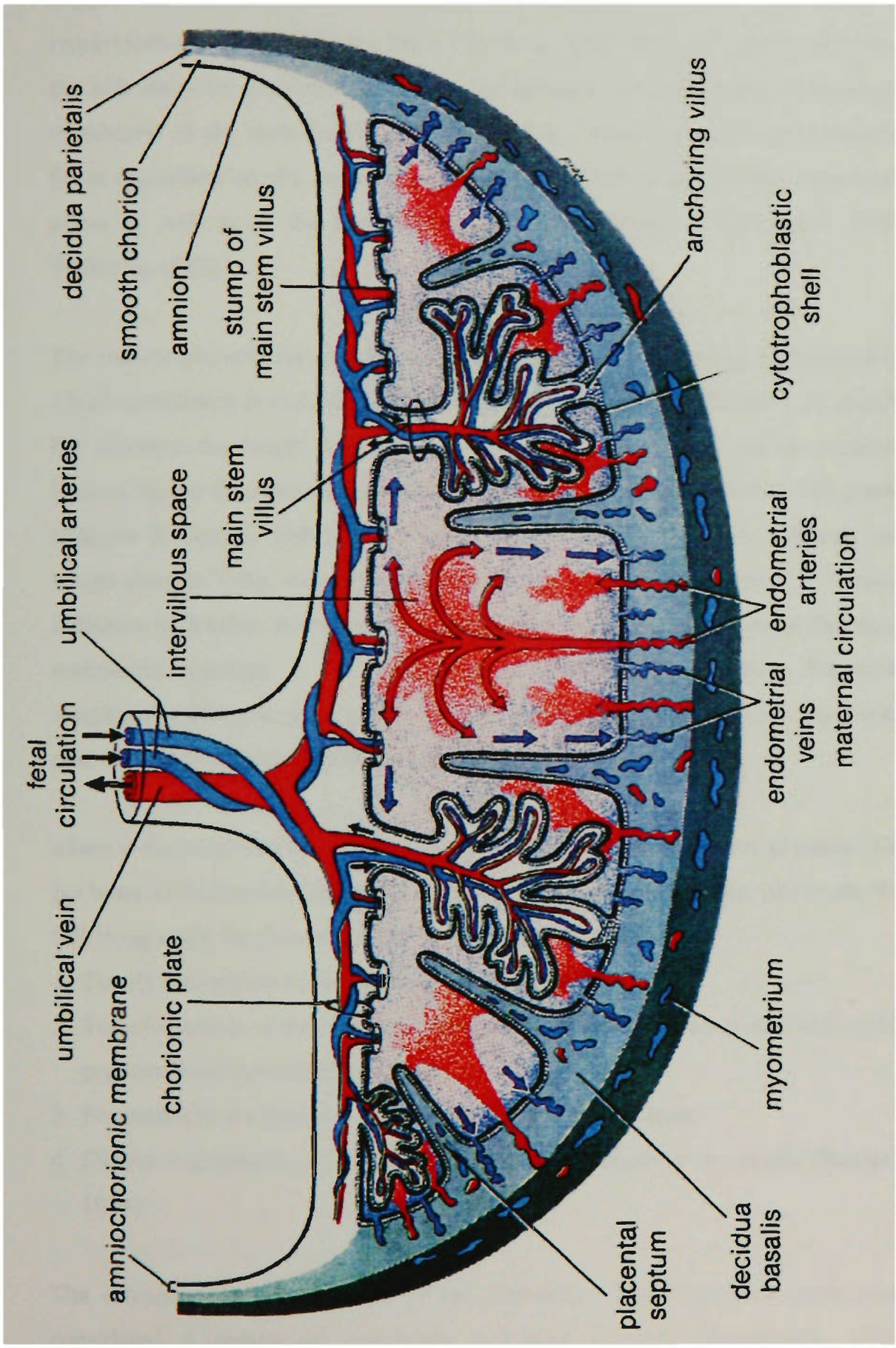
1.4. The Maturing Placenta

In early pregnancy the growth of the placenta is rapid compared to that of the fetus, but later in pregnancy, whilst the fetus is growing relatively rapidly, the placenta increases only slightly in size. Placental growth after the fourth month is achieved by the enlargement of the formed cotyledons. Here, the villi continue to branch (Kaufmann, 1981; Castellucci & Kaufmann, 1982; Kaufmann *et al.,* 1987) and the volume and prominence of cytotrophoblasts in the villi diminish, while they remain obvious in the placental floor. Quantitative studies using stereological techniques (Mayhew & Simpson, 1994) show that the number of trophoblast, as measured by the number of nuclei, increases with gestation (Mayhew *et al.,* 1994; Mayhew, 1997) and that packing density of the cells within the tissue decreases during gestation (Mayhew *et al.,* 1994). The expansion of the villous surface area and the thinning of trophoblasts may be an explanation. By four months of gestation, the apparent continuity of the cytotrophoblast is broken and the syncytium thins and forms knots (Cantle *et al.,* 1987). Capillary vessels become more prominent, increasing in number and lying closer to the syncytial surface. Changes are also noted in the stroma of the villi (Castellucci & Kaufmann, 1982).

Early in placental development an abundant loose intercellular matrix separates the branching connective tissue cells. Later, the stroma becomes denser and the cells become more spindly and compacted. At term, the villous covering may be reduced focally to a thin layer of syncytium with minimal connective tissue and fetal capillaries that apparently abut the trophoblast. In the villous stroma the Hofbauer cells and cytotrophoblasts are markedly reduced and the number of thin-walled capillaries has noticeably increased (Castellucci & Kaufmann, 1982

Figure 1.3. A schematic drawing of a section through a full term placenta.

This figure shows the relation of the villous chorion (fetal placenta) to the decidua basalis (maternal placenta), the fetal placental circulation, and the maternal placental circulation. Maternal blood flows into the intervillous spaces where exchanges occur with the fetal blood as the maternal blood flows around the villi. Deoxygenated fetal blood carried in the umbilical arteries to the placenta is shown in blue while oxygenated blood carried in the umbilical veins to the fetus is in red. Each cotyledon consists of two or more main stem villi and their branches. This diagram shows only one main stem villus in each cotyledon. Diagram taken from Moore, K.L. (1989) Before We Are Born, Basic Embryology and Birth Defects (3rd ed) W.B. Saunders Co., Philadelphia.


decidua parietalis
smooth chorion
amnion
stump of main stem villus
anchoring villus
cytotrophoblastic shell
umbilical arteries
intervillous space
main stem villus
endometrial arteries
endometrial veins
maternal circulation
fetal circulation
umbilical vein
amniochorionic membrane
chorionic plate
myometrium
placental septum
decidua basalis

The histological changes accompanying the growth and aging of the placenta suggest an increase in the efficiency of transport to meet the metabolic requirements of the developing fetus. However, other changes appear to decrease the efficiency for placental exchange, for example, the thickening of basement membrane of the endothelium and trophoblast, obliteration of certain vessels, fibrin deposition on villi surfaces, and deposits of fibrin in the basal and chorionic plates as well as in the intervillous space. (Castellucci & Kaufmann, 1982; Williams, 1985)

The mature placenta has an almost circular disc shape measuring approximately 15-20 centimetres in diameter and is thinner towards the edge (Figure 1.3). Inside the placenta the height varies greatly and the weight for a normal placenta (excluding the fetal membranes and umbilical cord) is approximately 500 grams (Ringler & Strauss, 1990). This varies, and a normally developed placenta can weigh close to 700g, varying according to ethnicity. A weight below 300 grams indicates retardation in the morphological and functional maturation of the organ commonly resulting in the inadequate development of the fetus. Placentae weighing $\geq$1,000 grams, such as in diabetic pregnancies, are often associated with macrosomic infants (>4kg) (Pedersen, 1977).

There is no consensus as to how to morphologically define a mature placenta. On the basis of anatomical findings on five hundred fetuses and their placentae, the following signs for placental maturity were initially set out:

1. Timely diminution of the diameter of the resorption villus;
2. Transformation of the capillaries into large capillary vessels (sinusoids) under pressure from the stroma of the villus;
3. Formation of the syncytio-capillary metabolic membranes;
4. Fibrous degeneration of the main villus and the fibrosis of its vessels. (Panigel, 1974)

The ultrastructural architecture of the placental villous tree has since been considered a means of measuring placental maturity (Kaufmann, 1981; Castellucci & Kaufmann, 1982; Kaufmann *et al.,* 1987). Not only has this led to distinguishing differences between immature (premature) placentae and those

seen in prolonged ('hypermature') pregnancies, but has also supplemented knowledge on the growth and structure of the placenta under abnormal conditions such as pre-eclampsia (Fox, 1978), diabetes mellitus (Jones & Fox, 1976) and acute peripheral hypoxia (Kaufmann *et al.,* 1987).

In the normal mature placenta, long mature intermediate villi with budding terminal branches of trophoblastic outgrowths predominate (Kaufmann *et al.,* 1987). The immature placental villous tree, in contrast, comprises uniformly thick and bulbous immature intermediate villi with irregular outer surfaces. Several syncytial sprouts are evident but terminal branches are almost absent. In placentae resulting from prolonged pregnancies, numerous terminal villi are seen that are long, branched and twisted. The villous surface is even more irregular with many transverse grooves and the irregular appearance of knot-like thickenings. No evidence of excessive syncytial sprouting was observed (Castellucci & Kaufmann, 1982).

The structure of the placenta in pre-eclampsia is very similar to that seen in prolonged pregnancy (Kaufmann *et al.,* 1987). Additional structural alterations of the villi (differentiating this abnormality from that of prolonged pregnancy) have been described, including stromal fibrosis and sinusoidal dilatation (Jones & Fox, 1980). Anatomical examinations reveal that cytotrophoblast invasion of the uterus is shallow and that endovascular invasion is incomplete (Zhou *et al.,* 1998). The placental villi in maternal diabetes mellitus are described as immature, with increased numbers of trophoblastic sprouts (indicating increased proliferative activity) (Kaufmann *et al.,* 1987). However, other characteristic features include stromal fibroblastic proliferation, excessive numbers of cytotrophoblasts as well as reduced fetal vascularisation (Jones & Fox, 1976). In the case of acute peripheral hypoxia, there is a deficit of terminal villi (Kaufmann *et al.*, 1987). Mature intermediate villi, almost devoid of branching predominate in placentae of such conditions. Small capillaries prevail, stroma with few connective tissue cells and fibres are in abundance and trophoblasts lacking vasculosyncytial membranes are present (Kaufmann *et al.,* 1987).

1.5. Trophoblasts: Villous Cytotrophoblast, Extravillous Trophoblast and Syncytium

Studies have demonstrated that the chorionic villus of the placenta comprise two distinct epithelial cell types distinguishable by certain physical and biochemical traits: the cytotrophoblast and the syncytiotrophoblast. The cytotrophoblast is described as a small, round, mononuclear cell that is identified by its large nucleus, prominent nucleolus and vesicular cytoplasm filled with Golgi bodies and mitochondria (Okudaira *et al.,* 1966). Refer to Figure 1.4 for different types of trophoblast cells.

Methods for isolating human placental (villous and extravillous) trophoblast populations have improved since the simple trypsinisation method of Thiede (1960) where cultures were found to include other placental cell types such as fibroblasts and stromal cells. Highly enriched trophoblast populations can now be achieved through the combination of trypsinisation and filtration processes with gradient centrifugation (Kliman *et al.,* 1986) and/or negative selection techniques (Schmon *et al.,* 1991). Sometimes selective culture conditions are adopted, such as the use of enriched or specified medium (Douglas & King, 1990; Starreveld *et al.,* 1998) or different extracellular matrices such as gelatin, fibronectin or collagen (Morrish & Siy, 1986). Variable results of purity have been obtained. Other investigators have used magnetic microspheres (Douglas & King, 1989) and monoclonal antibodies specific for trophoblasts (Contractor & Sooranna, 1988; Beham *et al.,* 1988; Ribitsch *et al.,* 1990; Hartmann *et al.,* 1997; Proll *et al.,* 1997). Although these techniques result in purer populations of trophoblasts (free of stromal cells, endothelium and fibroblasts) antibody binding may effect cell function *in vitro*. Monoclonal antibodies for specific cell structures and products are now used extensively as cell type markers. The use of immunolabelling in conjunction with flow cytometry (Bloxam *et al.,* 1997) has proven to be a powerful tool for isolating trophoblast populations and subpopulations.

Such purification techniques have led to the isolation of distinct trophoblast populations, differing in phenotype and function (Benirschke & Kaufmann, 1990;

Graham & Lala, 1992; Genbacev *et al.,* 1993; Rachmilewitz *et al.,* 1993; Aboagye-Mathiesen *et al.,* 1996). These are:

1. Mononuclear villous cytotrophoblasts (trophoblast stem cells), which proliferate, fuse and differentiate to form syncytium of floating villi (responsible for absorption, exchange and endocrine function); and
2. Extravillous trophoblasts (EVT), which break out of chorionic villi as discrete cell columns, migrate and invade the decidua and its vasculature.

The EVT cells are highly proliferative and invasive (Graham & Lala, 1992; Lala *et al.,* 1998), particularly at the base of the cytotrophoblast columns during first trimester (Genbacev *et al.,* 1993), after which they segregate to form several subset populations of trophoblast cells. These subpopulations include the following:

a. A population that differentiates morphologically to form mononuclear cells;
b. A population that fuses to form multinucleated, non-invasive giant cells that secrete hPL;
c. A floating intermediate EVT population that fuses to form cell clumps, which then further differentiate to mononuclear anchoring intermediate EVT.

The EVT are round cells with smooth surface cell membranes. The cells are rich in glycogen, contain large lipid droplets and flattened cisternae of rough endoplasmic reticulum. *In vitro*, the EVT retain their mitogenic and migratory capacity (Genbacev *et al.,* 1993).

Each trophoblast type exhibit different biochemical and structural properties. These include the presence/absence of a broad range of cytokeratin-intermediate filaments (Beham *et al.,* 1988), the epithelial specific adhesion molecule E-cadherin (Aboagye-Mathiesen *et al.,* 1996) and desmosomes around their entire circumference when isolated (Beham *et al.,* 1988; Douglas & King, 1990). The extravillous trophoblasts stain intensely for proliferative cell nuclear antigen (PCNA) (Genbacev *et al.,* 1993), indicating high proliferative activity in these cells. They also express high levels of integrin subunits ($\alpha 1$, $\alpha 3$, $\alpha 5$ and $\beta 1$), major histocompatibility complex class I molecules (Aboagye-Mathiesen *et al.,* 1996) and human placental lactogen (hPL) (Genbacev *et al.,* 1993) compared to

villous trophoblasts which express high levels of integrins α6, and β4 and reduced levels of β1 (Aboagye-Mathiesen *et al.,* 1996).

In culture, stem cell cytotrophoblasts spontaneously differentiate both biochemically and morphologically to form syncytiotrophoblasts (Kliman *et al.,* 1986; Morrish *et al.,* 1987; 1997), a process that requires relatively pure populations of cytotrophoblasts plated at an adequate density (Bloxam *et al.,* 1997a). The differentiation of cytotrophoblasts to form syncytiotrophoblasts is a multistep process that generates several types of intermediate trophoblasts.

Syncytiotrophoblasts are giant multinucleated cells that are granular in appearance. They contain smaller mitochondria with dense matrix (Martinez *et al.,* 1997) and possess desmosomes located on the membranes except the basal and lumen-oriented membrane (Beham *et al.,* 1988). The morphological changes that occur during differentiation *in vitro* reflect events that occur *in vivo.* These include the development of multinuclear syncytial cells with apical microvilli (Kliman *et al.,* 1986) and characteristic changes in the appearance of nuclei (Douglas & King, 1990) and that of structures of other cytoplasmic organelles such as mitochondria and rough endoplasmic reticulum and cell membrane (Bax *et al.,* 1989; Genbacev *et al.,* 1993; Martinez *et al.,* 1997). Functional changes include the production of polypeptides (Vettenranta *et al.,* 1986; Kliman *et al.,* 1986) such as pregnancy-specific β1-glycoprotein (SP_1), inhibin and interferons (Aboagye-Mathiesen *et al.,* 1993) as well as the synthesis and secretion of hormones (human chorionic gonadotropin (hCG) and hPL (Richards *et al.,* 1994), oestrogens and progesterone (Chaudhary *et al.,* 1992).

Energy turnover and metabolism in the isolated syncytiotrophoblast approximate those observed for the whole placenta (Bax & Bloxam, 1997). Results reveal that mostly aerobic glycolysis occurs before and after differentiation, but that during differentiation cytotrophoblasts depend on both glycolytic and aerobic energy production whilst syncytiotrophoblasts rely upon aerobic metabolism. It was also revealed that glucose uptake, lactate output and oxygen consumption occurs

largely from the basal side of the syncytiotrophoblast (corresponding to the fetal facing side *in vivo*) (Bax & Bloxam, 1997).

The role of growth factors and serum in the differentiation and function of the syncytiotrophoblast have been investigated. Medium supplemented with human maternal serum was found to enhance hCG and hPL secretion from isolated trophoblasts compared to that supplemented with heat inactivated fetal bovine serum (Richards *et al.,* 1994) suggesting that certain factors present in maternal serum may be important for the full functional differentiation of the trophoblast. It is also believed that such sera may provide 'mobility factors' (Babalola *et al.,* 1990) necessary for the aggregation and fusion of cytotrophoblast to from syncytium. However, variations in plating efficiency, cell morphology and viability it has been reported and may be attributed to differences in batches or sources of fetal calf serum (Branchaud *et al.,* 1983). Some researchers have found growth factors such as insulin, epidermal growth factor (EGF) and the insulin-like growth factors (IGF-I and IGF-II) have shown to be necessary for syncytial differentiation (Morrish *et al.,* 1997). Others have had success with basal medium without the addition of these factors (Kliman *et al.,* 1986; Douglas & King, 1990; Richards *et al.,* 1994).

1.6. Other Cell Types in the Human Placenta

The stroma of the human placental villi comprises mesenchymal cells, small and large reticulum cells and fibroblasts (Kaufmann *et al.,* 1977) as well as collagen fibres and microfibrillar elements containing elastic fibres (Graf *et al.,* 1996) and fibronectin (Aplin & Campbell, 1985). The stroma is formed by a lattice of mesenchymal cells that are connected (but not fused) together by long, interdigitated cytoplasmic processes (Kaufmann *et al.,* 1977). These connective tissue cells develop the ultrastructure characteristics of ordinary fibroblasts, i.e, they are spindle-shaped cells with multiple, delicate cell processes and an oval nucleus, and contain prominent rough endoplasmic reticulum, Golgi complexes and numerous filaments in the cytoplasm. The functional significance of these cells is mainly support and maintenance of the villous architecture. The reticulum

cell, a connective tissue cell of the reticular stroma of the intermediate villous also serves this function (Kaufmann *et al.,* 1977). These cells are small and possess highly branched cytoplasmic extensions. They have a large nucleus, a few narrow cisternae of rough endoplasmic reticulum, occasional polyribosomes, small vesicles and, in most cases, distended Golgi apparatus (Kaufmann *et al.,* 1977).

Several subpopulations of stromal cells, distinguished by typical co-expression patterns of cytoskeletal proteins are present in the chorionic villi of the human placenta (Graf *et al.,* 1996; Kohnen *et al.,* 1996). In term placenta the cells are arranged in concentric layers with clearly defined spatial differentiation gradient, around the fetal stem vessels. Increasing cytoskeletal complexity from the superficial trophoblast towards the blood vessels located centrally in the stem villi is noted (Kohnen *et al.,* 1996). The extravascular stromal cells that lie beneath the trophoblast are positive for vimentin and desmin and ultrastructurally resemble mesenchymal cells and are mitotically active (Sparn *et al.,* 1994; Kohnen *et al.,* 1996). The next layer of cells expresses vimentin, desmin and markers for smooth muscle (i.e., sm-α-actin) (Sparn *et al.,* 1994; Kohnen *et al.,* 1996). Further central towards the fetal vessels are stromal cells that co-express vimentin, desmin and markers for smooth muscle actin as well as markers for myofibroblasts and these cells develop myofibroblast features (Kohnen *et al.,* 1996). Finally, cells closest to the fetal vessels additionally express smooth muscle myosin and reveal a smooth muscle cell-related ultrastructure (Kohnen *et al.,* 1996).

In addition to these cells, there are Hofbauer cells (Enders & King, 1970) that are morphologically similar to macrophages (Castellucci *et al.,* 1987). These round cells are characterised by numerous cytoplasmic vacuoles and are found randomly scattered in the villi (Castellucci & Kaufmann, 1982). A potential role for these cells in protein ingestion and water balance regulation has been suggested (Enders & King, 1970). However, the motility of the Hofbauer cells along developing villous channels (Castellucci & Kaufmann, 1982) implies macrophage-like behaviour, important in maintaining host defence. In addition to this, Hofbauer cells are also believed to control direction of fluid movement through the

connective tissue channels of the developing placenta, thereby influencing the movement of substances important for fetal nutrition.

The placenta also contains numerous other cell types, eg, endothelial cells, which line the blood vessels of the placenta, fibroblasts and the red and white blood cells such as lymphocytes, macrophages and monocytes.

1.7. Regulators of Placental Growth and Function

Many factors have been implicated in the regulation of placental growth and function. These include nutrients, hormones, growth factors and cytokines, which will be discussed in detail in the following chapter. The cytokines and hormones are often endogenous to the placenta and/or surrounding fetal membranes and are thought to act in an autocrine and/or paracrine manner in controlling placental cell proliferation, differentiation, invasion and function.

The elucidation of the endocrine function of the placenta was difficult in the past as study of such a function encountered many experimental obstacles. Such difficulties arose due to:

1. The unique location of the placenta;
2. Marked species differences in placental structure; and
3. The impossibility of applying classical endocrine experiments, i.e., the removal of the placenta followed by transplantation or hormone replacement without interrupting pregnancy and resulting in fetal death.

The evolution of the knowledge on placental hormonal function began with the injection of emulsions or extracts of human placentae into different species (as cited by Williams, 1985). Recent studies using techniques such as whole placental infusion, immunohistochemistry, hybridisation histochemistry, receptor binding assays, immunocytochemical staining and studies of expression of mRNA as well as cell culture and biochemical assays have shown that the human placenta produces and secretes both protein and steroid hormones. What is known is discussed below, however, for many of these factors the functions are not completely understood.

Human chorionic gonadotrophin (hCG) is synthesised and secreted by the cytotrophoblast cells of the early placenta (Maruo *et al.,* 1992) and the syncytiotrophoblasts in term placenta (Kliman *et al.,* 1986; Nulsen *et al.,* 1988). The receptor for hCG has been localised to villous syncytiotrophoblast and endovascular intermediate trophoblasts with low levels found in villous cytotrophoblasts (Tao *et al.,* 1995). This hormone has been implicated in the autocrine control of invasion (Zygmunt *et al.,* 1998) and differentiation of trophoblast cells and is often used as an *in vitro* marker of differentiation (Cronier *et al.,* 1994).

Neuropeptides, such as gonadotrophin-releasing hormone (GnRH) (Wolfahrt *et al.,* 1998), and corticotrophin-releasing hormone (CRH) (Perkins & Linton, 1995; Wadhwa *et al.,* 1998) are expressed in first trimester and term placenta by both cytotrophoblast and syncytiotrophoblast cells. Due to the abundance of hormone expression at these times of trophoblast proliferative activity and differentiation, these hormones are generally believed to play a role in placental growth. Abundant levels of mRNA for parathyroid -related peptide and its receptor (Bruns *et al.,* 1995) are found in the chorion-decidua suggesting possible paracrine interactions within fetal membranes.

Components of the extracellular matrix in the surrounding microenvironment of the trophoblast cell have also been shown to influence cell growth and/or migration. Variation in the type of extracellular matrix (fibronectin, collagen, polylysine and gelatin) has been shown to influence cell growth (Morrish & Siy, 1986; Truman & Ford, 1986). Proteoglycan substances found within the extracellular matrix are also believed to influence cell growth, for example, decorin (Lysiak *et al.,* 1995a) a substance secreted by first trimester decidual tissue. Decorin is thought to bind transforming growth factor β (TGFβ; synthesised and secreted by syncytia) and inhibits its actions.

The TGFβ (isoforms 1, 2 and 3), important in regulating placental growth and function, have been shown to inhibit trophoblast proliferation (Lysiak *et al.,* 1995a) and invasion (Caniggia *et al.*, 1999), reduce cell migration by promoting

cell adhesion to the extracellular matrix (Graham & Lala, 1991; Irving & Lala, 1995) and to augment trophoblast fusion to form multinucleated cells (Graham *et al.,* 1992). It remains a contentious issue as to whether $TGF\beta_3$ overexpression may play a role in preeclampsia (Caniggia *et al.*, 1999) or growth restriction (Lyall *et al.*, 2001).

Other important factors include the inhibin-related proteins, activins A, inhibin A and inhibin B which are involved in the control of the feto-maternal communication required to maintain pregnancy (Florio *et al.*, 2001) and initiate labor (Riley *et al.*, 2000).

1.8. The Surrounding Fetal Membranes: Amnion and Chorion

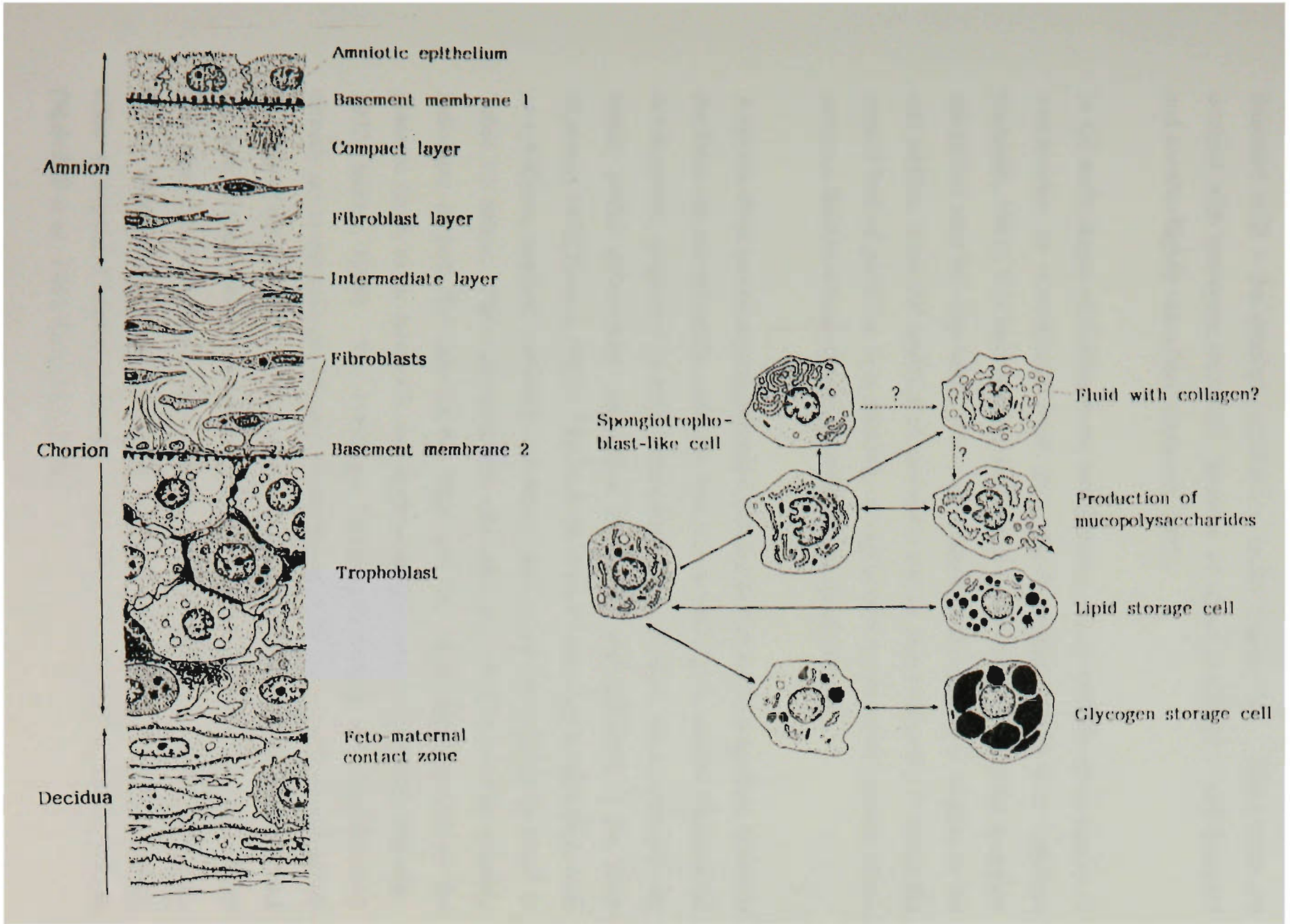
In humans, the amnion arises by the cavitation of the inner cell mass as a result of the confluence of intercellular spaces in the portion closest to the covering trophoblast (Williams, 1985). The subsequent development of the amnion also varies considerably. The membrane may remain an independent nonvascular structure until full term, as in most mammals (eg. rodents) with vestigial allantois, or it may expand to obliterate almost completely the extraembryonic coelom. The mesoderm covering the amnion may fuse with that of the chorion, as in human, or it may become surrounded by the allantois to form a vascular allantochorion, as in many carnivores. Generally, however, early implantation is accompanied by cavitational amniogenesis whilst late implantation is associated with the formation of amnion by folding (Williams, 1985; King, 1993).

The amnion is made of 3 differentiated parts: the placental amnion, the amnion of the umbilical cord and the reflected amnion (where the wall of the embryonic sac is not covered by placenta). The reflected amnion is a translucent membrane of high tensile strength. Its thickness measures 45-80μm, which varies between the differentiated parts.

Figure 1.4. Schematic representation of the cellular components of fetal membranes at term

Diagram shows the relationship of the amnion, chorion and decidua and the cell types that comprise these tissues. There is also a diagram of the different types of trophoblast cells found in the chorion.

(Diagrams taken from Schmidt (1992) Adv Anat Embryol cell Biol 127:1.)


Amnion
Chorion
Decidua
Amniotic epithelium
Basement membrane 1
Compact layer
Fibroblast layer
Intermediate layer
Fibroblasts
Basement membrane 2
Trophoblast
Feto-maternal contact zone
Spongiotropho-blast-like cell
?
?
Fluid with collagen?
Production of mucopolysaccharides
Lipid storage cell
Glycogen storage cell

Non-fixed cryocut sections have shown that at the placental margin, the amnion can measure 0.8mm the amnion consists of a single layer cluster of polygonal, flattened cells - the amniotic epithelium (refer Figure 1.4). These cells are covered with numerous microvilli (absence of which is related to cell damage) and are also highly vacuolised (Schmidt, 1992).

In the early stages of development, the amniotic cavity contains fluid similar in composition to intercellular fluid after which amniotic fluid then develops (Schmidt, 1992). It is believed that the large number of vacuoles of the amniotic epithelium may be responsible where the vacuoles are believed to migrate to the cell surface, burst and empty their contents into the amniotic cavity. During the second half of gestation, it is generally accepted that further development of the amniotic fluid involves some transepithelial transport of fluid.

Amniotic fluid serves several important functions: it provides a medium in which the fetus can move readily, cushions against possible injury, allows symmetrical development, maintains an even temperature and provides, when appropriately tested, useful information concerning the health and maturity of the fetus (Ramsey, 1972; Moore, 1989). The amniotic fluid contains many substances such as cytokines, steroids, metabolic substrates, hormones and enzyme as listed in Table 1.1 below. The fetal metabolic substrates can often be used as valuable indicators of both the state of the fetus and the functioning capacity of the placenta (such as in metabolic and haemolytic diseases or hypoxia) (Ramsey, 1972; Moore, 1989). The cytokines, steroids, hormones and enzymes may directly act to enhance growth and development of the fetus and may also act on the cells of the placenta and surrounding tissues to promote or inhibit growth and function of these tissues. Binding proteins and other cytokine-like factors have been shown to be secreted into the amniotic fluid (Han *et al.,* 1996; Soh *et al.*, 2000). Many of their functions remain unclear but it is believed that they may act either as trophic or inhibitory factors on the cells of the neighbouring fetal tissues (Mulvihill *et al.,* 1989; Soh *et al.*, 2000).

Table 1.1. Components of Amniotic Fluid

	Component	Examples of substances
Inorganic	Sodium, potassium, zinc, calcium, magnesium, iron chloride, inorganic phosphate, carbonate	
Organic	Carbohydrates and metabolic products	Glucose (5-20mg/100mL) lactic acid, pyruvate
	Amino acids (20)	
	Proteins (tot. 280-780mg/100mL)	Prealbumin, α_1, α_2-albumin, β-globulin, χ-globulins, α-fetoprotein, lipoproteins
	Glycoproteins	Pregnancy specific β_1 glycoprotein
	Catecholamines	
	Lipids (tot. 40mg/mL)	Cholesterol, triglycerides, free fatty acids, phospholipids
	Prostaglandins	Prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$
	Steroids	Cholesterol, pregnenolone
	Products of metabolism	Urea, uric acid, creatinine, bilirubin
	Enzymes	Alkaline phosphotase, α-amylase, cholinesterase, pepsinogen, amino oxidase, lactate dehydrogenase etc
	Hormones	Testosterone, progesterone, estradiol, estriol, cortisol, aldosterone 17-ketosteroid
	Proteohormones	LH, FSH, hCG, hPL, ACTH, hGH, TSH, PTH, glucagon, oxytocin, insulin, neurophysin

A list of known organic and inorganic compounds found in amniotic fluid. (Taken from Schmidt, 1992).

The quantity of the amniotic fluid oscillates considerably as pregnancy advances until near term, where the volume decreases. The average volume at term is ~850mL although this may vary between individuals and under abnormal conditions (such as hydramnios). The continual urinating and swallowing of large amounts of fluid by the fetus modify the composition and volume of the amniotic fluid during pregnancy. Movement of this fluid, into and out of the respiratory tract may also affect the volume and composition (Williams, 1985).

The amnion and the chorion adhere to one another but are not linked for communication by neural or vascular tissue. The amnion does not contain any blood vessels, lymphatic channels or nerves at any stage of development (Schmidt, 1992). The chorion is between 30μm and 50μm thick and is rich in cells and collagenous fibrils arranged in characteristic undulating bundles. The extracellular matrix, similar to amniotic connective tissue, is made of collagen mucopolysaccharides and fluid.

The chorion comprises several layers of different forms of trophoblast cells (refer Figure 1.4). The trophoblast of the chorion is rounded, polyhedral in shape, is inconsistent in size and has numerous cell branches (Schmidt, 1992). Some of these trophoblasts contain many rough endoplasmic reticulum, free ribosomes and microfilaments, many mitochondria, Golgi bodies, lysosomes and vesicles within their cytoplasm. The cell surface reveals numerous microvilli. These cells, also found in the basal plate of placenta, have been shown to be active in endocytosis (Kaufmann & Stark, 1971; Schmidt, 1992). Vacuolised trophoblasts are also found in the chorion. Mitochondria are scarce in these cells. These cells are believed to be involved in storage of glycogen and lipid which is then transported to the amniotic fluid to provide for fetal metabolism (Schmidt, 1992).

The last layers of cells are syncytiotrophoblasts found scattered between decidual cells forming the feto-maternal contact zone. It should be noted that the viability of these trophoblast cells at the end of pregnancy has been assessed. Very few cells have been shown to be necrotic and are believed to be very active throughout pregnancy including at term (Schmidt, 1992).

These fetal tissues are within close proximity to one another and are therefore capable of affecting adjacent tissues including the placenta. Studies have shown that autocrine/paracrine interactions are involved. The following section will look into these interactions and review the knowledge of some of the factors involved in the regulation of fetal tissue growth and development.

CHAPTER TWO

FACTORS AFFECTING PLACENTAL CELL GROWTH

In this chapter, information regarding the effects of certain known growth factors and cytokines produced by the fetal tissues and acting in a paracrine/autocrine manner to regulate adjacent tissue function will be reviewed. This section will particularly concentrate on the source, structure and function of insulin and the insulin-like growth factors, their role in regulating 'normal' fetal growth and development and comparing these effects to those observed in diseased states ie, gestational diabetes.

2.1. Growth Factors of the Placenta

Growth factors produced either by the placental cells themselves or by the surrounding fetal membranes have been shown to regulate placental cell growth, migration and invasion in a positive or negative way, via independent or interdependent processes.

Factors that have been shown to effect proliferation of placental cells *in vitro* include the TGFβ family, EGF (Lysiak *et al.,* 1994; Morrish *et al.,* 1998) and related family members such as TGFα (Lysiak *et al.,* 1993; 1994) and amphiregulin (Lysiak *et al.,* 1995b), colony stimulating factor (CSF-1) (Hamilton *et al.,* 1995), placental growth factor (PGF) (Ziche *et al.,* 1997) and the vascular endothelial growth factor (VEGF) family (Ferrara *et al.,* 1992). EGF and related peptides are produced by the trophoblasts and TGFα is produced in the decidua (Lala *et al,* 1998). CSF-1 is a potent mitogen that is produced by the endometrium as well as the placenta during pregnancy (Kauma *et al.,* 1991). The mRNA, protein and receptor for CSF-1 are expressed by EVT (Pampfer *et al.,* 1992). VEGF mRNA is abundant in decidual macrophages (Charnock-Jones *et al.,* 1994) whilst that of PGF is found in villous and extravillous trophoblast cells (Clark *et al.,* 1997). Their common receptor is found on cytotrophoblast membranes (Clark *et al.,* 1996). These factors possess potent angiogenic ability and have been shown to stimulate proliferation of EVT *in vitro* (Lala *et al.,* 1998). Endothelins (ET) have also been shown to be synthesised by term placental trophoblasts, amnion and stromal cells of the maternal decidua (Cervar & Desoye, 1998), and their receptor is found to be expressed in trophoblasts throughout gestation. In cells derived from first trimester tissue, ET-1 has been shown to stimulate mitogenic and invasive effects (Cervar *et al.,* 1996). However, its function in term placental cells remains unclear.

The importance of the regulation of placental growth and function is exemplified by disturbances in fetal development as a result of placental malfunction and/or structural or metabolic disorders, such as in the case of gestational diabetes. Pre-existing diabetes (such as IDDM) in pregnant women has a profound influence on

the growth and body composition of the fetus and surrounding fetal tissues (Pedersen, 1977), particularly the placenta which has been shown to be glycogen laden and significantly heavier than those from non-diabetic mothers (Pedersen, 1977; Fox, 1978) and mothers with well controlled gestational diabetes. These effects, for which the mechanisms responsible are poorly understood, are believed to be the consequence of maternal hyperglycemia and peripheral hyperinsulinemia (Pedersen, 1977). The elevated circulating levels of insulin in diabetic pregnancies have been associated with an apparent hyperplacentation and trophoblastic morphofunctional pathology (Jones & Fox, 1976). This chapter will investigate the information regarding the role of insulin and the insulin-like growth factors in the regulation of trophoblast proliferation and differentiation *in vitro*, with particular interest in diabetes during pregnancy.

2.2. Insulin

2.2.1. Structure and Physiological Roles of Insulin

Insulin is a polypeptide hormone that comprises two chains - an A chain of 21 residues and a B chain of 30 residues, the two chains being covalently bonded by disulfide bonds. Insulin regulates cellular metabolism and development primarily in target tissues such as liver, adipose and muscle. In response to increased circulating glucose levels, insulin is released from the β cells of the pancreatic islets. When bound to its receptor, insulin stimulates glucose, ion and amino acid transport, gene expression, RNA and protein synthesis as well as a number of enzymatic processes (Rosen, 1987; O'Brien & Granner, 1996). It inhibits the breakdown of glycogen and fat and decreases gluconeogenesis by reducing the available levels of pyruvate carboxylase and fructose-1,6-diphosphatase (Rosen, 1987). Insulin has been shown to effect steroidogenesis in a number of tissues including the human placenta (Adashi *et al.,* 1984; Nestler & Williams, 1987; Nestler, 1989).

2.2.2. Insulin as a Growth Factor?

Of interest is the growth promoting effect of insulin and its possible function as a growth factor in both 'normal' and abnormal tissue developments. Studies have demonstrated the ability of insulin to stimulate cellular proliferation in fibroblasts and hepatoma cell lines (Lin *et al.,* 1992). Its positive and/or negative effects on the expression of specific growth promoting genes have also been shown (Tuab *et al.,* 1987; Messina, 1989; Messina, 1990; O'Brien & Granner, 1991). *In vivo,* changes in insulin secretion and/or activity (particularly perinatally) have often been associated with growth disorders and malformations such as decreased fetal sources of insulin and pro-insulin which have been associated with reduced fetal growth (Godfrey *et al.,* 1996). In conditions of hypoinsulinemia in pregnant women, with or without nutritional deprivation, circulating IGF levels were reported as low and fetal growth was often retarded. Pregnant diabetic women were often treated with insulin to maintain euglycemia, which often results in peripheral hyperinsulinemia. This leads to hyperplacentation and trophoblastic morphofunctional pathology (Jones & Fox, 1976; Fox, 1978; Bobkov, 1991). This, in turn, may contribute to fetal macrosomia and/or other growth anomalies, and an increased mortality rate (Eriksson *et al.,* 1998).

Evidence suggests that increased insulin binding may be associated with increases in fetal weight and size. In a study on well controlled type II diabetic women with appropriate-for-gestational-age (AGA) babies compared to those with large-for-gestational-age (LGA) babies (Takayama-Hasumi *et al.,* 1994), insulin binding was found to be significantly increased in placental membranes from women with AGA and LGA babies compared to non-diabetic. Insulin uptake of ^{32}P into the β-subunit of the insulin receptor was found to increase significantly in placentae from AGA and LGA mothers compared to non-diabetic. The tyrosine kinase activity was also found to be significantly greater in that of LGA pregnancies compared to the other two groups (ie, AGA and 'normal'). Interestingly, there were no significant differences found in the binding and activity of IGF-I between the three groups (Takayama-Hasumi *et al.,* 1994).

2.2.3. Other Members of the Insulin Family

Free maternal insulin does not cross the blood/placental barrier and fetal pancreatic insulin secretion starts only after week 14 of gestation. Further evidence has demonstrated the transplacental passage of complexed insulin (Menon *et al.,* 1990). Insulin bound to autoantibodies have been demonstrated in cord blood (Wellick *et al.,* 1995; Weiss *et al.,* 1998). There are conflicting results regarding the correlation of the antibody bound insulin and maternal and fetal insulin with birth weight. The significance of maternal insulin in fetal blood is unclear although antibody complexed insulin has been found to be significantly higher in insulin treated diabetic mothers compared to 'normal' (Weiss *et al.,* 1998).

Other sources of insulin or the IGFs are available. It has been found that first and third term placental tissue of 'normal' pregnancies as well as term placental tissue of diabetic pregnancies express polyadenylated RNAs of an insulin-related gene which has a sequence similar to that which encodes fetal pancreatic insulin (Liu *et al.,* 1985). Moreover, placentae of diabetic mothers exhibit enhanced expression of this RNA compared to 'normal'. Other members of the insulin family have been identified also. These include placentin (Koman *et al.,* 1996), isolated from first trimester human placenta and shown to increase trophoblast proliferation and differentiation *in vitro*, and the insulin-like peptides, EPIL (Bellet *et al.,* 1997), expressed in both cytotrophoblasts and syncytiotrophoblasts in early placenta and villous cytotrophoblast cells at term. These results suggest that the insulin-related genes are expressed in placental tissue during development and may be a source of growth-promoting hormones for the human fetus and the placenta. Fetuses developing in pregnant diabetic women are often exposed to a large influx of glucose that may, in turn, stimulate the expression of these insulin-related genes. This may result in greater utilisation of glucose, that may in turn contribute to macrosomia and associated complications at birth.

2.3. The Insulin Receptor and Placental Growth

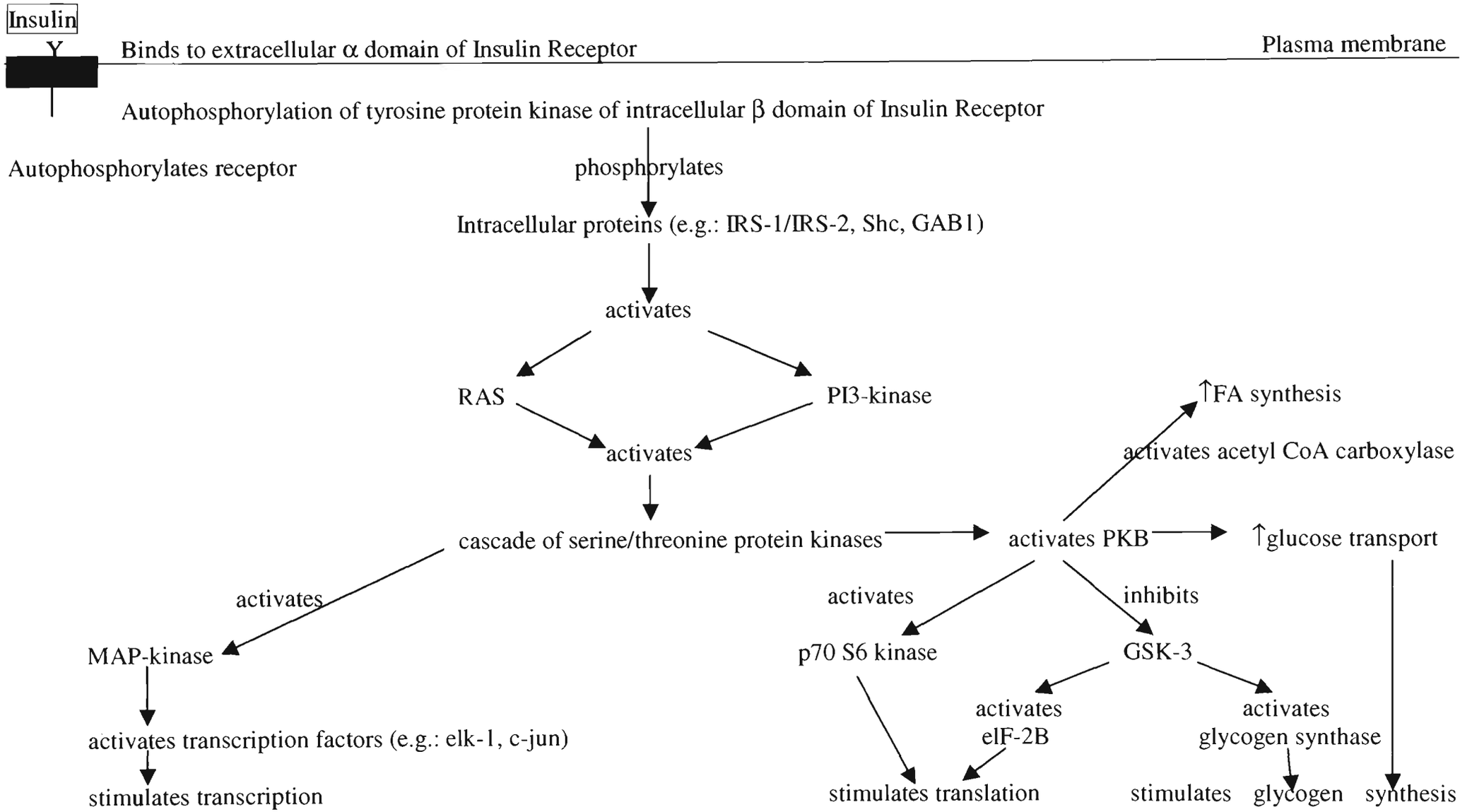
The placental insulin receptor is structurally and functionally similar to that found in its classic target tissues (Desoye, 1993). That is, it comprises two copies each of an exoplasmic α chain (719 amino acids) and a β chain (620 amino acids), linked by disulfide bonds. The α–subunit contains the insulin-binding domain, while the β–subunit contains a single transmembrane α–helix and a cytoplasmic domain of 391 amino acids. Apart from the tyrosine kinase domain (Machicao *et al.,* 1987) which phosphorylates an array of substrates *in vitro*, other proteins such as Ras (Mollat *et al.,* 1994), and the S6 kinase (Dennis & Masaracchia, 1993) have also been found to be involved in the signalling pathway (Figure 2.1). However, another mechanism whereby insulin mediates its effects may be via receptor internalisation and translocation to the nucleus, as was found to occur in intact hepatocytes isolated from rat tissues (Podlecki *et al.,* 1987).

In the placenta, insulin receptors are highly concentrated in mesenchymal villi and syncytial sprouts in early gestation, with distribution patterns changing during pregnancy (Desoye *et al.,* 1994). In term placental tissue, insulin receptors are found to be nonuniformly distributed (either as singleton or in groups of two or more (Nelson *et al.,* 1978)) in the villous brush border of syncytiotrophoblasts (Whitsett & Lessard, 1978; Desoye *et al.,* 1994) as well as on the endothelium of fetal circulatory vessels (Desoye *et al.,* 1994).

Evidence shows that insulin in the placenta does not necessarily act on the 'classic' insulin receptors alone in order to produce a response. It is possible that other types of insulin receptors may exist. For example, mRNAs for two different isoforms of the insulin receptor have been found in term placenta (Seino & Bell, 1989). The two isoforms, A and B, have been found to be expressed in almost equal amounts. However, the functions, localization of these proteins in different placental cell types and the regulations of their expression have yet to be elucidated.

Figure 2.1: Multiple intracellular signalling pathways of Insulin.

When insulin binds to its respective receptor, the interaction triggers a series of phosphorylation steps that proceed to activate a series of internal cellular factors that ultimately result in the stimulation of certain cellular responses such as translation, transcription and glycogen synthesis.
[Adapted from Moule & Denton, 1997, Am J Cardiol 80:41A].


Insulin
Binds to extracellular α domain of Insulin Receptor
Plasma membrane
Autophosphorylation of tyrosine protein kinase of intracellular β domain of Insulin Receptor
Autophosphorylates receptor
phosphorylates
Intracellular proteins (e.g.: IRS-1/IRS-2, Shc, GAB1)
activates
RAS
PI3-kinase
activates
cascade of serine/threonine protein kinases
activates PKB
↑FA synthesis
activates acetyl CoA carboxylase
↑glucose transport
activates
MAP-kinase
activates transcription factors (e.g.: elk-1, c-jun)
stimulates transcription
activates
p70 S6 kinase
inhibits
GSK-3
activates
elF-2B
activates
glycogen synthase
stimulates translation
stimulates glycogen synthesis

Another research laboratory independently located a group of 'atypical' insulin receptors in the human placenta. These receptors are believed to be located on trophoblast cells and have been shown to have unusually high affinities for insulin and IGF-II but not IGF-I (Jonas *et al.,* 1989). It is possible that their function is to regulate cellular growth by directly stimulating mitogenic signalling pathways. Studies using NIH-3T3 cells, which express high levels of wild-type insulin receptors, have found that mutations to the C-terminal end of the insulin receptor results in changes to cellular metabolism and mitogenic activity (McClain *et al.,* 1988; Takata *et al.,* 1991; Baron *et al.,* 1991).

In fact, the physiological significance of the insulin receptor in the placenta still requires some clarification. In human placentae, insulin has been reported to activate a number of processes. These include the increase of glucose (Gordon *et al.,* 1995) and amino acid uptake (Dancis *et al.,* 1968) as well as glucose oxidation and glycogen synthase activity (Demers *et al.,* 1972; Schmon *et al.,* 1991), lipogenesis (Coltart & Bateman, 1975) and glucose transport (Challier *et al.,* 1986). There is some evidence supporting insulin-dependent uptake of α-aminoisobutyric acid (Karl *et al.,* 1992). There is also evidence available suggesting the involvement of insulin in the regulation of protein and steroid hormone synthesis in the placenta (Hochberg *et al.,* 1983; Lavy *et al.,* 1987; Nestler & Williams, 1987; Nestler, 1989; Douglas & King, 1990). Yet other studies have failed to demonstrate any of these effects in the human placenta (Szabo & Grimaldi, 1970; Coltart & Bateman, 1975; Challier *et al.,* 1986; Schmon *et al.,* 1991).

Is it possible then that one important function of insulin in the placenta may be as a growth promoter during development? In some species, the results indicate a possible role for insulin as a growth factor during preimplantation and placentation (Desoye & Shafrir, 1994). Insulin binding studies have also shown a correlation between the number of insulin receptors in the human placenta and the weight of the fetus (Potua *et al.,* 1981). Here, placentae of infants that were small-for-gestational age (SGA) were shown to have significantly lower numbers of insulin receptors compared to those of preterm and full term adequate for

gestational age (AGA) infants. The collective evidence to date clearly suggests a role for insulin as a growth factor in the feto-placental unit. However, whether insulin directly effects the proliferation of placental cells and those of the fetus, or simply increases metabolic activity providing an increased supply of the necessary nutrients and energy for cell growth has yet to be demonstrated.

2.4. Glucose Transport in the Placenta

Studies in animals have shown an association of placental size with glucose transport capacity (Bell *et al.,* 1999). Glucose is carried across the placental barrier via specific glucose transport systems: GLUT1 and GLUT3 transporters (Illsley, 2000).

Insulin and increasing glucose concentration have been shown to modulate the transport of glucose by human placental cells (Brunette *et al.,* 1990; Gordon *et al.,* 1995; Hahn *et al.,* 1998; Osmond *et al,* 2000). Hyperglycemia has been shown to downregulate the GLUT1 transporter system (Hahn *et al.,* 1998) by redistributing these transporters intracellularly (Hahn *et al.,* 2000) whilst insulin treatment resulted in the increased expression of GLUT1 mRNA (Gordon *et al.,* 1995). The mechanisms include effects on glucose transporter (GLUT1) expression and activity (Gordon *et al.,* 1995; Illsley *et al.,* 1998) and subcellular redistribution (Hahn *et al.,* 2000).

Under normoglycemic levels, the density of basal GLUT1 transporters has been found to be almost 2-fold greater in diabetic groups compared to 'normal' (Gaither *et al.,* 1999). This has been associated with a 40% increase in glucose flux. The increase in GLUT1 transporter numbers may be explained by the elevated circulating levels of insulin often associated with gestational diabetes. This suggests that the impaired fetal development often linked with diabetes may be related not only to placental glucose uptake, but by the maternal circulating levels of insulin and glucose as well.

2.5. Insulin-like Growth Factors

2.5.1. IGF-I and IGF-II

Insulin shares structural and some functional similarities with the IGFs. IGF-I and IGF-II are single chain polypeptide growth factors comprising 70 and 67 amino acids, respectively, with a tertiary structure analogous to that of proinsulin (Humbel, 1984). They can mimic, both *in vivo* and *in vitro,* the biological actions of insulin (Froesch *et al.,* 1985; Rechler & Nissley, 1985), including the stimulation of glucose oxidation (Clemmons *et al.,* 1975), lipid synthesis and cell growth (Reddan & Dzeidzic, 1982). The IGFs have also been shown to have proliferative effects in a number of cell lines as well as demonstrating some differentiative effects (Schmid *et al.,* 1983; Adashi *et al.,* 1984; Florini *et al.,* 1986).

IGF-I (protein and mRNA) has been identified in a variety of fetal tissues (D'Ercole *et al.,* 1980; Han *et al.,* 1996). It has been shown to be expressed at a relatively high level during the first two trimesters of pregnancy (Wang *et al.,* 1988), corresponding to the proliferative phase of the growth of the placenta while human cord serum levels of both IGF-I and IGF-II strongly correlate with birth weight and gestational age (Bennett *et al.,* 1983; Reece *et al.,* 1994) and are believed to be regulated by a complex interaction of several factors including growth hormone and insulin (Yang & Yu, 2000). In addition, high affinity IGF receptors have been found in many fetal tissues (D'Ercole *et al.,* 1976; Owen *et al.,* 1980; Adams *et al.,* 1983) and found to be expressed in all cell types of the human placenta (Han *et al.,* 1996).

IGF-II has been specifically implicated as an important determinant of fetal growth (Moses *et al.,* 1980; Adams *et al.,* 1982). IGF-II mRNA has been shown to be expressed in trophoblasts of the cytotrophoblastic shell and Langhan's layer of placental villi only in first term (Han *et al.,* 1996). This suggests a possible autocrine role in cytotrophoblastic proliferation and differentiation. IGF-II mRNA was also found to be expressed abundantly in intermediate trophoblasts in anchoring villi and the chorionic and basal plates and identified in amnion and

chorion (Han *et al.,* 1996). It has been shown to suppress apoptosis (Hill *et al.,* 1998) and to be involved in trophoblast invasion early in pregnancy (Irwin *et al.,* 1999). However, the underlying mechanisms involved in the autocrine/paracrine role of IGF-II during invasion require further elucidation.

Recent studies have examined the relationship of the IGFs to fetal growth in diabetic pregnancies. In the diabetic state, cord levels of IGF-II are elevated whilst in cases of macrosomia (independent of diabetes) IGF-I levels have been found to be high (Roth *et al.,* 1996). With intra-uterine retardation both IGF-I and IGF-II levels were substantially reduced (McIntyre *et al.,* 2000). These data strongly point to the IGFs as being important regulators of fetal development but increasing evidence also suggests the IGFs as potentially important regulators in the growth and development of the placenta.

2.5.2. The IGF Binding Proteins

IGF-I and IGF-II are synthesised within the placenta, however, IGF bioavailability is determined at the cellular level by several specific proteins. At least six IGF binding proteins (IGFBPs) have been characterised and detected in blood, extracellular fluids, tissue extracts and cell-conditioned culture medium (Ooi & Herrington, 1988). In fetal tissue, the IGFBPs have been shown to be largely produced by the amnion, chorion and decidua and are believed to act in a paracrine manner to modify IGF activity in the placenta.

Of the IGFBPs identified, IGFBP-1 mRNA is expressed in greatest abundance (Han *et al.,* 1996) and is prominent in amniotic fluid (Chevallier *et al.,* 1998). Conflicting evidence exists on the correlation of IGFBP-1 levels in amniotic fluid and fetal growth retardation (Chevallier *et al.,* 1996; Heffner *et al.,* 1998). However, evidence does suggest that high levels of IGFBP-1 is associated with low placental weight (Verhaeghe *et al.,* 1997) and dysfunction (Holmes *et al.,* 2000) which may indirectly affect fetal growth. Table 2.1 outlines the different binding proteins, their mRNA sources and possible functions in fetal tissues during gestation.

Table 2.1. The insulin-like growth factor binding proteins (IGFBP).

IGFBP	size	mRNA source & detection	possible functions	references
IGFBP-1	28-30 kDa	Stroma of decidua, and Amniotic fluid	Inhibits IGF-I binding to endometrial cells, blocks bioavailability, ↓ IGF-I mitogenicity, stimulates EVT invasiveness	Rutanen *et al.*, 1988; Ritvos, 1988; Hill *et al.*, 1993; Han *et al.*, 1996; Lala *et al.*, 1998
IGFBP-2	34 kDa	Stroma, amnion and chorion	Modulates IGF-I mitogenicity	Binkert *et al.*, 1989; Han *et al.*, 1996
IGFBP-3	150 kDa complex	Stroma, IT of basal plate and anchoring villi, amnion and chorion, blood and sera	Cell to cell communication between trophoblasts and maternal decidua for placental development and/or function	Baxter & Martin, 1989; Han *et al.*, 1996
IGFBP-4	-	As for IGFBP-3 chorionic mesoderm	As for IGFBP-3	Han *et al.*, 1996
IGFBP-5	-	Stroma	As for IGFBP-3	Han *et al.*, 1996
IGFBP-6	-	Decidua parietalis	As for IGFBP-3	Han *et al.*, 1996

The Insulin-like Growth Factor Binding Proteins (IGFBP) currently identified, their size, mRNA source and possible functions in fetal tissues and the placenta during gestation.

2.6. IGF Receptors: Type I and Type II

IGF-I binds to specific cell membrane receptors, the type 1 IGF receptor (MacDonald *et al.,* 1988). This is a tetrameric transmembrane protein, similar in structure to the insulin receptor, composed of two α-subunits and two β-subunits joined by disulfide bridges. The α-subunits are entirely extracellular and appear to be involved in ligand binding, whereas the β-subunits traverse the cell membrane and contain a tyrosine kinase domain in their cytoplasmic moieties (Rechler & Nissley, 1985).

A high degree of similarity exists between the insulin and type 1 IGF receptor, both in structure and function. Both receptor types are transmembrane glycoproteins that are heterotetrameric in structure (LeRoith *et al.,* 1994). They both possess α- and β- subunits that are N-glycosylated with highly conserved amino acid sequences in the tyrosine kinase domain of the β-subunit. Following the formation of disulphide bridges, the receptors adopt an $(\alpha\beta)_2$ configuration which is the form expressed on the cell surface (LeRoith *et al.,* 1994).

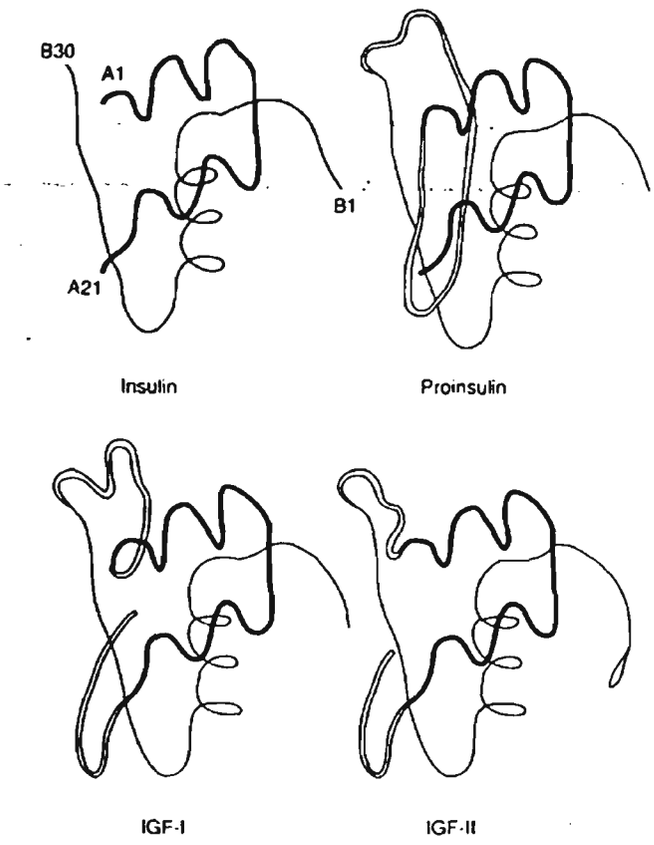
Although the structure of the insulin and type 1 IGF receptor are overwhelmingly similar, differences in both the α- and β- subunits between these two receptors do exist. Such differences may account for the different mechanisms of action which, in turn, may result in the generation of different biological effects of insulin and IGF-I in the same tissue. For example, insulin binds to the insulin receptor with high affinity whilst IGF-I does so at a much lower affinity and vice versa. These specific interactions are thought to be due to certain structural features of both ligands and their respective receptors. This interaction, in turn, determines the functions of these ligands. That is, IGF-I can mimic insulin responses but is not considered as potent a metabolic activator as insulin, while insulin, acting via the IGF-I receptor, may stimulate proliferation of cells but at a much lower capacity compared to IGF-I. Other factors such as the IGF binding proteins can affect IGF-I affinity producing either a synergistic or an antagonistic effect on IGF-I activity and/or bioavailability, which again alters the biological effects of this ligand.

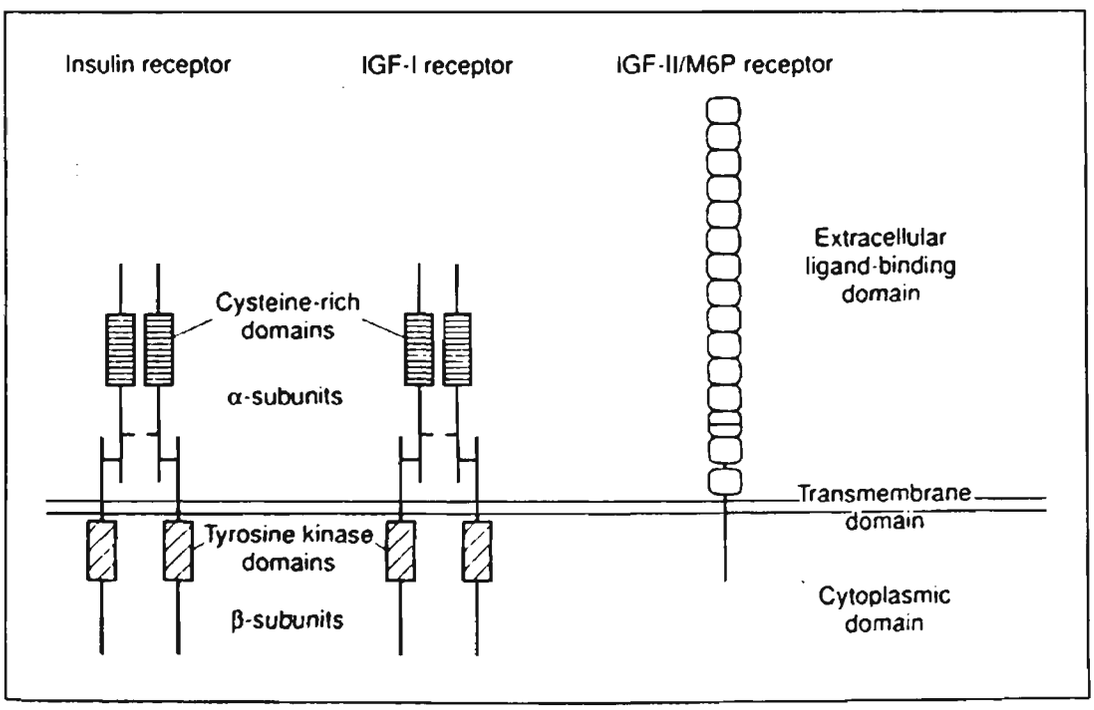
Figure 2.2: Insulin and the IGFs: Structure and receptors.

The tertiary structure of insulin and IGF-I and IGF-II peptides.
The heavy line represents the A chain, double lines the C chain joining A and B chains or the D extension of the A chain found in the IGFs.

The structure of insulin and the type 1 and type 2 IGF receptors.
The insulin and IGF receptor are both heterotetrameric complexes composed of extracellular a-subunits that bind the ligands and b-subunits that anchor the receptor in the membrane. These contain tyrosine kinase activity in their cytoplasmic domains. The IGF-II receptor is very different with a short cytoplasmic tail and no tyrosine kinase activity.

(Taken from: Le Roith *et al*, (1994) Horm Res 41:74)


B30
A1
B1
A21
Insulin
Proinsulin
IGF-I
IGF-II


Insulin receptor
IGF-I receptor
IGF-II/M6P receptor
Extracellular ligand-binding domain
Cysteine-rich domains
α-subunits
Transmembrane domain
Tyrosine kinase domains
β-subunits
Cytoplasmic domain

The IGF-II receptor is very different in structure and its function has yet to be determined. This receptor binds IGF-I at low affinity and does not bind insulin. *In situ* hybridisation of mRNA reveals that IGF-II is produced by both the villous cytotrophoblast and the EVT (Han *et al.,* 1996). IGF-II also binds to the type 1 IGF receptor with less affinity than IGF-I. In addition, IGF-II has its own distinct receptor, the type 2 receptor, equivalent to the mannose-6-phosphate receptor (MacDonald *et al.,* 1988). It is widely believed that the proliferative effects of IGF-II are mediated via the type 1 IGF receptor. IGF-II has been shown to stimulate EVT invasion (Lala *et al.,* 1998), a process believed to be mediated via the type 2 IGF receptor (Hamilton & Lala, 1996).

2.7. Insulin/IGF-I Hybrid Receptors of the Placenta

Insulin receptors and the type 1 IGF receptors have been shown to form insulin/IGF-I hybrid receptors in tissues that express both receptor types (Bhaumick *et al.,* 1988; Treadway *et al.,* 1989; Kasuya *et al.,* 1993; Soos *et al.,* 1993; Siddle *et al.,* 1994). Classic insulin and IGF-I receptors exist as α2 β2-heterotetrameric complexes that are assembled from two identical αβ heterodimeric half receptor precursors (Rechler & Nissley, 1985; Desoye, 1993). The insulin/IGF-I hybrid receptors are believed to be formed from the heterologous pairing of the insulin and IGF-I half receptors complexes that are readily assembled both *in vivo* and *in vitro* (Soos & Siddle, 1989; Treadway *et al.,* 1989; 1991).

It has been shown that when cells are treated with either insulin or IGF-I *in vitro* (Treadway *et al.,* 1989) different types of insulin/IGF-I hybrid receptors are formed demonstrating different binding potencies for insulin and IGF-I (Cara *et al.,* 1990; Soos *et al.,* 1993; Siddle *et al.*, 1994). With insulin treatment, insulin receptors on membranes are induced to form α2β2-heterotetrameric insulin hybrid receptors (which bind IGF-I at greater levels than insulin) whilst IGF-I treatment was found to be completely ineffective in inducing such hybrids. Yet IGF-I treatment was found to induce the formation of α2β2-heterotetrameric IGF-I hybrid receptors (which bind insulin at greater levels than IGF-I) with insulin

treatment being completely ineffective in this system. When both insulin and IGF-I are simultaneously administered to cell membranes containing both insulin and IGF-I receptors, both receptor types can be induced to formation at varying proportions depending on the ratio of ligands administered (Soos & Siddle, 1989; Federici *et al.,* 1997; Bailyes *et al.,* 1997).

The numbers of insulin and IGF-I hybrid receptors have been shown to alter under abnormal conditions (Bailyes *et al,* 1997). The number of hybrid receptors was found to increase significantly in the placentae of hyperinsulinaemic women complicated with hypertension, compared to control and that of normoinsulinaemic and was found to correlate positively with the degree of insulineamia. Increasing blood insulin levels was associated with decreasing affinity and binding of insulin to placental membranes whilst the binding of IGF-I was found to increase. However, IGF-I affinity for the receptor was found to be similar between control, normoinsulinaemic and hyperinsulinaemic mothers (Valensise *et al.,* 1996).

2.8. Maternal Diabetes: Effects on Placental and Fetal Development.

The rate of embryonic dysmorphogenesis increases in diabetic pregnancies and has been found to correlate with the severity and duration of hyperglycaemic levels during early gestation (Eriksson *et al.,* 1998). It has been demonstrated that the disturbances in embryonic development resulting from increased glucose concentrations may be partly mediated via altered metabolism of arachidonic acid and radical oxygen species in the embryo (Wentzel & Eriksson, 1998). In the rat, increased concentrations of glucose were shown to inhibit oxygen uptake into embryonic neural tissue and alter glucose utilisation significantly (Yang *et al.,* 1997). Such glucose-altered metabolism led to the production of superoxides by the tissue resulting in diminished metabolic sensitivity to increased glucose exposure suggesting damage to mitochondrial function (Yang *et al.,* 1995). Despite changes in glucose concentrations, *in vitro* studies have shown that insulin biosynthesis in fetal pancreatic cells remains unchanged in diabetic pregnancies (Aoyagi *et al.,* 1997). However, studies have shown that insulin

treatment in diabetic rats caused greater embryonic maldevelopment (Wentzel & Eriksson, 1996). Other serum factors such as increased levels of triglycerides, branched-chain amino acids, and proline, alanine, asparagine, orthinine and tyrosine may also contribute to congenital malformations in diabetic pregnancies (Styrud *et al.,* 1995).

In pregnancies of women with pregestational diabetes, macrosomia is a significant problem. Diabetes is believed to contribute to an increased perinatal morbidity leading to difficult delivery, higher rates of birth trauma and a range of fetal growth anomalies (Fox, 1978; Zanardo *et al.,* 1983; Mimouni *et al.,* 1992; Van Allen *et al.,* 1994). The risk of congenital malformations is almost 3X greater in women with pregestational diabetes compared to women with well-controlled gestational diabetes and non-diabetic women (Ray *et al.*, 2001). Despite the current improvements in methods of maintaining glycemic control in pregnant women, macrosomia remains prevalent in pregnancies complicated by insulin-dependent diabetes mellitus (IDDM) (Small *et al.,* 1987; Berk *et al.,* 1989). However, the rate of incidence may vary depending upon additional factors considered when the population is examined. These include: sample size, age, body mass index, parity, complications, country, ethnicity, income level and educational level of the population examined (Engelgau *et al.*, 1995; Sermer *et al.,* 1998; Hardy, 1999; Vambergue *et al.,* 2000).

Reports have indicated that fetal and placental macrosomia do not correlate with the degree of maternal hyperglycemia. This is supported by studies performed on intact pregnant mothers of different animal species (Heding *et al.,* 1980) where the lack of fetal production of insulin resulted in no appreciable reduction in fetal size or marked fetal growth retardation. In a human study (Small *et al.,* 1987) it was found that a significant proportion of IDDM pregnancies resulted in macrosomic infants, despite there being no significant differences in overall glycemic control, throughout pregnancy, between non-diabetic and IDDM mothers. It is possible that limited sources of other fuels may effect placental and/or fetal development by affecting glucose disposal and insulin production. For example, protein-energy malnutrition has been shown to reduce glucose disposal and increase nutrient transfer to the fetus (Eriksson & Swenne, 1993).

This results in the stimulation of pancreatic β-cell growth and the development of macrosomia in the offspring.

When comparing placental morphology between those obtained from diabetic mothers and placentae of normal, healthy mothers, distinct differences are noted. Placentae from overt diabetic mothers, especially when associated with macrosomic fetuses, are generally heavier than those obtained from 'normal', healthy mothers (Laurini *et al.,* 1987; Clarson *et al.,* 1989). In cases of insulin-dependent diabetes mellitus (IDDM), the placenta comprises a preponderance of cytotrophoblastic cells, similar to that seen in the early stages of normal placental growth (Desoye & Shafrir, 1996). These have been shown to have increased cellular DNA content (Diamant *et al.,* 1982) and reduced levels of epidermal growth factor (EGF) receptors and transcripts (Fujita *et al.,* 1991). Morrish and colleagues (1987) have demonstrated the differentiative properties of EGF on cytotrophoblasts of normal human placenta. Therefore, it is possible that a decline in the EGF receptor number could lead to a possible increase in proliferative activity, as differentiation would be limited. These observations clearly suggest that the placentomegaly associated with maternal diabetes may be the result of increased proliferative activity of the trophoblast cells. However, the mechanisms for the increased cellular proliferation are still poorly understood. It is generally believed that this may be the result of increased supply of maternal metabolic fuels or the increased release of growth factors (such as the IGFs) from fetal tissues and the resultant increased growth factor activity, or both.

In cases of maternal diabetes with poor glycemic control, the placentae have thickened trophoblastic basement membranes (Jones & Fox, 1976; Fox, 1978), attributed to increased amounts of collagen (predominantly type IV) (Laureti *et al.,* 1982), and villous oedemas are not uncommon. Maternal diabetes has been shown to alter the extracellular component (reduced laminin deposits and increased fibronectin levels) in the fetal-maternal interface (Cagliero *et al.,* 1993; Forsberg *et al.,* 1998), which may affect placental development. This is also associated with enlargement of the villous and capillary surface areas (Bjork & Persson, 1982; 1984), increasing exchange between maternal and fetal tissues, and

found to correlate with maternal glucose levels during the first and second trimesters (Bjork & Persson, 1982). However, in placentae of well controlled diabetic pregnancies, differences in placental anatomy and structure, compared to normal, healthy placentae, do not appear to be so pronounced, even though they may be considerably (but not always significantly) heavier (Mayhew *et al.,* 1993a; 1993b). Such results indicate the adaptive properties of the placentae to maintain sufficient function for fetal growth and development.

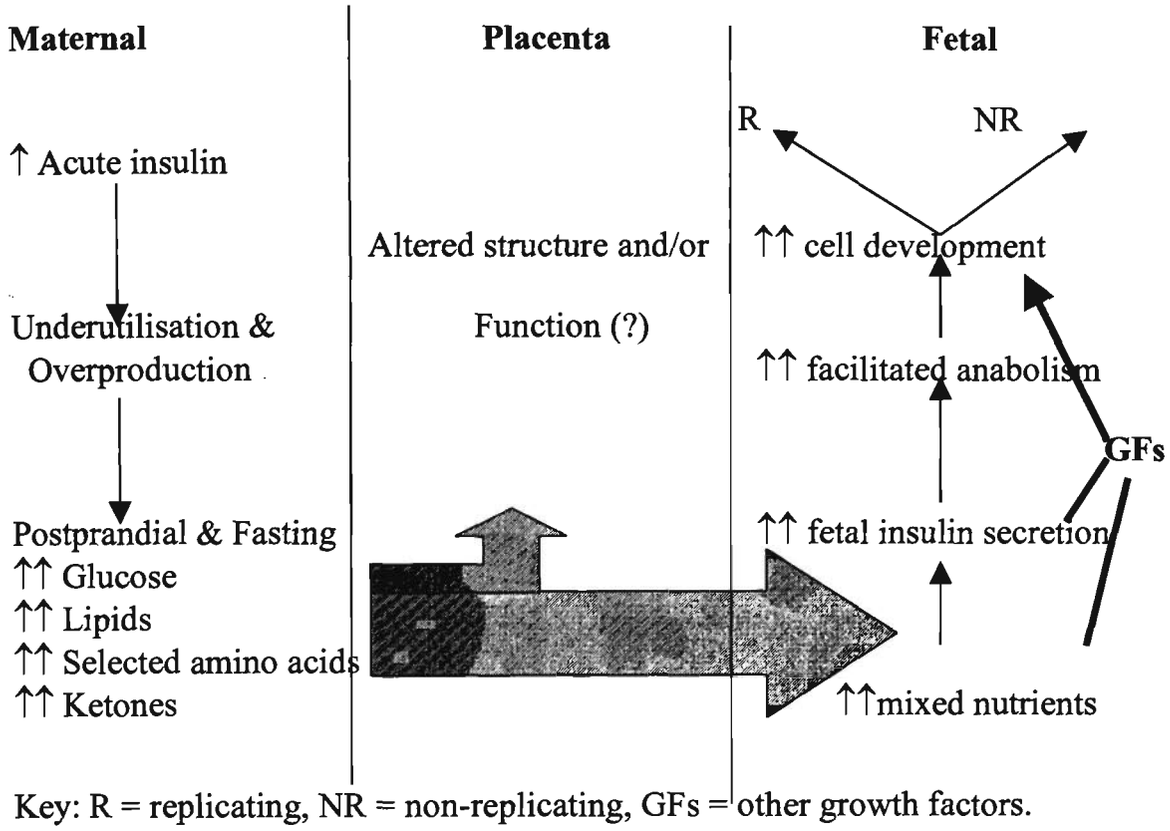
2.9. Pedersen's Theory

Diabetes is a well-defined metabolic disorder that complicates pregnancy. The incidence of fetal malformations in such a state is up to three times higher than the 'normal' rate (Beard & Hoet, 1979). The conceptus depends on the nutrients derived entirely from the mother and its health is, therefore, ultimately determined by what she eats, how she handles these nutrients, and how they are stored and recalled. It is, therefore, believed that the abnormal metabolism of the mother at the time of implantation and organogenesis may, in some way, be responsible for fetal abnormalities.

Current theory put forward by Barker (1998) has suggested that one may be predisposed to developing diabetes, which may be determined from the environment *in utero* during fetal life. A stimulus or insult encountered during a critical period in fetal development influences the programming of the human body and has life long consequences. Links have been shown to exist between birth weight and placental weight and in particular birth weight, the deficiency of insulin and the onset of type II diabetes. These are believed to be associated with the undernutrition of the fetus at a critical stage in early life, which may result in the persistent changes in insulin response to glucose and other metabolic and endocrine function important in this disease. Refer to Figure 2.3 for an adapted model explaining the development of macrosomic infants and the onset of diabetes using Barker's theory.

Fig 2.3 : A comparison of Pedersen's theory and Barker's theory.

a: Fetal development in more severe diabetes in pregnancy (maternal underutilisation and overproduction) using Pedersen's theory.



Key: R = replicating, NR = non-replicating, GFs = other growth factors.

b. Familial predisposition to macrosomic infants and the onset of diabetes using Barker's theory.

Thin mother

↓↑ Undernourished child/young adult

Low birthweight baby

'Insulin resistance"

↓ Adult obesity

More insulin resistance

↓

Mother

↓ Hyperglycaemia

Macrosomic baby

Reduced B-cell function

↓

NIDDM

The macrosomic effects in diabetic pregnancies have been explained by the classical 'Hyperglycaemia-Hyperinsulinism' hypothesis of Pedersen (1977) ie, diminished insulin secretion in the mother leads to maternal hyperglycaemia. The glucose crosses the placenta freely causing hyperglycaemia in the fetus, stimulating fetal insulin secretion, increased deposition of fat and glycogen and the formation of larger babies (Figure 2.3). The role of glucose is thus readily encompassed by Pedersen's hypothesis.

However, it is becoming increasingly clear that increased glucose uptake is not the only factor contributing to placental and fetal malformations in diabetic pregnancies. Other factors include, for example, increases in triglycerides, branched-chain amino acids and asparagine, proline, tyrosine associated with decreases in glutamic acid, glutamine, cysteine and lysine (Styrud *et al.,* 1995). Temporary severe protein-energy malnutrition is believed to affect glucose disposal, which in turn increases the transfer of other nutrients to the fetus, thereby stimulating fetal pancreatic B-cell growth and contributing to macrosomia (Eriksson & Swenne, 1993). Also during gestational diabetes, increases in growth factor synthesis and secretion by fetal and placental tissues has been demonstrated leading to abnormal growth and function of the placenta and thus abnormal growth of the fetus.

Similarly, concentration-dependent transfer of amino acids across the placenta could provide the fetus with more building blocks for protein anabolism, gluconeogenesis, or even direct insulinogenic stimulus. FFA may serve as alternative oxidative fuels in the mother. They could retard the utilisation of ingested glucose and prolong its availability for transplacental delivery to the fetus. They may even slow hydrolysis of esterified lipids in the maternal circulation, or within the placenta could effect a sustaining infusion of fatty acids and glycerol for direct utilisation in the fetus (Koren & Shafrir, 1964).

2.10. Insulin and maternal diabetes

Due to its many biological and metabolic effects and the abundance of insulin receptors in the human placenta, insulin is often administered in the control of placental function in diabetic pregnancies. However, it is as yet unclear which insulin source exerts control over placental function and metabolism during gestation - fetal or maternal. It is generally believed that during the first trimester maternal insulin promotes placental growth and metabolism and that this control is increasingly overtaken by insulin from fetal sources from mid first trimester onwards (Adam *et al.,* 1969). However, as the insulin receptors are largely located on the maternal surface of the placenta, it is highly likely that these receptors are stimulated by maternal insulin. Regardless, circulating insulin concentrations from either fetal or maternal sources are believed to be dependent upon maternal glycemic levels.

Alterations in insulin receptor mediated-responses have been implicated in maternal diabetic pregnancies. Extent of insulin binding to placental cell membranes obtained from 'normal', healthy mothers and to those with well-controlled GDM, treated with diet alone, or diet and insulin, was examined (Desoye *et al.,* 1992). It was found that the maximum binding of insulin to its receptor was unchanged in the total collective of the gestational diabetic group but was decreased by 30% in diet treated diabetics, and increased by 90% in insulin treated diabetics compared to control. No significant differences were observed in receptor affinity. There was only a fraction (40%) of the number of receptors per milligram of protein in diet treated GDM compared to control whilst in GDM subjects treated with insulin, the placental samples were found to have >2X the number of receptors compared to control (Desoye *et al.,* 1992).

These differences in receptor numbers and specific binding may be explained by exposure to altered environment. It has been shown in rat fibroblast cells (Haring *et al.*, 1994) that high levels of glucose impair insulin receptor (isoform B) function. The hyperglycaemia is believed to induce the inhibition of tyrosine kinase activity by mediating certain protein kinase C isoforms to form stable

complexes with the insulin receptor (Haring *et al.*, 1994). This interaction results in the activation of serine phosphorylation of the β-subunit of the receptor that, in turn, modulates its tyrosine kinase activity. This mechanism is believed to contribute to the pathogenesis of insulin resistance seen in non-insulin dependent diabetes mellitus (NIDDM). It may also contribute to the alterations observed in insulin receptor activity in maternal diabetic pregnancies that lead to macrosomic fetuses and heavier placentae. As stated above, macrosomia remains a common problem in diabetic pregnancies despite good glycemic control. Is it possible then that insulin, itself, may contribute to increased growth rate of fetal tissue and the placenta?

Insulin is known to stimulate RNA and protein synthesis in cells (Rosen, 1987). It is, therefore, possible that insulin may stimulate the synthesis and release of growth factors that may act in an autocrine manner to stimulate proliferation. In rat hepatocytes, insulin administration stimulates IGF-I production (Boni-Schnetzler *et al.,* 1991). Hence, it may be possible that in placental cells (such as trophoblasts) insulin may increase IGF-I expression and synthesis which, in turn, may result in increased proliferation of these cells. In the diabetic state, animal models have shown that the associated hyperinsulinism is usually linked to an elevated serum level of IGF-I (D'Ercole, 1987), whilst in cases of insulin deficiency, serum levels of IGF-I remain low. However, the mechanisms involved in the interaction of these two factors still needs clarification.

The abnormal metabolism (involving substrates apart from glucose) observed in gestational diabetes is believed to invoke fetal hyperinsulinemia (Eriksson & Swenne, 1993). As previously discussed in section 2.2.3., increased glucose levels may also stimulate the expression of insulin-like genes such as placentin (Koman *et al.,* 1996) and EPIL (Bellet *et al.,* 1997). Increased levels of insulin, IGF-I and IGF-II have been reported in the cord blood of infants of diabetic mothers (Froesch *et al.,* 1985). As these factors are known stimulators of cell proliferation in a variety of cell types, this may explain the macrosomic factors of fetal tissue and the placentae in diabetic pregnancies. However, it is difficult to ascertain whether these effects are primarily metabolic or not.

From the review of the literature, much information is available on the different stages of development of the maturing placenta and surrounding fetal tissues, the architecture of the developing villous tree of the placenta and the different cell types and their functions of the constituent cellular components. It is also known that the fetal tissues are in close proximity to one another and are capable of interacting in an autocrine/paracrine manner to regulate the proliferation, differentiation and function of adjacent cells/tissues. Abnormal production, release and activity may contribute to abnormalities in growth and increased mortality. However, the roles of certain factors in cellular proliferation, differentiation and function are not fully understood, nor are their underlying mechanisms or their contribution to fetal outcome in diseased states, such as gestational diabetes.

Based on this, this study plans to undertake the examination of the effects of insulin, IGF-I and IGF-II on proliferation and differentiation of cells isolated from human term placentae. This study also aims to elucidate the possible mechanisms involved in the regulation of cellular proliferation and differentiation when insulin and the IGFs bind to their respective receptors in human placenta. Comparisons will be made between the 'normal' and diabetic placentae (gestational diabetic and insulin-dependent diabetic). It is hoped that this study will provide an insight into the roles of insulin and the IGFs in perinatal growth and development.

Specific Aims

The specific aims of this study:

To investigate and compare the *in vitro* effects of insulin and the IGFs (IGF-I and IGF-II) on the proliferation and differentiation of cytotrophoblasts isolated from human term placentae.

To investigate and compare the differences in the effects of insulin and the IGFs on cytotrophoblasts isolated from term placental tissue obtained from diabetic and non-diabetic pregnancies.

To examine the presence of any specific insulin binding sites on cytotrophoblast membranes and to examine whether differences occur between binding of insulin to the cytotrophoblasts obtained from term placentae of diabetic and non-diabetic pregnancies.

To elucidate the mechanisms involved in regulation of the proliferative and/or differentiative processes when insulin and the IGFs bind to their respective receptors in human placenta.

To examine any paracrine effects involved in the proliferation and differentiation of cytotrophoblasts with cells isolated from other human term gestational tissues, i.e, amnion and chorion.

CHAPTER THREE

MATERIALS AND METHODS

The following chapter details the overall methods used for the study. Individual protocols will be outlined in each chapter.

METHODS

3.1. Ethical Considerations and Patient Consent

3.1.1. Ethics

Ethical clearance followed National Health and Medical Research Committee (NH & MRC) guidelines and was obtained from the Victoria University of Technology Human Research Ethics Committee and was approved by the Royal Women's Hospital and Mercy Hospital Research and Ethics Committees. Guidelines are available on The Australian Health Ethics Committee web page at http://www.health.gov.au/nhmrc/ethics/contents.htm. Each subject was required to sign an informed consent form at which time any risks of participation were explained. Refer to Appendix One.

3.1.2. Patient consent

As placentae were procured from two different hospital sources, each required a separate informed consent form. Briefly, consent forms described the nature of the research in simple terms (Appendix One), the requirement of the placental tissue at the time of delivery and the need for patient information regarding smoking habits and medical information relating to the pregnancy. The patients who had agreed to participate in the study were reassured that the collection procedure would not interfere with the delivery process nor cause harm to the patient or baby. Patient confidentiality was maintained at all times during the research.

3.1.3. Patients

The control group comprised a random selection of women whose placentae were collected from the maternity ward of the Werribee Mercy Hospital. These women presented with the following: normal oral glucose tolerance test results, normal course and outcome of pregnancy, no complications were studied (eg:

hypertension), no smoking, no diet restrictions and no medication other than epidural anaesthesia. This group was designated as 'normal'.

Placentae from pregnant women with gestational diabetes mellitus (GDM) treated with diet (high in protein, low in carbohydrate and fat) or insulin-dependent diabetes mellitus (IDDM) were collected from the Department of Perinatal Medicine at the Royal Women's Hospital, Melbourne. For GDM mothers the measurements of their circulating glucose levels (i.e., HbA1C results obtained from hospital records) were within normal range (4.7-6.4%). Placentae from IDDM pregnant women presenting no complications apart from hypertension were studied. Treatment aimed to achieve fasting serum glucose levels <6.4 mmol/L and there was no glucose administration during delivery. Table 3.1 provides a summary of the subject information.

Table 3.1. Characteristics of 'normal' (n=30), GDM (n=12) and IDDM (n=10) mothers and their offspring.

	Subjects		
Parameters	'Normal'	GDM	IDDM
n	30	12	10
Length of gestation (weeks)	39.4 ± 0.6	38.2 ± 0.6	37.0 ± 1.1[a]
Parity	1.9 ± 0.7	2.3 ± 1.0	1.6 ± 0.5
Birth weight (g)	3350.6 ± 515.5	3204.6 ± 435.9	4174.3 ± 259.1[b]
Placental weight (g)	549.2 ± 139.6	642.8 ± 97.5	911.4 ± 49.6[b]
Fetal/placental weight ratio	6.1 ± 0.9	5.0 ± 0.7	4.6 ± 0.3[a]
Fasting blood glucose (M)	4.9 ± 0.7	5.6 ± 0.5	7.7 ± 1.8[c]
HbA_{1C} (%)	5.1 ± 0.7	6.0 ± 0.6	7.4 ± 0.8[a]

Fetal, placental and maternal blood glucose details of patients that have consented for this study. ([a] $p<0.001$ compared to 'normal' data only; [b] $p<0.01$ compared to 'normal' and GDM data; [c] $p<0.05$ compared to 'normal' and GDM data).

3.2. Cell isolation, purification and culture

3.2.1. Isolation of placental cells

Prior to experimentation, the midwives or the appropriate research nurses of the respective hospitals were contacted and a request was made for placental tissue from pregnant women. The day of experimentation was dictated by the time of arrival of placentae. Patient consent for placental tissue was confirmed at the time of collection of the placenta from the appropriate ward or research unit of the respective hospitals and the experimentation for cell isolation was initiated immediately.

Placentae (34-41 weeks) from 'normal', GDM (diet control) and IDDM pregnant women were obtained, with consent, at Caesarean section immediately after delivery. At the hospital, approximately 200-250 grams of tissue was dissected from various parts of the one placenta placed directly into 50mL Hanks Balanced Salt Solution (HBSS, pH 7.4, Sigma) supplemented with 1mg/L gentamycin sulphate (Sigma) and agitated thoroughly to wash the tissue sample. This was then transported (no more than 20 minutes) on ice to the cell culture laboratory. Under aseptic conditions, using a Cytotoxic 1200 cabinet (Email Westinghouse P/L), the tissue sample was again washed in HBSS so as to remove fungal and blood contamination. Washing was then continued in saline solution. The cells were dispersed as described by Jones and colleagues (1989), with minor modifications. With sterile dissecting instruments, villous tissue was dissected free of blood and membrane components. The resulting placental tissue fragments were added to 50mL Joklik's Modified Essentials Medium (JMEM, pH 7.4, ICN Biomedicals Inc), supplemented with 2g/L sodium bicarbonate, 2.5g/L dextrose, 0.2g/L EDTA, 2.6g/L HEPES, 5mg/L transferrin (Boehringer Mannheim) and 1.56μg/L selenous acid. These tissue fragments were enzymatically dispersed with 5g/L trypsin and 0.05g/L DNAse I (Grade II, Boehringer Mannheim), heated to 37^0C in a water-filled beaker and mechanically dispersed with rapid stirring for 60 minutes. During tissue disaggregation, unwanted cell clumping occurred due to the release of large DNA fragments from lysed cells, making dispersion of single cells difficult (Kaighn, 1987; Prop &

Wiepjes, 1987). DNAse I, used here in noncytotoxic concentrations (<1mg/mL) was essential to prevent this unwanted clumping of cells (Fischer, 1982). The resulting cell suspension was filtered through a 0.16mm nylon filter, loosely fitted onto the wide end of a funnel placed into a 50mL plastic, centrifuge tube (Greiner). To inhibit further enzyme digestion of the cells 10% (v/v) fetal calf serum (FCS, Sigma) was added to the filtered solution. This suspension was then centrifuged at 3000x g in a benchtop centrifuge (Hettich Universal) for 15 minutes at room temperature. The cells were resuspended with Eagle's Minimum Essential Medium with Earles salts (EMEM, pH 7.4, ICN Biomedicals) supplemented with 2g/L sodium bicarbonate, 5mg/L transferrin and 1.56μg/L selenous acid, and the centrifugation process repeated twice.

3.2.2. Separation of placental cells using Density Gradients

The dissociated cells obtained from the isolation procedure were purified using a modified version of the Percoll gradient method, which isolates cells based upon cell density described by Kliman and colleagues (1986). The discontinuous Percoll (Sigma) layers of 60%, 50%, 40%, 30%, 20% and 10% Percoll were prepared in JMEM on the day of the experiment. 7mL of each Percoll solution was layered carefully in a descending order of concentration in 50mL plastic centrifuge tubes held on ice. The cell pellets obtained in section 3.2.1 were resuspended in 7mL EMEM and carefully layered over the discontinuous Percoll gradient. The tubes were centrifuged at 1000x g in a refrigerated centrifuge (Beckman GS-15R) at 4^0C for 20 minutes. The cell populations that settled between the 60% and 40% Percoll layers (1.048-1.062g/mL density) and were removed carefully using a Pasteur pipette and placed into another 50mL tube. Ten volumes of EMEM were then added and the mixture centrifuged at 3000x g in a benchtop centrifuge for 10 minutes at room temperature. The resultant cell pellet was resuspended in 10 volumes of EMEM. This washing procedure was repeated twice. The cells were left to stand, on ice, in 50mL tubes containing EMEM supplemented with 10% (v/v) FCS for 30-60 minutes prior to seeding. During this time, the number of cells isolated was determined and the viability assessed using Trypan Blue (0.4%) exclusion (section 3.5).

3.2.3. Selective enrichment of cells using HLA antibody

To obtain a cell population that comprised mainly trophoblast cells with little contamination of any other cellular type of the placenta (eg., fibroblasts, stromal cells, circulating cells and endothelial cells) a negative selection procedure, as described by Schmon and colleagues (1991), was adopted with some modification. The antibody used (anti-human HLA-ABC common antigen Mab PHM-4, ICN Biomedicals Inc) is a monoclonal antibody directed against HLA-A, -B, and -C monomorphic antigens (Parham *et al*, 1979) which reacts to most cell types including stromal cells, endothelium and extravillous trophoblast cells. This antibody does not react with villous cytotrophoblasts and syncytiotrophoblasts, where HLA class-I antigens are absent (Butterworth & Loke, 1985).

Plastic petri dishes (35mm diameter) were sterilised by an ethanol wash, coated with 1.5mL of diluted antibody (1:50 in PBS) and allowed to stand for 60 minutes at 37^0C. The supernatant liquid was aspirated and the inner surface of the petri dishes was washed several times with sterile PBS solution. EMEM medium containing 5% FCS was added to the petri dishes, which were incubated at 37^0C for a further 60 minutes. This wash reduced the nonspecific binding of cells to the surface of the petri dish (Schmon *et al*, 1991). The dishes were then washed three times with PBS and once with EMEM.

Approximately 2mL of the isolated placental cells (0.5-1.0 x 10^6 cells/mL) were transferred to the precoated petri dishes and allowed to incubate at 37^0C for 15 minutes. The non-adherent cells, which were HLA class-I negative and therefore assumed to be trophoblast cells, were recovered by gently agitating the dishes and carefully aspirating the cells using a Pasteur pipette. The purification procedure was repeated and the cells harvested and used for cell culture experimentation. The adherent cells were discarded.

3.2.4. Isolation of amnionic cells and cells of the chorion laeve

When collecting the placental tissue at Caesarean section, the attached amnion and chorion were also collected. These adherent membranous tissues were

dissected free of the placenta and placed directly into HBSS supplemented with 1mg/L gentamycin sulphate. This was quickly transported on ice to the laboratory where the tissue was processed under aseptic conditions.

Primary cultures of human chorionic cells and cells of the amnion were obtained as described by Gibb and colleagues (1986) with minor modifications. Any attached decidua was scraped carefully from the chorion laeve and discarded and the chorion and amnion sheets were separated. The tissue (amnion or chorion) was finely minced with scissors and then placed into a schott bottle (with a magnetic stirring bar) containing 50mL Ham's F12-Dulbecco's Modified Essential Medium (1:1 v/v, pH 7.4, Sigma) supplemented with 1g/L BSA and 5.4g/L HEPES. The tissue was enzymatically digested with 1.25g/L collagenase (Boehringer Mannheim) with the schott bottle submerged in a water-filled beaker heated to 37^0C. Cells were mechanically dispersed for 2-3 hours with rapid stirring. The mixture was then filtered through two layers of 0.16mm nylon mesh, as described for trophoblast cells (section 3.2.1). The enzyme digestion was stopped by the addition of 10% (v/v) FCS to the filtered suspension. The cell suspension was centrifuged at 1000x g for 15 minutes at room temperature. The cell pellets were resuspended in Ham's F12-DMEM (1:1) medium and recentrifuged. The resultant cell pellets were resuspended in 10mL EMEM alone and carefully layered onto a Percoll gradient comprising 10mL each of 60%, 40%, 20% and 5% Percoll prepared in EMEM. These gradients were prepared in a 50mL plastic centrifuge tube on ice, just prior to use. The Percoll gradient with the cell layer was centrifuged at 800x g for 20 minutes in a refrigerated benchtop centrifuge set at 4^0C. Cells of the chorion sedimented at the 20% and 40% interface of the Percoll gradient. These were removed carefully into another 50mL tube using a Pasteur pipette. To the harvested cells, 10 volumes of EMEM were added and the cell suspension was centrifuged at 3000x g for 10 minutes at room temperature. The cell pellet was washed again with 10 volumes of EMEM alone and the centrifugation procedure repeated twice. The cells were left to stand, on ice, in 50mL tubes containing EMEM supplemented with 10% (v/v) FCS for between 30-60 minutes prior to seeding. During this time, the number of cells isolated was determined and the viability assessed using Trypan Blue exclusion.

3.2.5. Cell culture

Trypan Blue exclusion was used to determine the number of cells isolated and cell viability (section 3.3). The cell fraction obtained, comprising a relatively pure population of mononuclear cells, was appropriately diluted and the cells were seeded at an initial (t=0) density of 0.5 x 10^6 cells/mL/well in 24 well, flat-bottom culture plates (Linbro Flow Lab). These plates were previously coated with collagen (section 3.2.7) and fitted with loose lids. The cell cultures were grown in media supplemented with 10%FCS (control) and treated with 0.1nM insulin (bovine; Boehringer Mannheim Biochemica), 1.0nM IGF-I (24-41; Auspep), 10.0nM IGF-II (33-40; Auspep) or any combination of two of these factors. The cultures were then incubated for 96 hours in a CO_2 water-jacketed incubator (Nuaire TS Autoflow) set at 37^{0}C and supplying 5% CO_2 in air. Nutrients were provided by the addition of 1mL EMEM supplemented with 10% (v/v) FCS which was replaced every 48 hours. For immunocytochemical staining, cells were cultured in petri dishes containing sterile glass slides (section 3.5).

All cultures contained 0.5μCi/ml ^{3}H-thymidine (Amersham, UK) which was added to the cell suspension mixture immediately prior to seeding. Observations and repeated measures were performed in quadruplicate and samples taken every 2h for 24h and then at 48, 72 and 96h after seeding. At the appointed times, cultures were counted (section 3.3), assayed for thymidine uptake (section 3.4) and the glass slides removed for immunocytochemical staining (section 3.5.2). The media supernatant was collected and centrifuged (and cell pellets removed) for either hCG analysis (section 3.6) using direct chemiluminescence techniques (performed in duplicate) or stored at -20^{0}C for further protein analysis (section 3.7). The cells were harvested and stored at -80^{0}C until further analysis was required.

3.2.6. Co-culture of cells

For co-culture experiments cell isolation and purification procedures adopted were as described above. However, during the culture, combinations of cell types (i.e.: cells of the amnion, chorion and/or placenta) were grown together within the

same well of the culture plate using translucent cell culture inserts (Falcon, Becton Dickinson Labware) of high pore density with 0.45μm diameter pores. The insert membranes were highly permeable allowing for adequate rates of basolateral diffusion of nutrients and molecules involved in cellular processes such as transport, secretion and ligand binding. In this system, each cell types studied was seeded at a density of 0.5 x 10^6 cells/mL/well in EMEM supplemented with 10% (v/v) FCS in both control and growth factor treated groups. One cell type was seeded directly into the well forming a layer of cells that coated the bottom surface of the well. The other cell type was seeded into the cell culture insert that was securely positioned directly above the first layer of cells. Refer to Appendix Two for the set up of the cell culture insert system.

3.2.7. Collagen coating of culture plates

Tissue culture plates were coated with collagen and dried prior to cell seeding. Here, 1mL of collagen solution (refer to Materials section) was added to each well and incubated for approximately 30 seconds after which the solution was removed quickly. The plates, with lids left slightly ajar, were allowed to dry at room temperature within a cytotoxic cabinet until a thin translucent film could be detected on the inner surface of each well.

3.2.8. Sterility of apparatus

Glassware and dissecting apparatus required for the isolation and purification procedures described here were sterilised by autoclave the day before the experiment whilst any equipment used was washed with 70% alcohol on the day of the experiment just prior to use. Plasticware such as the culture plates, cell culture inserts, centrifuge tubes and plastic petri dishes were obtained presterilised by gamma irradiation and were used only once.

3.3. Cell counts and viability

Viability was determined using Trypan blue exclusion (0.4% (w/v) Trypan Blue solution, Sigma). Cells were lifted from the culture plates using 100μL trypsin

treatment which was stopped after 30 seconds with 10% (v/v) FCS. A cell suspension was prepared in HBSS. Trypan Blue solution (0.5mL) was transferred to a vial, to which 0.3mL HBSS and 0.2mL of the cell suspension (where cells were vigorously dispersed using a Pasteur pipette) was added. This was then mixed thoroughly and allowed to stand for 5 minutes. The cells were counted using the grid of a cytometer and light microscope (Olympus) at 40X magnification. Cell counts were performed in quadruplicate and the results expressed as mean $\pm$ SEM x 10^6 cells/mL. Aggregates were defined as a cluster of individual cells ($\geq$3 cells) not yet fused. Where the individual cells were no longer distinct within an aggregate formation, this was counted as one multinucleated cell.

All cells in the 1mm centre square and four 1mm corner squares of the cytometer as well as the top and left touching middle line of the perimeter of each square were counted. Each square of the cytometer, with coverslip in place, represented a total volume of 0.1mm^3 or 10^{-4}cm^3. Refer to Appendix Three. Since 1cm^3 is equivalent to approximately 1mL the subsequent cell concentrations were calculated as follows:

1. Cell viability (%) = total viable cells/total cells x 100;
2. Cells/mL = ave. cell count/square x dilution factor x 10^4;
3. Total cells = cells/mL x mL of fluid from which cell sample was removed;
4. % Aggregates = # aggregates (>3 cells)/total cells x 100.

Cell size was measured using a calibrated slide, which contained a ruler marking every 10μm for 100μm.

3.4. Thymidine incorporation analysis

Thymidine uptake was used to assess proliferation in cultured cells and to confirm cell count data. Thymidine uptake was assessed following a method described by Staples and colleagues (1981), with modification. Here, cells were harvested onto glass fibre sheets (Whatman) by suction using a semi-automated device (Titretek, Flow) which could harvest from 12 wells on the culture plate simultaneously. This device collected the cells into small compact circles that allowed the glass

fibre sheet to be cut into discs. The sheets were washed 10-12 times with fresh media and then allowed to dry in a radiation cabinet. When dry, the discs were cut from the sheet using fine forceps and transferred to vials containing scintillation fluid (Emulsifier-safe, Packard). Samples were counted using a liquid scintillation counter (Wallac 1410, Pharmacia). Thymidine uptake measurements were expressed as the mean (of quadruplicate replicates) $\pm$ SEM counts per minute (cpm).

3.5. Immunocytochemical staining

Immunocytochemical staining was performed on cells cultured on glass slides in petri dishes. All antibody incubations and washings were performed in a laminar flow hood (HWS Series, Gelman Sciences).

3.5.1. Fixing of cells

At the appointed times, the glass slides were removed from culture and washed three times in ice cold PBS buffer prior to fixing. The cells were fixed by immersing the slides in 1:1 acetone: methanol for at least 10 minutes in petri dishes that were kept on ice, and then dipping them several times in ice cold 1:1 ethanol: acetone solution. At this stage, the fixed slides could be stored at -20^{0}C.

3.5.2. Characterisation of cells using cytokeratin7, vimentin and desmoplakins I and II

In this study differentiation was defined as the fusing of single unicellular trophoblasts to form giant multinuclear syncytia. This process involved the aggregation of the cells prior to fusion. Before staining, the fixed slides were removed from -20^{0}C and washed 10-15 times, for 5 minutes each wash, with phosphate-buffered saline solution (PBS; pH 7.4). The cells were incubated with the first monoclonal antibody (Mouse anti-cytokeratin peptide 7, Sigma Immunochemicals; mouse antidesmoplakin I and II; ICN Biomedicals, Inc) for 30 minutes at room temperature. Cells were then washed several times with excess PBS. The second antibody (FITC-labelled goat anti-mouse IgG, Fab specific;

Sigma Immunochemicals) was then added and the slides incubated for 30 minutes at room temperature followed by washing with PBS. The slides were mounted using PBS containing 10% glycerol. Slides were examined using an Olympus microscope equipped with epifluorescence optics. Coverslips of cells were also stained with antihuman-vimentin (LN-6; ICN Biomedicals, Inc) for 30 minutes at room temperature and examined.

3.5.3. Confirmation of DNA replication with PCNA staining

PCNA (Proliferating Cell Nuclear Antigen) is a 36kD nuclear protein essential for cellular DNA synthesis (Maeda *et al.,* 1994). Staining for this protein was used to confirm proliferative activity in trophoblast cells *in vitro*. Techniques adopted for staining were identical to those outlined above in section 3.5.2. The first monoclonal antibody used was mouse anti-PCNA (1:200, Zymed Laboratories, Inc).

3.5.4. Apoptosis: the TUNEL assay

Adherent cells were stained for apoptosis using the In Situ Cell Death Kit (Boehringer Mannheim). Cells were fixed as described in section 3.5.1. Slides were rinsed twice with PBS. The TUNEL reaction mixture (50μL) was then added and covered with a coverslip so as to allow uniform spread of the reaction mixture and to minimise evaporative loss. The samples were then incubated in a humidified incubator for 60 minutes at 37^0C in the dark. Slides were then washed 3X with PBS and then analysed under a fluorescent microscope.

3.6. hCG analysis

The quantitative determination of hCG in media was performed using the Chiron Diagnostics ASC:180® Automated Chemiluminescence Systems (Chiron Diagnostics, USA). This assay detects the intact hCG molecule and free β subunits of the hCG molecule by a two-site sandwich immunoassay with the minimal detectable level of 2.0 mIU/mL.

3.7. Protein analysis of media samples

3.7.1. Lowry Assay

Protein concentrations in media harvested from control or growth factor treated cultures, at varying time intervals, were determined by a modification of Lowry method (Petersen, 1977). This method is effective in estimating protein concentrations in the range of 0-100μg of protein. Harvested medium was first diluted 1:2 with distilled water and 10μL of this diluted medium was processed for the experiment. The diluted media were made to 0.5mL with distilled water and 0.5mL of solution A [0.1mL 5% (w/v) copper sulphate, 0.9mL 1% (w/v) potassium tartrate, 10mL 10% (w/v) sodium carbonate in 0.5M sodium hydroxide] were added to it. The mixture was vortexed thoroughly, incubated at 37^0C for 10 minutes, 1.5mL of solution B [1mL Folin-Ciocalteu's reagent, 10mL double distilled water] was added and the mixture immediately mixed by vortex. This changed the colourless sample to yellow. This mixture was then incubated at 52^0C for 20 minutes. The samples proceeded to change in color from yellow to blue. If the blue color was too intense (almost black) the samples had greater than 0-100μg of protein and were discarded in these cases. The media were then rediluted (to a smaller protein concentration range) and reprocessed. Absorbance was recorded using an Ultrospec III spectrophotometer (Pharmacia Biotech), at 680nm against a reagent blank. Standards containing 0-100μg of protein were prepared from a 0.1% BSA solution. Solutions A and B were prepared immediately prior to use. Protein concentration in medium was also determined by reading absorbance of untreated, diluted samples at 215nm (indicative of the amount of peptide bonds) and at 280nm (relative index of the amount of tryptophan and tyrosine residues) and the values compared to that obtained from Lowry assay.

3.7.2. SDS PAGE

3.7.2a: Sample and acrylamide gel preparation:

A volume of protein sample (media from cultured cells) corresponding to 0.2mg/mL protein was diluted in loading buffer to give a final concentration of 0.1mg/mL. The samples were placed in a boiling water bath for 5 minutes and allowed to cool to room temperature. The samples were then centrifuged in a high-speed bench centrifuge for 2 minutes prior to loading on to a 12% resolving gel. See table 3.2 for gel components. LMW marker (Amersham Pharmacia Biotech) was used.

The gels used for separation of proteins were electrophoresed at a constant current of 20 milliamps on mini Protean Gel II set up until the bromophenol blue dye just ran off the gel (approximately 1 hour).

Table 3.2: SDS-PAGE composition

	Resolving gel (15mL)	Stacking gel (5mL)
% acrylamide	12.0%	5%
30% acrylamide stock solution (mL)	3.2	0.8
Distilled water (mL)	3.09	3.6
1.875 M Tris-HCL (pH 8.8) (mL)	1.6	
1.25 M Tris-HCL (pH 6.8) (mL)		0.5
Degas the solution, then add		
10% SDS (μL)	80	50
TEMED (μL)	5	22
10% ammonium persulfate (μL)	27	9
Mix carefully and cast the gel immediately		

Composition of a standard 1.5mm thick gel comprised of a stacking gel and a 12% resolving gel.

3.7.2b: Protein detection:

For detection of protein bands, gels were silver stained according to Heukeshoven and Dernick (1988) with some modifications. All steps were performed with gentle shaking of the gel.

After electrophoresis, the gel was fixed by immersing immediately into fixing solution [80mL ethanol, 20mL acetic acid, 100mL distilled water] for 30 minutes. The fixing solution precipitated the proteins and allowed the SDS to diffuse out of the gels. The gel was then transferred to incubation solution [60mL ethanol, 8.2g sodium acetate x $3H_2O$, 1.04mL gluteraldehyde, 0.4g sodiumthiosulfate, 139mL distilled water] for 30 minutes. The gel could be left overnight in this solution, at this stage. The gel was then washed three times in distilled water at 5 minutes each. The gel was then placed into the silver solution [0.2g silver nitrate, 40μL formaldehyde, 160mL distilled water] for 40 minutes, after which it was then developed in developing solution [5g sodium carbonate, 20μL formaldehyde, 180mL distilled water] where very intensely stained dark bands appeared. To terminate this reaction the gel was placed in stop solution [2.92g EDTA-Na_2 x $2H_2O$ in 200mL distilled water] for 5-10 minutes, followed by a wash and destaining in distilled water with 1 change for 5-10 minutes. The silver stained gel was then preserved by placing it in preserving solution [20mL glycerol in 180mL distilled water] for 20 minutes then wrapping in cellophane and allowing to dry at room temperature.

3.8. Receptor binding analysis

3.8.1. Preparation of the PD10 column for iodination

Immediately preceding the iodination of the ligand, a sterile PD10 Sephadex G-25 column (Pharmacia) was prepared. Here, the lid and tip ends of the column were removed and the preserving solution discarded. The column was washed with 20mL PBS, followed by 20mL PBS/0.5% BSA and then 10mL of PBS, which were allowed to flow through the column to waste.

3.8.2. Iodination of ligand

Insulin was iodinated using IODO-GEN® Iodination Reagent (1,3,4,6-tetrachloro-3α-6α-diphenylglycouril, Pierce, Perstorp Biotec Company) as per the manufacturer's instructions. IODO-GEN® radioiodinates the exposed tyrosine residues on the protein. The procedure is similar to that used for Chloramine T but results in less damage to the protein and loss of activity. Briefly, IODO-GEN® Iodination Reagent was dissolved in chloroform to give a final concentration of 0.1μg/μL reagent. 50μL aliquots of this solution were transferred into microfuge tubes and then slowly evaporated off using a gentle stream of dry nitrogen gas, sealed and stored at 4^0C, desiccated, until required. The tube was rinsed with PBS before 20μg of ligand (0.1M PBS, pH 7.2) and 100-200μCi $Na^{125}I$ (Amersham) were added to the tube and incubated at room temperature for 7 minutes, with intermittent agitation. The iodinating mixture was then transferred to a pre-prepared PD10 Sephadex G-25 column that separated the radiolabelled protein from free $Na^{125}I$. The iodinated ligand was extracted from the column with several washes of 500μL of PBS/0.5%BSA solution. Each 500μL aliquot was collected. From each aliquot 5μL of sample was counted using an automatic Gamma counter (Wallac 1470 WizardTM, Pharmacia). The results were graphed. The samples with the highest counts were pooled and used for receptor binding analysis. The other samples were appropriately discarded as it was assumed that these samples contained degraded protein.

3.8.3. Competitive Binding Assay (Insulin)

For this assay, non-cooperative models were assumed comprising either one class of binding sites or two classes of independent, non-interacting binding sites. These models described the dependent, observed variable (labelled insulin counts bound) as a function of the independent variable (unlabelled insulin concentrations) and were analytically defined by non-linear regression analyses.

The method adopted was based on that described by Desoye and colleagues (1992) with some modifications. Cytotrophoblasts (~10^7 cells/mL) were

incubated with iodinated insulin (~70,000cpm) in 96 well, round-bottom plates (Falcon) in EMEM at 4^0C for 16-18 hours in the presence of increasing concentrations of unlabelled insulin, ranging from 6 x 10^{-13}M to 3 x 10^{-8}M. A minimal nutrient medium (EMEM) was used to maintain the structure and function of the cells during the assay. This was used without additives or supplements so as to reduce the number of possible factors that may block or interact with binding of the ligand to the receptor. The total assay volume was 0.15mL. Cells were then removed from incubation, gently resuspended and layered onto a 0.2mL mixture of phthalate oils (60% dibutyl phthalate: 40% bis(2-ethylhexyl) phthalate; FLUKA) in 0.4mL microtubes (Greiner) and centrifuged. The pellets, comprising bound insulin, were cut free from the supernatant, containing unbound insulin, and then transferred to assay tubes to be counted using an automated Gamma counter (WALLAC 1470 WizardTM). Measurements were performed in duplicate.

3.8.4. Saturation Binding Assay (blocking with IGF-I, IGF-II or both)

Freshly isolated cytotrophoblasts (~10^7 cells/mL) were blocked for a minimum of 2 hours with 10mg insulin, 200μg IGF-I, 200μg IGF-II or 200μg IGF-I and IGF-II in EMEM prior to incubation at 4^0C for 16-18 hours in the presence of increasing concentrations of iodinated insulin (~70,000cpm), ranging from 20ng/mL to 40μg/mL. The cells were then assayed as described above in section 3.8.3.

3.8.5. Calculations

3.8.5.a. Receptor Ligand Interactions:

The following equation represents the interaction between ligand and receptor:

$R + L \Leftrightarrow RL$ where: R = receptor and L = ligand

3.8.5.b. Half Life and Rate Constants:

The half life of the above interaction is defined by simple first order decay process as given below:

$\mathbf{N = NOe^{-kt}}$

Where NO = number of complexes at time 0

N = number of complexes remaining at time t

k = rate constant and is dependent upon temperature being constant.

= if k is large the reaction is fast, and if k is low the reaction is slow.

The association rate constant is k^{+1}(forwards; $M^{-1}sec^{-1}$)

The dissociation rate is k^{-1}(reverse; sec^{-1})

Therefore,

$k^{+1}[R][L] = k^{-1}[RL]$ or $k^{+1}/k^{-1} = [R][L]/[RL]$

where [R] = concentration of receptor

[L] = concentration of ligand

3.8.5.c. Equilibrium and Affinity Constants:

The equilibrium constant (K) is defined as equal to the rate constants

Therefore: equilibrium association constant (K_A)

$$K_A = k^{+1}/k^{-1} = [R][L]/[RL]$$

And equilibrium dissociation constant (K_D)

$$K_D = k^{-1}/k^{+1} = [RL]/[R][L]$$

The following calculations were made using data collected from the receptor binding assays:

1. Specific activity of the iodination of the ligand:

   μg ligand x 10^6/molecular wt = moles iodinated ligand

   Moles iodinated/total counts of bound fractions = moles/cpm = specific activity.

2. Assay counts:

   Counts bound x 10^4 x specific activity = moles/L

   This formula was used to convert cpm counts to moles/L.

3.9. Statistical and data analyses

All data is expressed as the mean $\pm$ SEM. A MANOVA modified for repeated measures was performed on the data collected from cell counts and thymidine uptake analyses using the SPSS statistical package. The level of significance was at $p<0.01$. General linear model *post hoc* multiple comparisons of the observed means was performed using a series of multivariate tests including the Scheffe, Dunnett's T_3, Dunnett's C and the Tamhane's T_2 test. A Student T test was adopted to analyse hCG data between mean values at zero time and at the end of the culture period.

Insulin binding data was analysed by non-linear regression functions, which described the dependent, observed variable (bound insulin, cpm) as a function of the independent variable (concentrations of unlabelled insulin). This was achieved using a curve fitting program "Curve Expert" which determines the 'line of best fit' by minimising the squares of the residuals for the data using the following formulae:

$y = (a*x)/(b+x)$; for single site binding , or

$y = ((a*x)/(b+x))+((c*x)/(d+x))$ for two-site binding

where y = insulin bound, and x = unlabelled insulin.

$a=B_{max1}$, $b=K_{D1}$, $c=B_{max2}$ and $d=K_{D2}$

where B_{max} = maximum binding = indicates the number of specific receptors bound by ligand.

K_D = dissociation constant = indicates the affinity of the receptor.

The equilibrium binding dissociation constant (K_D) was obtained from this data, and an average K_D from three experiments with a correlation coefficient of >0.9 was obtained. Non-cooperative models were adopted which assumed either one class of binding sites or two classes of independent, non-interacting binding sites. Non specific binding was calculated in all receptor-binding experiments from saturation binding experiments blocked with an excess of insulin (~100-fold) and subtracted from the obtained data. The concentration of the insulin-receptor

complex was calculated from the determined values of receptor concentrations and affinities. Receptor occupancy was expressed as the fraction of receptors occupied by insulin and calculated from the total number of receptors and the concentrations of insulin-receptor complexes.

3.10. Simple Flow diagram of overall experiment:

Human Term Placental Tissue (Consent and Collection)
[34-41 weeks, n=36 -- 'normal'; diabetic: GDM and IDDM]

↓

Mechanical and Enzymatic Digestion

↓

Filtration and Centrifugation
(Jones *et al*, 1989)

↓

Discontinuous Percoll Gradient
[5%, 10%, 20%, 40%, 60%, 80%]
(Kliman *et al*, 1986)

↓

HLA Purification
(Schmon *et al*, 1991)

↓

Incubation at 37^0C with 5% CO_2 in air

Plated in 24-well culture dishes flasks
EMEM and 10% FCS with:

1. 0.1nMInsulin;
2. 1.0nM IGF-I;
3. 10.0nM IGF-II;
4. 0.1nM Insulin and 1.0nM IGF-I;
5. 0.1nM Insulin and 10.0nM IGF-II;
6. 1.0nM IGF-I and 10.0nM IGF-II.

[Cells seeded at 0.5 x 10^6 cells/mL]

maintained in

Plated in 75mL culture flasks
Suspension culture of cells

JMEM alone

Receptor Binding Assays

Protocol

Time (h)	0	12	24	48	72	96
Cell number	**********			*	*	*
Thymidine uptake	**********			*	*	*
Cytochemical stains	*	*	*	*	*	*
hCG analysis	*	*	*	*	*	*
Protein Assay	*	*	*	*	*	*
RBA analysis	*		*			

SOLUTIONS AND MEDIA

All reagents used in the making up of solutions and media are of analytical grade.

Collagen solution

Collagen solution was aseptically prepared as described by Ehrmann and Gey (1956). Rats' tails were freshly obtained and immediately stored in the freezer. Prior to collagen extraction, the frozen tails were soaked for 15 minutes in 95% ethanol in 50mL tubes. The tails were then fractured into small pieces by means of two gross forceps. Each fractured piece was pulled free from the remainder of the tail exposing long, silvery tendons that were cut with scissors and placed into a petri dish containing sterile water. Using fine forceps, the finer filaments were teased out of each tendon. This was then transferred, en masse, to a sterile 250mL centrifuge bottle containing 150mL of 1:1000 acetic acid solution. The bottle was sealed, shaken thoroughly and stored for 48 hours at 4^0C. This allowed the tendon strands to swell forming a translucent jelly-like mass. This was then centrifuged at 2,300 rpm in a Beckman J2-HS centrifuge for 2 hours. The first supernatant (~40mL of viscous fluid) was collected and stored in 500mL medicine flat bottles at 4^0C. Another 30mL of 1:1000 acetic acid solution were then added to the remaining residue, shaken vigorously and the mixture stored at 4^0C for 24 hours. The centrifugation process was repeated and the second supernatant collected and added to the previously collected supernatant. This was the collagen solution. The remaining residue in the 250mL centrifuge bottle was then shaken (without any further addition of acetic acid) and stored for another 24 hours where the centrifugation process was repeated. The final supernatant was removed and the remaining residue discarded. The supernatant was then added and mixed with the previously harvested collagen solution. The resulting cloudy solution was clarified by ultracentrifugation at 25,000rpm for 30 minutes. The total volume of supernatant obtained from one rat tail was ~140mL with a collagen yield of ~0.20g/100mL. The solution was stored at 4^0C without deterioration for 7-10 months.

Cell Culture Media

The following cell culture media were prepared. Media was supplied in powdered form, the components of which are listed in Table 3.3. All media solutions were prepared without insulin or the IGFs. The fetal calf serum used was stripped of any cytokines and growth factors.

Hanks Balanced Saline Solution (HBSS; Sigma)

This was supplemented with 0.35g sodium bicarbonate and 1mg gentamycin sulphate (Sigma). The pH was adjusted to 7.2 with 1M HCl. The solution was then filter sterilised through 0.22μm filters (Micro Filtration Systems) using an autoclave sterilised Millipore filtration unit (500mL) in a laminar flow hood. This solution was stored in a sterile 1L schott bottle at 4^0C for a maximum of 3 months.

Eagle's Minimum Essentials Medium (EMEM; ICN Biomedicals)

This was supplemented with 2g sodium bicarbonate, 5mg transferrin (Boehringer Mannheim) and 1.563μg selenous acid. The pH was adjusted to 7.2 with 10M HCl. The solution was then filter sterilised through 0.22μm filters, using a Millipore filtration unit inside a laminar flow hood. This solution was stored in sterile bottles at 4^0C for a maximum of 3 months.

Joklik's Modified Minimum Essential Medium (JMEM; ICN Biomedicals)

This was supplemented with 2g sodium bicarbonate, 2.5g dextrose, 0.2g EDTA, 2.6g HEPES, 5mg transferrin and 1.563μg selenous. The pH was adjusted to 7.4 with 10M HCl and the solution was then filter sterilised through 0.22μm filters using a Millipore filtration unit inside a laminar flow hood. This solution was stored in a sterile bottle at 4^0C for a maximum of 3 months.

Dulbecco's Modified Minimum Essential Medium (DMEM; Sigma)

This was supplemented with 1g BSA, 22.5mM HEPES, 16mg gentamycin sulphate, 10^5U Penicillin G sodium salt, 5mg transferrin, 0.2mg α-tocopherol acetate, 584mg L-glutamine and 3.5g dextrose (D-glucose) was added. The pH was adjusted to 7.2 with 10M HCl. The solution was then filter sterilised through

0.22μm filters using a Millipore filtration unit in a laminar flow hood. This solution was stored in a sterile bottle at 4^0C for a maximum of 3 months.

Ham's F12 (Flow Laboratories)

This was supplemented with 1g BSA (ICN), 22.5mM HEPES (Flow Laboratories), 16mg gentamycin sulphate, 10^5U Penicillin G sodium salt (Glaxo Labs), 5mg transferrin and 0.2mg α-tocopherol acetate (vitamin E, Sigma Chemical Co). The pH was adjusted to 7.2 with 10M HCl and the resulting solution was filter sterilised through 0.22μm filters using a Millipore filtration unit in a laminar flow hood. This solution was stored in a sterile 1L schott bottle at 4^0C for a maximum of 3 months.

Phosphate-buffered saline solution (PBS)

Components	**Concentration (g/L)**
NaCl	8.00
KCl	0.20
Na_2HPO_4	1.44
KH_2PO_4	0.24

The pH was adjusted to 7.4 with 1M HCl then sterilised by autoclaving and stored at room temperature for 6-8 months.

Trypsin for lifting cultured cells

Components	**Concentration (g/L)**
NaCl	6.80
KCl	0.40
glucose	1.00
EDTA	0.20
$NaHCO_3$	2.20
trypsin	1.50

This solution was prepared aseptically, mixed using a magnetic stirrer and 10mL aliquots stored in sterile microfuge tubes at -20^0C.

Trypan Blue stain

Trypan Blue (Sigma) is a stain used in dye exclusion procedures for viable cell counting and facilitates observations of cell morphology. The observation, on which this method is based, is that viable (live) cells do not take up the dye whilst non-viable (dead) cells do stain. The Trypan Blue stain used was a 0.4% (w/v) in isotonic saline solution.

Table 3.3. Components of media.

Components	Types of Media and their Component Concentrations (mg/L)				
	HBSS	EMEM	JMEM	DMEM	Ham's F12
Amino Acids					
L-Alanine					8.90
L-Arginine		126.40	105.00	84.00	211.00
L-Asparagine					15.01
L-Aspartic Acid					13.30
L-Cysteine.HCl.H_2O		28.42	29.60	56.78	35.12
L-Glutamic Acid					14.70
L-Glutamine		292.30	294.00	584.00	146.00
Glycine				30.00	7.50
L-Histidine.HCl.H_2O		42.00	42.00	42.00	20.96
L-Isoleucine		52.50	52.00	104.80	3.94
L-Leucine		52.50	52.00	104.80	13.10
L-Lysine.HCl		73.06	72.50	146.20	36.50
L-Methionine		14.90	15.00	30.00	4.48
L-Phenylalanine		33.02	32.00	66.00	4.96
L-Proline					34.50
L-Serine				42.00	10.50
L-Threonine		47.64	48.00	95.20	11.90
L-Tryptophan		10.20	10.00	16.00	2.04
L-Tyrosine		36.22	37.80	72.00	5.40
L-Valine		46.90	46.00	93.60	11.70
Vitamins					
Biotin					0.0073

D-Ca Pantothenate		1.00	1.00	4.00	0.48
Choline Chloride		1.00	1.00	4.00	13.96
Folic Acid		1.00	1.00	4.00	1.30
i-inositol		2.00	2.00	7.20	18.00
Nicotinamide		1.00	1.00	4.00	0.04
Pyridoxal.HCl		1.00	1.00	4.00	0.06
Pyridoxine.HCl					0.06
Riboflavin		0.10	0.10	0.40	0.04
Thiamine.HCl		1.00	1.00	4.00	0.34
Vitamin B12					1.36
Inorganic Salts					
$CaCl_2.2H_2O$	140.00	264.90		264.90	44.00
$CuSO_4.5H_2O$					0.0025
$Fe(NO_3)_3.9H_2O$				0.10	
$FeSO_4.5H_2O$					0.834
KCl	400.00	400.00	400.00	400.00	223.60
KH_2PO_4	60.00				
$MgCl_2.6H_2O$	100.00				122.00
$MgSO_4.7H_2O$	100.00	200.00	242.20	200.00	
NaCl	8000.00	6800.00	6500.00	6400.00	7599.00
$NaHCO_3$	350.00	2000.00	2000.00	3700.00	1176.00
$Na_2HPO_4.7H_2O$	90.00	158.30	1500.00	141.30	268.00
$ZnSO_4.7H_2O$					0.863
Other					
Dihydrostreptosulfate			50.00		
Glucose	1000.00	1000.00	2000.00	4500.00	1802.00
Hypoxanthine					4.10
Lipoic Acid					0.21

Linoleic Acid					0.08
Penicillin G			75000 IU		
Phenol Red	10.00	17.00	10.00	15.00	12.00
Putrescine.$2H_2O$					0.161
Sodium Pyruvate			110.00	110.00	110.00
Thymidine					0.73

Table of components of media used in cell culture. (Information extracted from Methods in molecular biology Vol 5: Animal cell culture, 1989, Pollard J.W. & Walker J.M. (eds), Humana Press, Clifton, New Jersey, pp692-700.)

CHAPTER FOUR

TROPHOBLAST CELL CULTURE: A MODEL FOR THE STUDY OF TROPHOBLAST PROLIFERATION AND DIFFERENTIATION *IN VITRO*

This chapter is the first experimental chapter of the thesis. In this chapter, different purification techniques, culture conditions and sera on the growth and maintenance of placental cells isolated from 'normal' placenta will be examined. Relative purity, growth rates and protein assays will be compared under different culture conditions. The appropriate ligand concentrations used for further study in the later chapters will also be determined.

4.1. Introduction

The information regarding available cell culture techniques and the growth and maintenance of placental cells *in vitro* has been discussed in detail previously in Chapter One, section 1.5. Briefly, *in vitro*, stem cytotrophoblast cells spontaneously differentiate to form syncytiotrophoblast cells (Kliman *et al.,* 1986; Douglas & King, 1990; Morrish *et al.,* 1997). Successful differentiation of cultured trophoblasts depends upon purity of the population and adequate plating densities (Bloxam *et al.,* 1997). Many methods have been developed for the isolation of homogenous populations of placental villous trophoblast cells. The most successful combination of techniques involves trypsinisation and filtration with purification processes. These processes include purification by sedimentation or density gradient (Kliman *et al.,* 1986), immunomagnetic microspheres (Douglas & King, 1989) or immunological binding (Contractor & Sooranna, 1988), negative selection (Schmon *et al.,* 1991) and/or selective disaggregation of tissue (Bax *et al.,* 1989).

Culture conditions are also important for the maintenance, growth and function of the cells *in vitro* to resemble that *in vivo*. Basal medium that may be enriched or have specified nutrients may be used to select for particular subpopulations of trophoblasts or improve cell survival or differentiation (Truman and Ford, 1986; Douglas & King, 1990; Starreveld *et al.,* 1998). Some investigators have had success by using different extracellular matrices (Morrish & Siy, 1986); yet others have used different types of culture for tissue at different gestations including explant culture (Graham *et al.,* 1992), monolayer (Kliman *et al.,* 1986) and suspension cultures (Nestler *et al.,* 1987). However, success in terms of purity, differentiation and function has varied.

Of importance are the effects of certain media supplements, particularly sera and growth factors. Sera can be obtained from different sources. To date several groups use fetal calf serum (FCS). FCS between sources has resulted in different sera components resulting in different effects. Treatment of sera, to minimise these effects, has varied from charcoal stripping to boiling and filtering. Serum has proven to be important for complete differentiation of the trophoblast *in vitro,*

not only functionally but morphologically as well (Richards *et al.,* 1994). However, serum contains a wide array of factors, for many of which the functions remain unknown. Many studies have looked into the effects of certain factors on cell proliferation, differentiation and function. These include EGF (Morrish *et al.,* 1997; Mochizuki *et al.,* 1998) and related peptides (Lysiak *et al.*, 1995a; 1995b; Lala *et al.,* 1998), neuropeptides - gonadotrophin releasing hormone (Wolfahrt *et al.,* 1998) and corticotropin-releasing hormone (Perkins & Linton, 1995; Wadhwa *et al.,* 1998), cytokines, such as interleukins (de Moraes-Pinto *et al.,* 1997) and lymphohemopoietic cytokines (Garcia-Lloret *et al.,* 1994)), leptin (Henson *et al.,* 1998), activin (Caniggia *et al.,* 1997), angiogenic growth factors - namely VEGF and PGF (Shore *et al.,* 1997), hCG, hPL, oestrogen and progesterone. But with differing isolation methods and culture conditions results have been variable.

4.2. Aims

The aims of this chapter:

1. To obtain a pure population of cytotrophoblasts for *in vitro* study;
2. To investigate and compare the effects of different types of culture (monolayer vs suspension) and conditions (sera vs serum starved) on the morphological differentiation of cytotrophoblasts;
3. To determine the best model for investigating proliferation and differentiation of isolated cytotrophoblasts;
4. To determine the minimum concentration of ligands (insulin and the insulin-like growth factors) required to produce an effect on cytotrophoblasts *in vitro.*

4.3. Materials & Methods

The materials procured and methods adopted are as detailed previously in Chapter Three: Materials and Methods. These include trophoblast isolation, purification and cell culture (3.2.1-3.2.3, 3.2.5, 3.2.7, 3.2.8), cell counts and viability (3.3.), thymidine incorporation (3.4.), immunocytochemical staining (3.5.), hCG analysis (3.6.) and protein analysis (3.7.).

4.3.1. Experimental Protocol

Cells were isolated and purified using Percoll gradients and negative selection for HLA antigen. The cells were then assessed for relative purity (cytokeratin 7 and vimentin staining) and then seeded into 24-well culture plates for monolayer culture (n=6). For suspension culture (n=6), cells were maintained in 50mL upright tubes that were regularly gently shaken. In experiments for serum starvation (n=6), cells were seeded and grown in serum-free media for 5d then stimulated to re-enter the cell cycle with PDGF for 2d prior to supplementation with FCS and treatment with insulin or the IGFs. Cells were then cultured in humidified atmosphere in 5% CO_2 in air at 37^0C where preliminary observations were made every 2h for 24h and then again at 48, 72 and 96h after seeding. Cell identification, cell number and thymidine incorporation were assessed at the appointed times and media was harvested for preliminary protein investigation. Cells isolated were then grown in the presence of varying concentrations of ligand (insulin or the IGFs) to determine the lowest, effective concentration in terms of cellular proliferation and differentiation. Statistics and data analyses were performed as outlined in Chapter Three, section 3.9.

4.4. Results

4.4.1. Comparison of cell isolation and purification techniques.

Cells were dispersed as described in Chapter Three, section 3.2. The cell population obtained comprised of cells of mixed morphology. Populations consisted of small, round and mononuclear cells, with some spindle-shaped, bipolar cells and the occasional polymorphonuclear cells. Cells exhibiting macrophage-like and fibroblast-like structural features were also present (Figure 4.1).

The various steps involved in the isolation procedure (temperature during enzymatic digestion, time of digestion, concentration of trypsin and the time taken to collect tissue) were optimised in terms of percentage cell viability (Table 4.1).

At higher temperatures, enzyme concentrations and greater times of digestion, cells were found to disintegrate resulting in poor yields whilst at lower temperatures, enzyme concentrations and shorter periods of digestion, tissue disaggregation was found to be inefficient with large cell clumps observed.

Table 4.1. Comparison of isolation conditions and their effect on cell viability

Condition tested	Optimal	% viability	Test 1	% viability	Test 2	% viability
Temperature	37^{0}C	>99	42^{0}C	50	30^{0}C	90 ID
Digestion	45	>99	60-70	~95	30	85 ID
[trypsin]	5g/L	>99	7g/L	72	2.5g/L	80 ID
Collection	20-30	>99	60-90	<40%	Directly	>99

Percentage viability of cells when testing isolation conditions such as enzymatic digestion temperature, time (minutes), concentration of trypsin (g/L) and delay in collection of tissue (minutes). Test 1 when conditions are higher than optimal levels, test 2 when conditions are lower and at optimal levels. ID = inefficient dispersal, cell clumping.

After filtration and centrifugation in the isolation procedure, the cell population remained relatively mixed comprising round, mononuclear cells, blood elements and large granulated and polymorphonuclear cells (Figure 4.1). After Percoll gradient purification, cell population consisted mainly of round, mononuclear cells with the occasional macrophage-like cells. Immunocytochemical staining showed that >95% of cells isolated were positive for cytokeratin 7.

With HLA negative selection, the resultant cell suspension comprised only small, round, mononuclear cells that measured 8-10μm in diameter as determined by microscopic ruler. The addition of HLA negative selection increased the percentage of cytokeratin 7 positive cells to >99%. The high level of purity of the cell population was consistent throughout the culture period. Vimentin staining showed that no greater than 5% of the cell population were non-trophoblastic in nature. This was consistent over the entire culture period (Figure 4.2). The isolated cells stained positive for the desmoplakins. Initially cells stained intensely about the periphery of each individual cell. As the cells began to

aggregate (48h) the peripheral staining for desmoplakin intensified until the cells began to fuse together forming one multinucleated cell. The staining was found around the border of the large cell mass (>72h) which was then observed to disappear over time. The cells obtained after purification with either Percoll gradients or HLA negative selection were morphologically (Figure 4.1) and immunocytochemically identical. The comparative characteristic traits in cell growth and differentiation were not significantly different either as the cells purified by either method were observed to proliferate, aggregate and fuse to form giant, multinucleated cells in monolayer culture.

Table 4.2: Comparison of numbers of cells purified by the two different methods

Time (h)	Control PG $x10^6$ cells/mL	Control HLA $x10^6$ cells/mL
24	0.84 ± 0.01^a	0.80 ± 0.01^a
48	0.81 ± 0.02	0.78 ± 0.02
72	0.79 ± 0.02	0.77 ± 0.02

The number of cells over 72h grown in monolayer culture in EMEM supplemented with 10% FCS. Cells were isolated and purified using Percoll gradients (PG; n=12) or with the addition of HLA negative selection (HLA; n=12). (^{a}=significance, $p<0.01$, over time)

When the majority of individual cells were indistinguishable within a cluster of cells (where membranes appeared to be fused as confirmed by desmoplakin staining), they were assessed as one multinucleated cell.

Figure 4.1.Photographs of cells during isolation and purification procedures.

(a) Dispersed mixed cell population after mechanical and enzymatic digestion; (b) Cells after filtration and washing in EMEM alone; (c) Centrifugation; (d) Purified trophoblast cells obtained from Percoll gradient; and following (e) HLA negative selection techniques.

[Mag. (a, b, c) X20; Mag. (d, e) X40] For details of the isolation, digestion, centrifugation, purification and staining procedures refer to sections 3.2.1. to 3.2.3 and section 3.5. of Chapter 3 Materials and Methods.

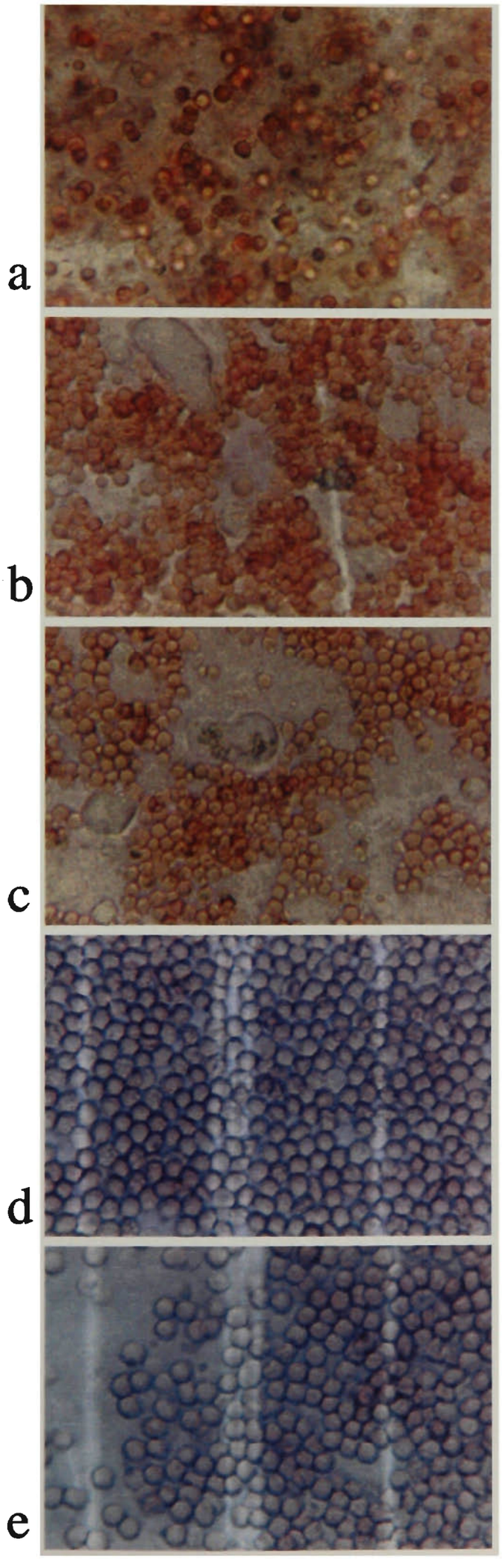

a
b
c
d
e

Figure 4.2. Staining of cells with cytokeratin 7 and vimentin at 24h of culture.

Photographs of cytotrophoblasts isolated from 'normal' placenta and stained with cytokeratin 7 and vimentin.

(a) Positive staining for cytokeratin 7

(b) Negative control for cytokeratin 7

(c) Positive staining for vimentin

(d) Negative control for vimentin.

[Mag: 20X (a, c); 40X (b, d)]

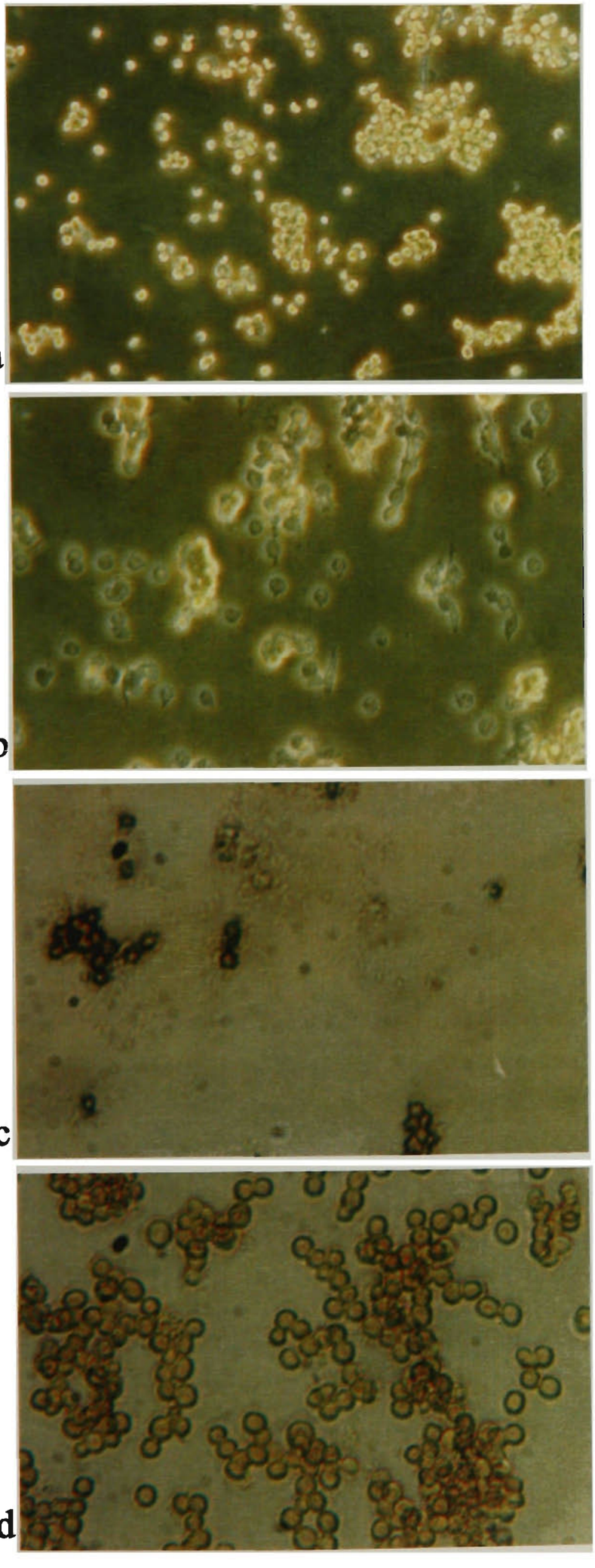

a
b
c
d

Figure 4.3. Differentiating Cytotrophoblasts *In Vitro*.

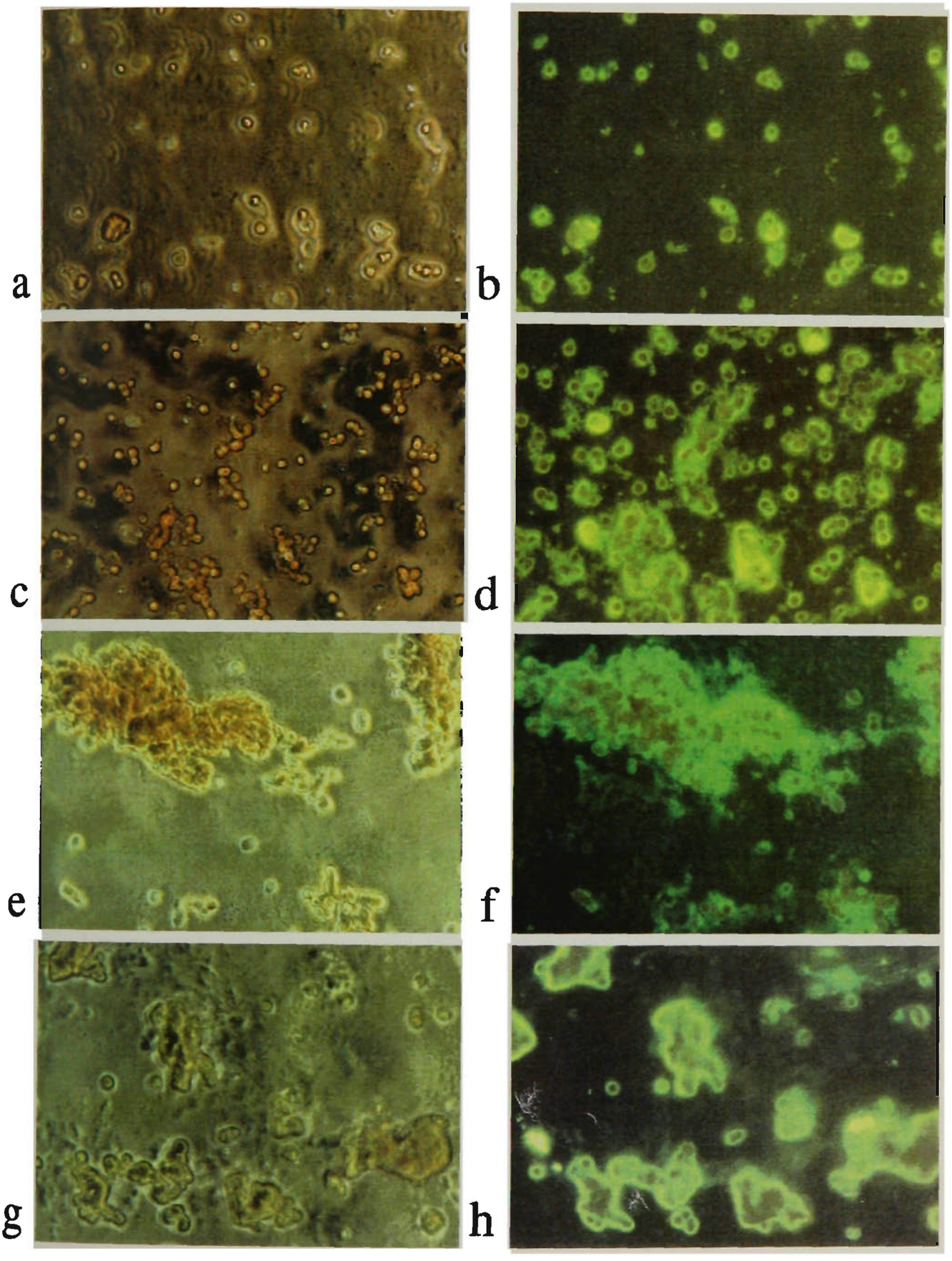
Cells were viewed under LM (a, c, e, g) and stained with antidesmoplakin I and II (b, d, f, h). Mag: X20.

(a, b): Cells in culture at 24h.

(c, d): Cells in culture at 48h.

(e, f): Cells in culture at 72h.

(g, h): Cells in culture at 96h.


a
b
c
d
e
f
g
h

4.4.2. Culture conditions:

4.4.2.a. The monolayer cell culture

Cells were seeded at 0.5 x 10^6 cells/mL and grown in EMEM supplemented with 10%FCS in monolayer culture on a collagen matrix as described in Chapter Three, section 3.2.5. Within the first 4-6 hours after seeding cells were observed to adhere well to the matrix. Over time, cells increased in number peaking at 24h (0.80 ± 0.01 x 10^6 cells/mL). This data was supported by thymidine incorporation studies where thymidine uptake in cell cultures was found to increase significantly ($p<0.01$, $F=2.67$) over time.

A "plateau" followed where cell numbers did not appear to change significantly and after 96h the number of cells decreased slightly. The changes in the cell numbers for cytotrophoblasts grown in EMEM with 10% FCS coincided with the morphological changes observed in the cell population under the light microscope. After 96h a significant proportion of the cells were visualised to aggregate with some observed to fuse together forming giant multinucleated cells. The degree of apoptosis was found to be minimal. Very few cell fragments were observed in the cell population and was estimated to be <5%. The content of hCG in the media was found to increase from non-detectable amounts to 30.0 ± 5.6 mIU/mL at 96 hours. This data, and that of the effects of insulin and the IGFs, is further discussed in Chapter Five.

Lowry assays of samples show that over time the amount of protein found in the media increases (Table 4.3). The protein levels were found to be higher in insulin treated cultures compared to control and those treated with IGF-I. Reading samples at 215nm and 280nm, which measured peptide bonds and the amounts of tryptophan and tyrosine residues, respectively (data not shown), reaffirmed these results.

Table 4.3. Lowry assay of media samples: monolayer culture

Time (h)	Control	0.1nM Insulin	1.0nM IGF-I
0	60.00 ± 1.00	62.50 ± 2.00	62.00 ± 2.05
24	71.00 ± 4.40	83.00 ± 4.50	80.50 ± 3.01
48	92.50 ± 5.20	122.50 ± 4.80	102.50 ± 5.50

Protein concentration as determined by Lowry assay in the medium of cells grown in EMEM supplemented with 10% FCS alone (control), and with the addition of insulin or IGF-I at 24 and 48h of culture (n=6; mean±SEM μg/mL protein).

Proteins secreted into culture media were separated on 12% SDS resolving gels and compared within treatments as well as between treatments over time. Refer to photographs in Figure 4.4. The same volume (20μL) of media for each treatment was applied to each well so as to observe any differences in the amounts of proteins expressed. The arrows in Figure 4.4 show differences in the density of protein bands within treatments and the absence/presence of protein bands between treatments. The gels show that in all treatments, overall protein expression increased over time with notable changes observed between the 45,000 MW (ovalbumin) and 66,200 MW (serum albumen) protein bands. With insulin and IGF-I treatment, the LMW proteins (below 45,000 MW) were observed to diminish over time.

Figure 4.4. Media protein separation using SDS-PAGE

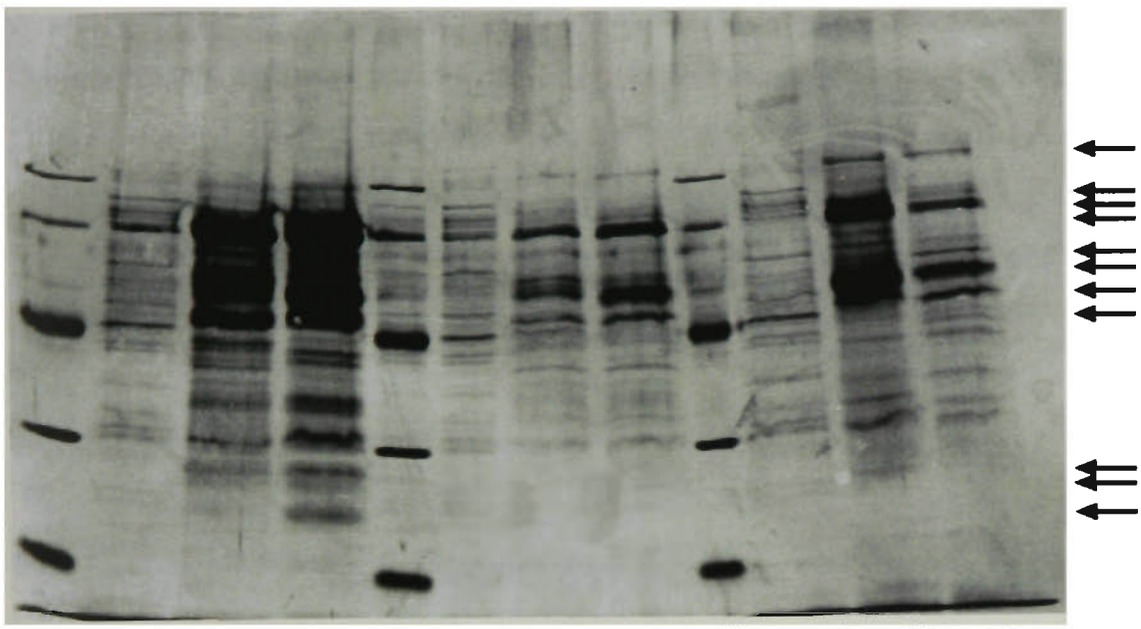
Proteins from media samples collected at various times from cytotrophoblast cultures maintained in EMEM and supplemented with 10%FCS (control) and treated with 0.1nM insulin or 1.0nM IGF-I.

Gel One:
Lane 1, 5, 9:LMW standard
Lane 2: Media alone
Lane 3: Control @ 24h
Lane 4: Control @ 48h
Lane 6: Media + 0.1nM insulin
Lane 7: insulin @ 24h
Lane 8: insulin @ 48h
Lane 10: Media + 1.0nM IGF-I
Lane 11: IGF-I @ 24h
Lane 12: IGF-I @ 48h

Gel Two:
Lane 5, 9, 13: LMW standard
Lane 2: Media alone
Lane 3: Media + 0.1nM insulin
Lane 4: Media + 1.0nM IGF-I
Lane 6: Control @ 24h
Lane 7: insulin @ 24h
Lane 8: IGF-I @ 24h
Lane 10: Control @ 48h
Lane 11: insulin @ 48h
Lane 12: IGF-I @ 48h

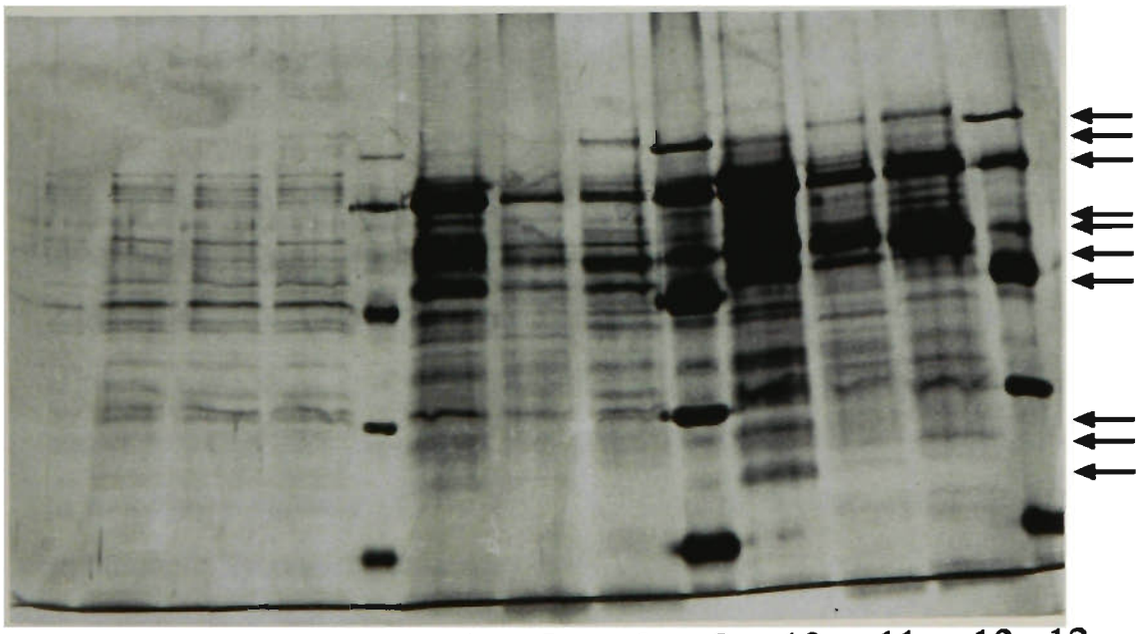
LMW standard	97,400	Phosphorylase B
	66,200	Serum albumen
	45,000	Ovalbumin
	31,000	Carbonic anhydrase
	21,500	Trypsin inhibitor
	14,400	Lysozyme

Gel One



1 2 3 4 5 6 7 8 9 10 11 12

Gel Two



1 2 3 4 5 6 7 8 9 10 11 12 13

4.4.2.b. The suspension culture: Comparisons to the monolayer culture.

Initially in suspension culture the majority of cells maintained cell structure. However, over time cell morphology was observed to deteriorate in all treatments. The numbers of cells in suspension cultures, regardless of treatment, were not significantly different at any time. Supplementation of media with insulin or the IGF's (IGF-I, IGF-II or a combination of these factors with insulin) had no significant effect on cell number. IGF-II administration resulted in a more rapid decrease in cell numbers compared to those of control cultures and insulin or IGF-I treated cultures (Figure 4.5).

Table 4.4. Lowry assay of media samples: suspension culture

Time (h)	Control	0.1nM Insulin	1.0nM IGF-I
0	60.50 ± 2.00	61.00 ± 3.00	60.00 ± 2.50
24	63.00 ± 3.50	63.20 ± 6.50	60.50 ± 3.01
48	58.50 ± 7.20	60.50 ± 8.00	59.50 ± 7.50

Protein concentration as determined by Lowry assay in the medium of cells grown in EMEM supplemented with 10% FCS alone (control), and with the addition of insulin or IGF-I at 24 and 48h of culture (n=6; mean±SEM μg/mL protein).

The suspension cultures were not conducive to cellular proliferation and differentiation and function. Cells grown in suspension were not observed to differentiate, i.e., form multinucleated cells (hCG concentrations in media samples were very low over 72h, 3.0±2.0mIU/mL; data not shown). Lowry assays revealed little protein release into the culture medium over time compared to monolayer cultures (Table 4.4). Suspension cultures were not observed to significantly increase in cell numbers (Figure 4.5).

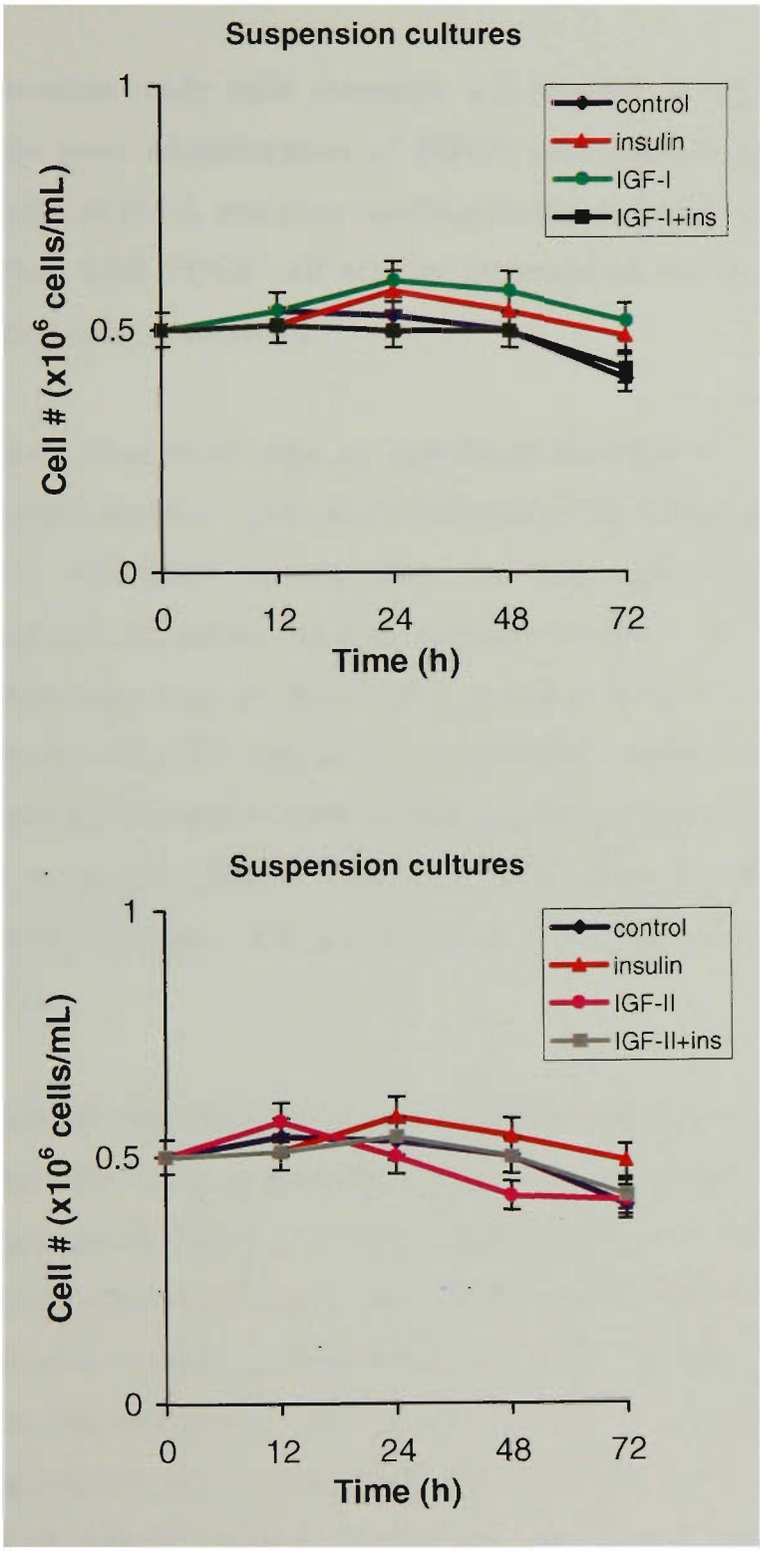
Figure 4.5. Cell number over time: Suspension culture.

Number of cells grown in control conditions over 72h in suspension culture and treated with:

a. Insulin, IGF-I and a combination of both factors.

b. Insulin, IGF-II and a combination of both factors.

(mean $\pm$ SEM x 10^6 cells/mL; n=6)


Suspension cultures
Cell # (x10^6 cells/mL)
1
0.5
0
0
12
24
48
72
Time (h)
control
insulin
IGF-I
IGF-I+ins
Suspension cultures
Cell # (x10^6 cells/mL)
1
0.5
0
0
12
24
48
72
Time (h)
control
insulin
IGF-II
IGF-II+ins

4.4.2.c. Serum starvation versus serum supplementation

Serum starvation made cells quiescent and resulted in reduced cell viability. Without the prior administration of PDGF, restimulation of cell growth with serum, insulin or IGF-I, was poor resulting in minimal effects on the proliferation of the cells. With PDGF cell activity increased as determined by thymidine incorporation (data not shown).

After restimulation, there were no significant differences in cell numbers over time in control cultures. This was confirmed by thymidine uptake data (Figure 4.6.) where thymidine incorporation was not significantly different from background and did not significantly change over time. With insulin treatment, cell numbers were seen to significantly increase ($p<0.05$, $F=19.59$) over time, peaking ($0.15\pm0.02 \times 10^6$ cells/mL) at 24h of culture. Insulin treatment resulted in cell numbers and thymidine uptake being significantly higher ($p<0.001$, $F=96.13$) than those of control cultures. After 24h cell numbers in insulin treated cultures were found to decrease. The percentage of fused cells observed in culture was low (<20%).

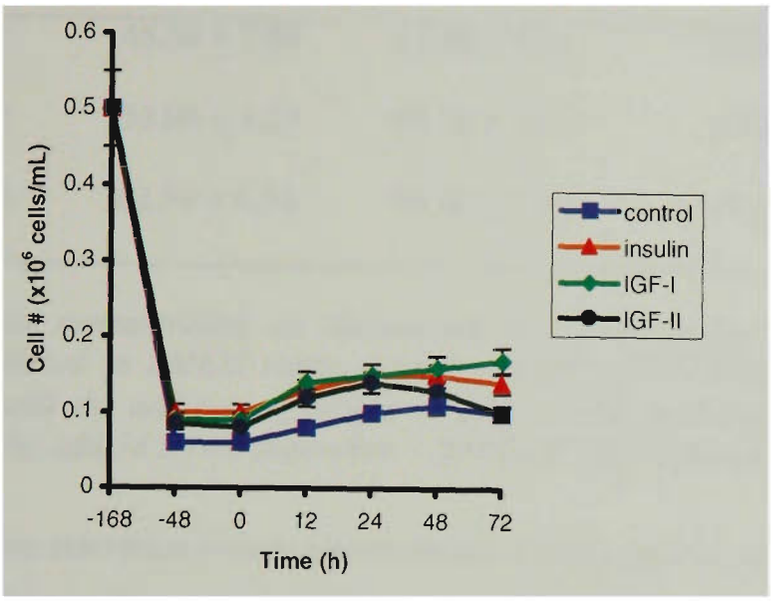
Cultures treated with IGF-I resulted in significant increases ($p<0.01$, $F=3.52$) in cell number over time, peaking at $0.16\pm0.02 \times 10^6$ cells/mL at 72h. This cell number increase was found to be significantly higher ($p<0.001$, $F=34.71$) than that of control and insulin treated cultures. Cells treated with IGF-II were found to be similar in trend to those of insulin treated cultures, peaking at $0.14\pm0.02 \times 10^6$ cells/mL at 24h followed by a decline in numbers. Cell numbers and thymidine uptake obtained for IGF-II treated cultures to 24h were not significantly different from that of cultures treated with insulin. However, they were found to be significantly higher ($p<0.01$) than those of control cultures over the first 48h and significantly lower ($p<0.001$) than those of IGF-I treated cultures.

Figure 4.6. Cell number and thymidine uptake over time: Serum starved culture.

Cells were maintained for 5 days in serum-free medium and then restimulated with PDGF for 2 days prior to supplementation with 10%FCS (control) and treatment with 0.1nM insulin, 1.0nM IGF-I or 10.0nM IGF-II. (mean $\pm$ SEM x 10^6 cells/mL, n=6).

(a) Number of cells over time.
(b) Thymidine uptake over time.
    (i) control cultures
    (ii) Insulin treated cultures
    (iii) IGF-I treated cultures
    (iv) IGF-II treated cultures

Serum Starvation: Cell Number



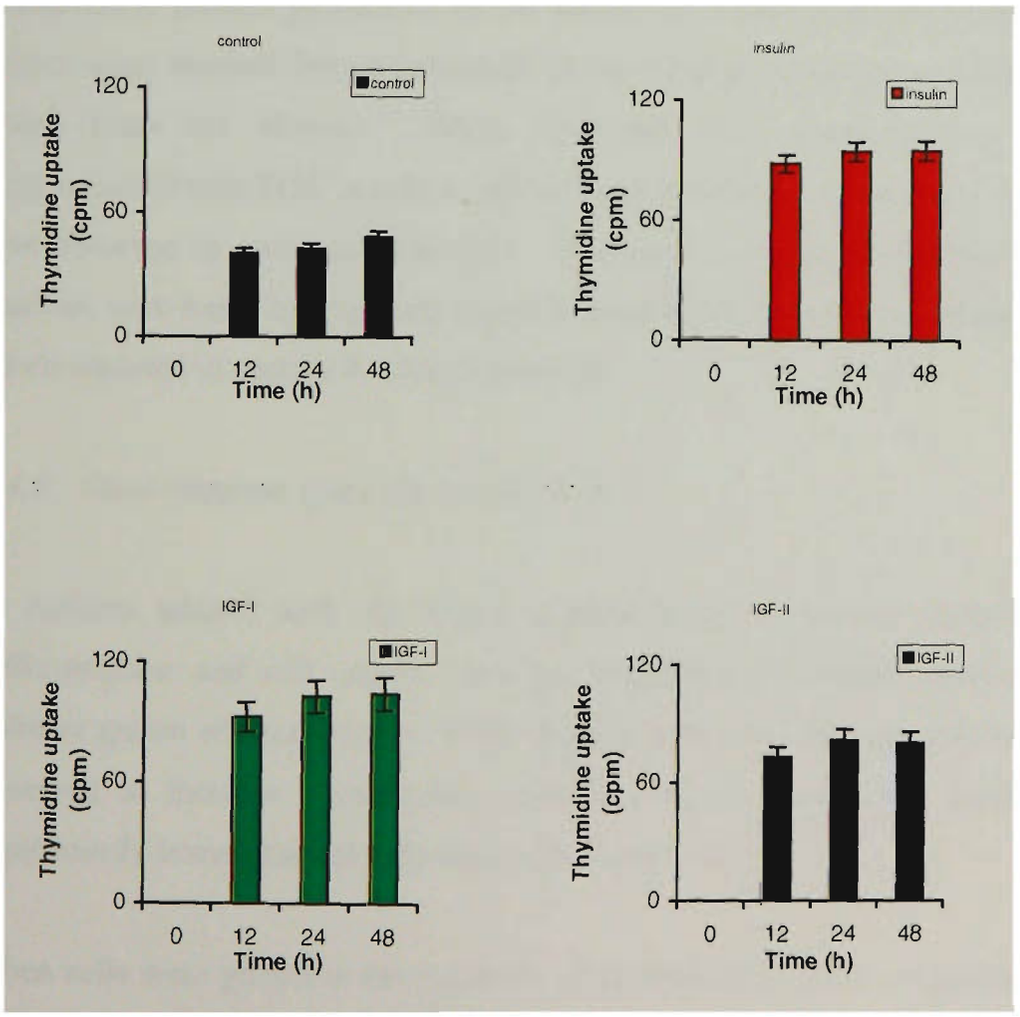


Table 4.5. Lowry assay of media samples: Serum starved culture

Time (h)	Control	0.1nM Insulin	1.0nM IGF-I
0	45.50 ± 7.80	51.00 ± 9.50	50.00 ± 8.50
24	53.00 ± 5.25	58.90 ± 7.30	55.50 ± 8.10
48	62.50 ± 6.20	69.50 ± 5.60	63.50 ± 6.00

Protein concentration as determined by Lowry assay in medium maintained in EMEM supplemented with 10% FCS alone (control), and with the addition of insulin or IGF-I (n=6; mean±SEM μg/mL protein; add 5d serum starvation + 2d PDGF restimulation to Time).

During serum starvation Lowry determination showed that protein production and secretion did not change significantly from initial readings, although the protein levels did decrease. Restimulation of the cells with PDGF did not appear to affect extracellular protein production or the secretion of any intracellular proteins as Lowry assay showed that protein levels did not change compared to serum starved levels (data not shown). When the cells were maintained in medium supplemented with FCS, insulin or IGF-I the concentration of protein in the media was observed to increase over time. The levels of protein secreted into the medium were found to be greatly reduced compared to those of monolayer culture levels reported in section 4.4.2.a (Table 4.3).

4.4.3. Dose-response of Insulin and the IGFs

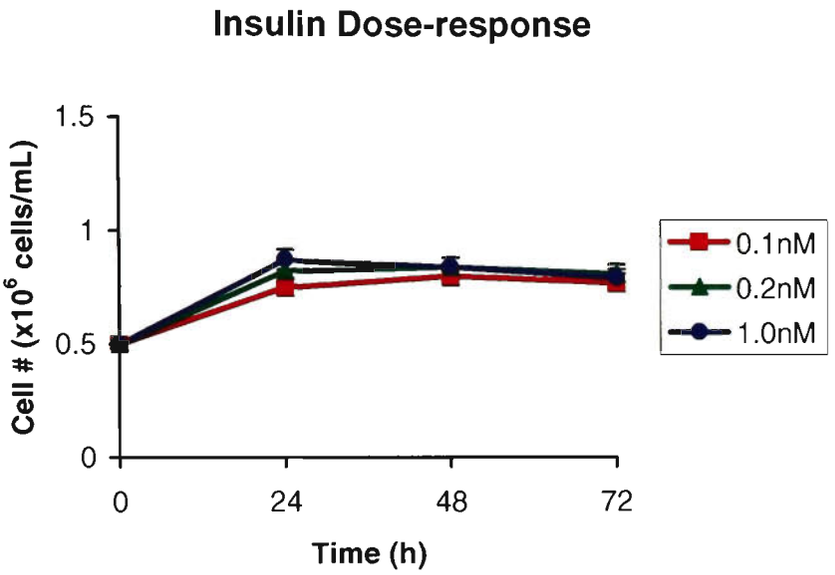
In cultures treated with the lowest concentration of insulin, morphological differentiation and cell counts were not significantly different from those of cultures grown without insulin. Over the first 24h, however, cell numbers were observed to increase significantly ($p<0.01$, $F=17.21$) and were found to be significantly lower than those treated with 1.0nM insulin.

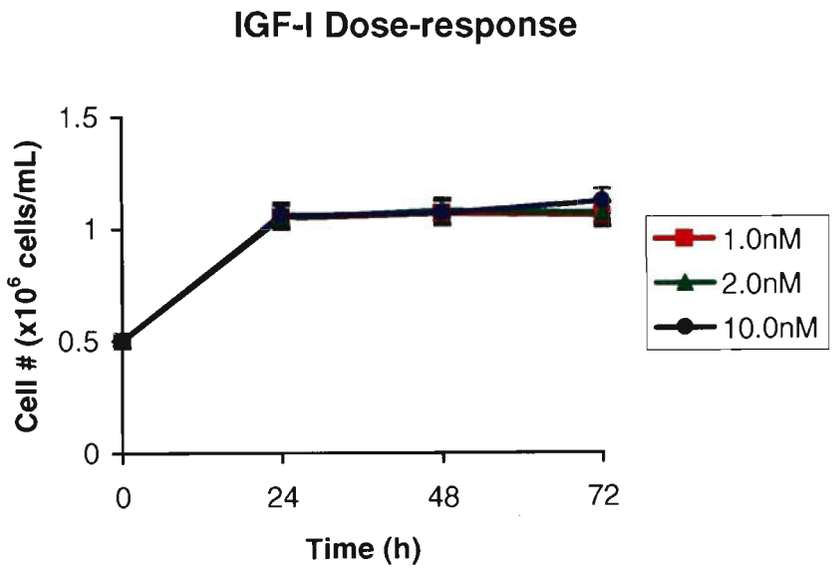
When cells were grown in the presence of increasingly higher concentrations of insulin the numbers of cells were observed to increase over time and found to be higher than those observed for the lowest insulin concentration.

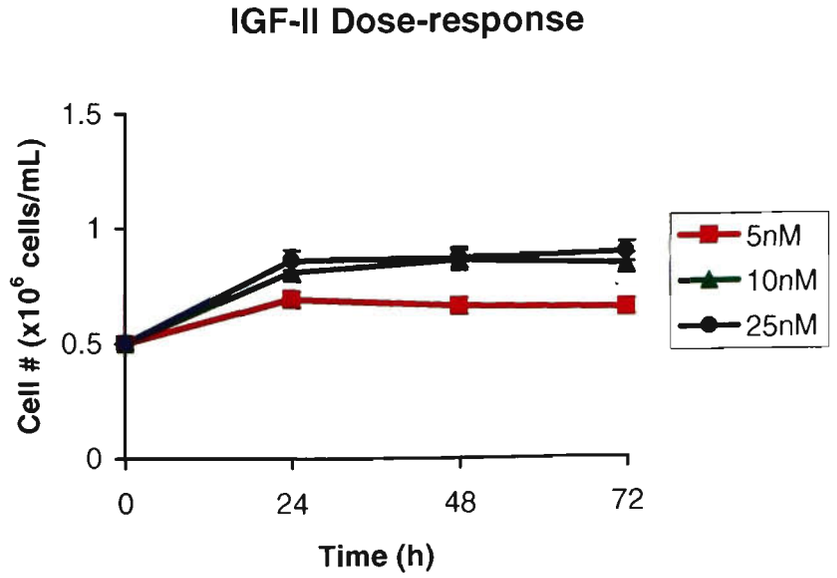
Figure 4.7. Dose-response curves: Insulin, IGF-I, IGF-II. (n = 4)

Numbers of cells grown in EMEM supplemented with 10%FCS alone and treated with

a) Varying concentrations of insulin,
b) Varying concentrations of IGF-I and
c) Varying concentrations of IGF-II.


Insulin Dose-response
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
Time (h)
0.1nM
0.2nM
1.0nM


IGF-I Dose-response
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
Time (h)
1.0nM
2.0nM
10.0nM


IGF-II Dose-response
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
Time (h)
5nM
10nM
25nM

In cultures treated with IGF-I, cells were observed to increase significantly ($p<0.01$; $F=222.28$) in number over time. There were no significant differences in cell numbers between cultures treated with varying IGF-I concentrations. Cell numbers were found to be significantly higher ($p<0.01$; $F=12.90$) in IGF-I treated cultures compared to those of control and insulin treated cultures. Very little aggregate and multinucleated cell formation was observed in these cultures. Cells exhibited a pavement-pathway arrangement in culture.

Cultures treated with IGF-II demonstrated similar effects to that observed with insulin treatment, i.e., cells were observed to proliferate over time followed by aggregation and cell fusion by the end of culture. Cells treated with the lowest concentration of IGF-II were not found to be significantly different in cell number compared to control cultures. However, at higher IGF-II concentrations, cell numbers were found to be significantly higher ($p<0.01$, $F=29.84$) compared to those for the lowest IGF-II concentration (Figure 4.7).

4.5. Discussion

After mechanical and enzymatic disaggregation of whole placental tissue, the cell population comprised trophoblasts, macrophages, fibroblasts, endothelial cells, stromal cells, connective tissue and blood elements. Several methods for purifying the cell population were adopted based on different principles i.e., differences in cell size (filtration), cell density (Percoll gradients) and antigenic identity (HLA negative selection). The most effective method for cell purification was found to be the use of all techniques combined. In this study relatively pure (>95%) cell populations could be obtained with Percoll gradients or HLA negative selection alone after filtration. Negative selection techniques were preferred to positive selection as positive selection may require further treatment of the cells to remove them from the selection material for culture, which may effect their viability and/or function.

Cell viability was dependent on a number of factors during the isolation and purification procedures. These included the temperature during cell dispersal, duration of enzymatic digestion, concentration of trypsin and the delay in

collection of the tissue. Any deviation from optimal levels of the abovementioned factors resulted in either reduced cell viability and deterioration of cell structure or inefficient cell dispersal and an increased likelihood of cell clumping. DNAse I was found to be a particularly important requirement during enzymatic digestion as it eliminated cell clumping.

Large tissue pieces and cellular debris were removed via filtration through fine gauze and subsequent washing and centrifugation. Cells were purified based on cell density using Percoll gradients (Kliman *et al.*, 1986). This method achieved relatively pure cell populations as assessed by morphological examination which revealed that the majority of the cell population comprised round, mononuclear cells that measured between 8-10μm in diameter. From information obtained from Sigma, macrophages share similar cell densities to cytotrophoblasts, cells were further purified by applying the HLA negative selection technique, as described by Schmon *et al* (1991), that increased cell purity to >99%.

The majority (~95%) of the cells isolated from the human placentae stained positive for cytokeratin 7 and the desmoplakins confirming that the cells were cytotrophoblasts (Blaschitz *et al*, 1997; Douglas & King, 1990). It has been suggested that cell populations purified by density gradient alone may be contaminated by macrophages (as these cells share similar densities to that of the trophoblast). However, in the present study, populations purified by density gradient or HLA selection were not morphologically different, nor were there any significant difference in proliferative activity suggesting that the populations were very similar. Any contaminants were probably present in very low numbers. Immunocytochemical staining showed that the percentage contamination in culture (regardless of purification method) was very low (5% were non-trophoblastic elements) and remained low (5%) throughout the culture period.

The model chosen for cell culture was the monolayer culture, where cells were plated within wells previously coated with a collagen matrix. Serum starvation, believed to synchronise cells, resulted in low yields. The monolayer culture is believed to resemble *in vivo*, where cells have been shown to adhere to a matrix of reticular, collagen (Castellucci & Kaufmann, 1982; Aplin & Campbell, 1985) and

elastic fibres (Graf *et al.,* 1996) in the stroma of human placental stem villi. These fibres are believed to provide structural support, elasticity, support to tensile and/or contracting forces within the stem villous as well as facilitating macrophage and immunological tasks of the Hofbauer cells (Castellucci & Kaufmann, 1982; Aplin & Campbell, 1985). Factors within the matrix allow cells to grow (Morrish & Siy, 1986; Truman & Ford, 1986), effect cell mobility (Graham & Lala, 1991; Irving & Lala, 1995), allow digestion of nutrients and cellular differentiation (Graham *et al.,* 1992). It has also been suggested that trophoblasts are active in matrix biosynthesis (Aplin & Campbell, 1985). This collective information demonstrates the relationship of the cells to the matrix, which is not obtained in a suspension culture. This may explain the reduced growth (proliferation and differentiation) and function of the cells (as assessed by reduced protein release and very low levels of thymidine uptake) within the suspension system regardless of the exogenous administration of stimulants such as insulin and the IGFs.

When cytotrophoblasts differentiate to form multinucleated cells, the cells take on new morphology and change in cellular processing. As reviewed in the literature (Chapter One, section 1.6), cytotrophoblasts and syncytiotrophoblasts express different proteins, hormones, cytokines and factors. Proteins and peptides released into the medium of cytotrophoblast cultures were assayed using Lowry assays and SDS-PAGE to provide preliminary information regarding any changes in the expression of proteins during cellular differentiation *in vitro*. Media samples taken at 24, 48 and 72h of culture within treatments and samples taken between treatments at 24 and 48h were assayed. The results obtained did not distinguish between cellular and extracellular proteins, which include cell secretion and/or cell degradation products. As the non viability of cells (as a result of apoptosis or cell lysis) was found to be minimal (<5%), proteins in the medium were indicative of expression/secretion and not largely due to cell breakdown, indicating cell activity.

Other factors proved to be important for cell growth and maintenance *in vitro*, particularly serum. Without serum, cells became quiescent (enter the G_0 phase) in culture and eventually lost viability. This supports work done by Vriz and

colleagues (1992). However, quiescent cells can be restimulated to continue proliferative activity and cellular functions. In the present study, exogenous administration of serum, insulin and the IGFs alone (without prior stimulation with PDGF) proved ineffective. It is possible that the ligand concentrations used *in vitro* were not sufficient to stimulate the entry into the cell cycle. It may also be that the cells were starved of nutrients (as minimal nutrient supply was provided via EMEM) necessary to sustain metabolic activity despite the addition of insulin. This suggests that the growth promoting effects of insulin may be indirect as a result of increased nutrient uptake. It may also be possible that insulin may act in unison with other growth factors (not present in EMEM) to promote cell proliferation *in vitro*. The serum used in the present study had been treated and may therefore lack the growth factors and/or nutrients required for restimulating the cell cycle.

Restimulation of the cell cycle, where cells re-enter the cycle at S phase (Vriz *et al.,* 1992), with PDGF was necessary. It has been shown that PDGF administration stimulates IGF-I secretion from placental cells (Fant *et al.,* 1986). This may explain the improved proliferative effects after PDGF restimulation of quiescent cells compared to no PDGF restimulation.

Finally, the concentrations of the ligands used were assessed. The final concentrations adopted for the following experiments were insulin (0.1nM), IGF-I (1.0nM) and IGF-II (10.0nM). The levels chosen fall within physiological levels (Houslay & Heyworth, 1983; Froesch *et al.,* 1985) and were the lowest concentrations of ligand to produce a response in terms of proliferative activity and differentiation *in vitro*. The concentrations used also indicate the relative potencies of each ligand where IGF-I was found to be more potent in producing a response than insulin whilst that of IGF-II was found to be at least 10X less potent than either insulin or IGF-I.

Thus, in this chapter the most appropriate isolation procedure and culture conditions to give optimum cytotrophoblast yield and allow observations into cellular proliferation and differentiation were determined. Appropriate concentrations that reflected physiological levels were also determined. The

following chapter will now utilise these techniques and ligand concentrations to examine the effects of insulin and the IGFs on the proliferation and differentiation of cytotrophoblasts isolated from placental tissue.

CHAPTER FIVE

THE EFFECTS OF INSULIN AND THE IGFs ON CYTOTROPHOBLASTS FROM "NORMAL" PLACENTAE

The following chapter will adopt the optimised cell isolation, purification and culture conditions established in the previous chapter, to investigate the effects of the administration of insulin and the IGFs on the proliferation and differentiation of cytotrophoblasts isolated from 'normal' placentae. Growth curves and hormone production will be examined and compared.

5.1. Introduction

As discussed previously in Chapter One, the primary function of the human placenta is to ensure an optimal environment for fetal growth and development. It provides the fetus with nutrients, oxygen and immunoglobulins whilst it removes waste and carbon dioxide. Essential to this role is the growth and development of the placenta itself, and the proliferation and differentiation of the constituent cell types that comprise the placenta. Little is known about the factors and the underlying mechanisms involved in the regulation of placental growth. Insulin and the Insulin-like Growth Factors (IGF-I and IGF-II) share structural and biochemical properties (D'Ercole, 1987) and are thought to play an important role in fetal growth and development. However, their roles in placental growth and differentiation have yet to be elucidated.

Insulin has demonstrated growth promoting effects in a variety of cell types. These include the ability of insulin to stimulate cellular proliferation in fibroblasts and hepatoma cell lines (Lin *et al.,* 1992) and to effect the expression of specific growth promoting genes in other cell types (Tuab *et al.,* 1987; Messina, 1989; Messina, 1990; O'Brien & Granner, 1991). In term placental tissue, insulin receptors have been localised to the villus brush border of syncytiotrophoblasts (Deal & Guyda, 1983), endothelial and macrophage cells (Desoye *et al.,* 1994).

The insulin-like growth factors, IGF-I and IGF-II, share structural homology with insulin (Humbel, 1984). They also share many insulin-like biological activities, including the stimulation of glucose oxidation (Clemmons *et al.,* 1975), lipid synthesis and cell growth (Salmon & Hosse, 1971). As outlined in Chapter 2, section 2.5, the IGFs have been shown to have proliferative effects in a number of cell lines as well as some differentiative effects (Florini *et al.,* 1986). Evidence to support a major role for the IGFs in the regulation of fetal growth include the correlation of serum IGF-I and IGF-II levels with birth weight and gestational age (Bennett *et al.,* 1983), the presence of insulin and high affinity Type 1 receptors on placental membranes and fetal tissues (D'Ercole *et al.,* 1980; Tally *et al.,* 1987) and the relatively high expression of IGF-I mRNA during pregnancy (Wang *et al.,*

1988). Increased levels of insulin, IGF-I and IGF-II have been reported in the cord blood of infants of diabetic mothers (Froesch *et al.,* 1985).

Although insulin has been shown to stimulate proliferation in other cell types (Lin *et al.,* 1992), it is not yet determined whether insulin directly effects this process in placental cells and those of the fetus, or simply increases metabolic activity. This chapter will address the issue of the effects of insulin and the IGFs on the proliferation and differentiation of cytotrophoblasts *in vitro*.

5.2. Aims

The aims of this study:

1. To examine the effects of insulin on the proliferation and differentiation of cytotrophoblasts isolated from placentae of 'normal', healthy pregnancies at term.
2. To examine the effects of the IGFs (IGF-I and IGF-II) on the proliferation and differentiation of cytotrophoblasts isolated from placentae of 'normal', healthy pregnancies at term.
3. To compare the relative proliferative effects of insulin and the IGFs on cytotrophoblasts isolated from placentae of 'normal', healthy pregnancies at term.

5.3. Materials and Methods

5.3.1. *Experimental protocol for culture conditions:*

Isolated cells were seeded at 0.5 x 10^6cells/mL and divided into the following treatment groups: EMEM supplemented with 10% FCS alone (control, n=30), and treated with

i. 0.1nM insulin (n=30);
ii. 1.0nM IGF-I (n=18);
iii. 10.0nM IGF-II (n=18);
iv. 0.1nM insulin and 1.0nM IGF-I (n=18);

v. 0.1nM insulin and 10.0nM IGF-II (n=18);

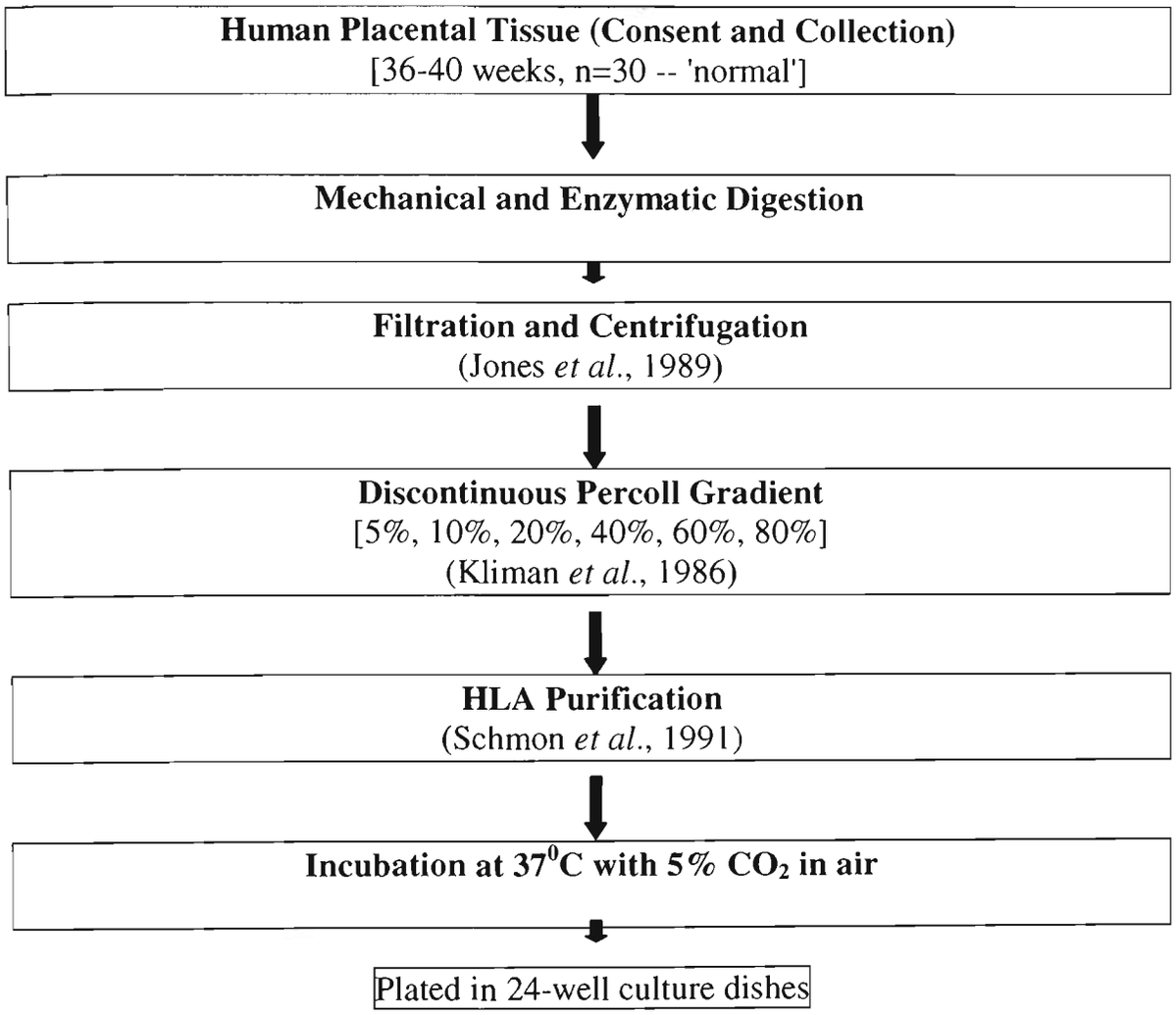
vi. 1.0nM IGF-I and 10.0nM IGF-II (n=12).

Methods and materials adopted are as described previously in Chapter Three. All cultures contained 0.5μCi/mL ^{3}H-thymidine (sections 3.2.1-3.2.3, 3.2.5, 3.2.7, 3.2.8). Observations and repeated measures were performed in quadruplicate and taken every 2h for up to 24h and then at 48h and 72h after seeding. At the appointed times, cultures were counted (section 3.3) and assayed for thymidine uptake (section 3.4). Slides were prepared of the cultured cells at the designated time intervals and immunocytochemically stained for cytokeratin 7, vimentin, desmoplakins, and PCNA (section 3.5). The proportion of cells staining positive was determined by the systematic counting of cells from 25 random photographs taken of each sample (prepared as per Chapter 3, section 3.5) which were performed in triplicate replicates. Apoptosis was also assayed using the In Situ Cell Death Kit (section 3.5). The media supernatant was collected after centrifugation (while the cell pellet was discarded) for hCG analysis using direct chemiluminescence (performed in duplicate, section 3.6).

5.3.2. *Statistical analysis:*

As described previously in Chapter Three, section 3.9, all data is expressed as the mean $\pm$ SEM. A MANOVA modified for repeated measures was performed on the data collected using the SPSS statistical package. The level of significance was at $p<0.01$. A Student T test was adopted to analyse hCG and cell aggregate data between mean values at zero time and at the end of the culture period.

5.3.3. Simple Flow diagram of overall experiment:



Protocol

Time (h)	0	12	24	48	72	96
Cell number	**********			*	*	*
Thymidine uptake	**********			*	*	*
Cytochemical stains	*	*	*	*	*	*
hCG analysis	*	*	*	*	*	*

5.4. Results

5.4.1. *Confirmation of cell identity*

Cells grown in culture were stained for cytokeratin 7 (trophoblast cell marker, Blaschitz *et al.,* 1997) to confirm the identity of the cells isolated and grown in culture. Results show that the majority of cells obtained after isolation with Percoll gradients (>95%) and HLA purification (>98%) stained positive for cytokeratin 7. Less than 5% of cells did not stain for cytokeratin 7 (Chapter 4, Figure 4.2). This percentage was consistent throughout the culture period and for all treatments and was supported by vimentin staining. At 96h the population was found to consist mainly of mononuclear trophoblasts found interspersed with multinucleated syncytium and other aggregating trophoblast cells.

5.4.2. *Cell counts and in vitro differentiation*

Cells cultured in EMEM supplemented with 10% FCS alone comprised small (8-10μm diameter), round, mononuclear cells, corresponding to 1.048-1.062g/mL density, which significantly increased ($p<0.05$, $F=2.01$) in number, peaking ($0.80\pm0.01 \times 10^6$ cells/mL) at 24h. The addition of insulin, IGF-I or IGF-II significantly increased ($p<0.0001$, $F=909.871$) cell numbers, throughout the culture period, compared to control (Figures 5.1 and 5.2). Cultures treated with IGF-I demonstrated significantly higher ($p<0.0001$, $F=3.68$) cell numbers over time compared to those treated with insulin or IGF-II. At 24h, cell size in insulin-treated cultures increased to 10-15μm diameter compared to control cultures (8-10μm) and cells treated with IGF-I alone (8-10μm).

Significant ($p<0.0001$, $F=3.68$) decreases in cell numbers were observed at 72h in cultures treated with insulin or IGF-II. This reduction coincided with the aggregation of cytotrophoblasts and their formation into giant, multinucleated cells (i.e., cellular differentiation as supported by immunocytochemical staining for the desmoplakins), which was pronounced in cultures treated with insulin or IGF-II (Figure 5.3).

5.4.3. *Combination of Insulin, IGF-I and IGF-II*

Cell numbers in cultures treated with the combination of any two of insulin, IGF-I and IGF-II were significantly higher ($p<0.0001$, $F=909.871$), throughout the culture period, compared to control and cultures treated with insulin alone, but were significantly lower ($p<0.0001$, $F=3.68$) compared to IGF-I treated cultures (Figures 5.1. and 5.2). There were no significant differences in the cell numbers between cultures treated with IGF-II, insulin and IGF-II, insulin and IGF-I and those treated with both IGF-I and IGF-II.

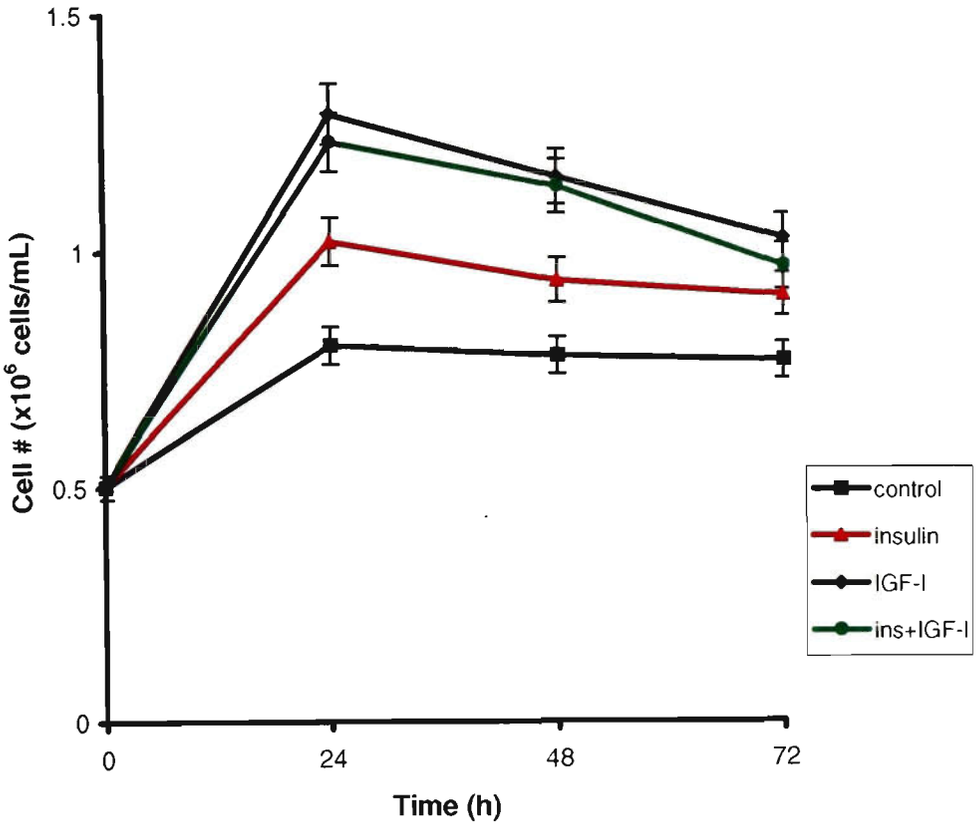
After 24h, cell numbers declined. However, the growth curves of cultures treated with a combination of insulin, IGF-I and IGF-II were not significantly different to those of trophoblast cells treated with IGF-II alone. The decrease in cell number coincided with the aggregation of cells and differentiation into syncytiotrophoblasts.

Figure 5.1: Cell number over time: Insulin and IGF-I.

The number of cytotrophoblasts grown in EMEM supplemented with 10%FCS alone (control) and treated with 0.1nM insulin, 1.0nM IGF-I or both for 72h (n=30; mean $\pm$ SEM x 10^6cells/mL).

Figure 5.2: Cell number over time: Insulin and IGF-II.

The number of cytotrophoblasts grown in EMEM supplemented with 10%FCS alone (control) and treated with 0.1nM insulin, 10.0nM IGF-II or both for 72h (n=30; mean $\pm$ SEM x 10^6 cells/mL).


1.5
1
0.5
0
Cell # (x10^6 cells/mL)
0
24
48
72
Time (h)
control
insulin
IGF-I
ins+IGF-I

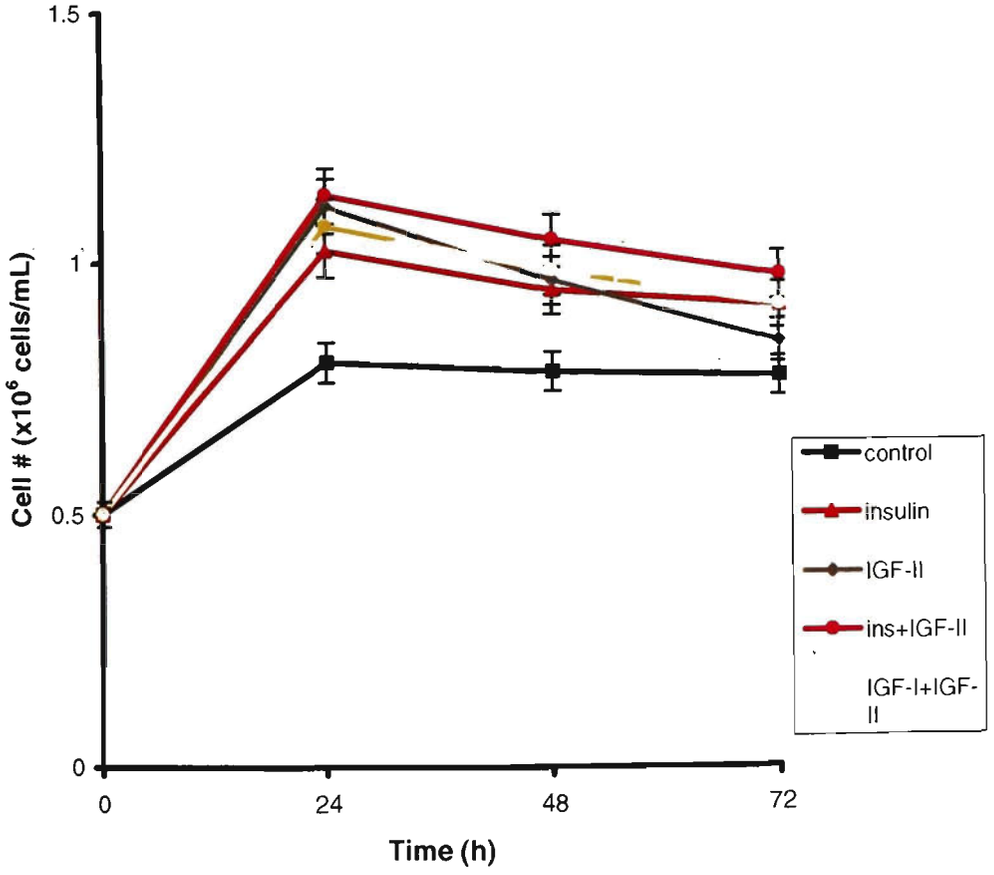

1.5
1
0.5
0
Cell # (x10^6 cells/mL)
0
24
48
72
Time (h)
control
insulin
IGF-II
ins+IGF-II
IGF-I+IGF-II

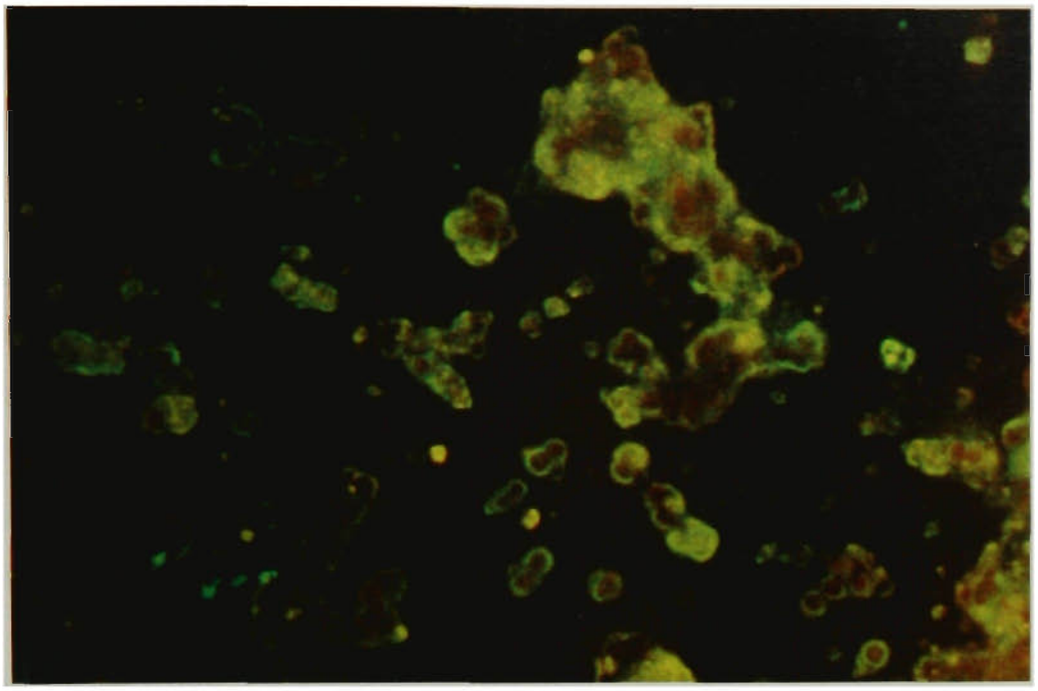
Figure 5.3: Differentiating cytotrophoblasts in culture: Comparison between treatments

Cultured cells at 72h were stained with antidesmoplakin I and II. Mag X40.

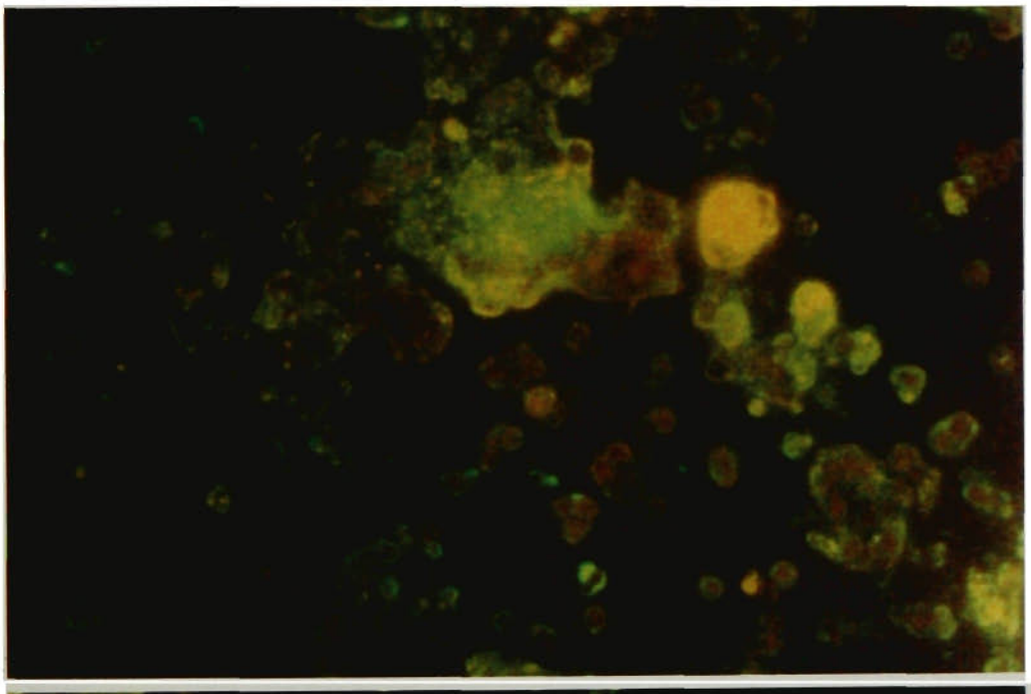
(a): Cells in control cultures.

(b): Cells in insulin treated cultures.

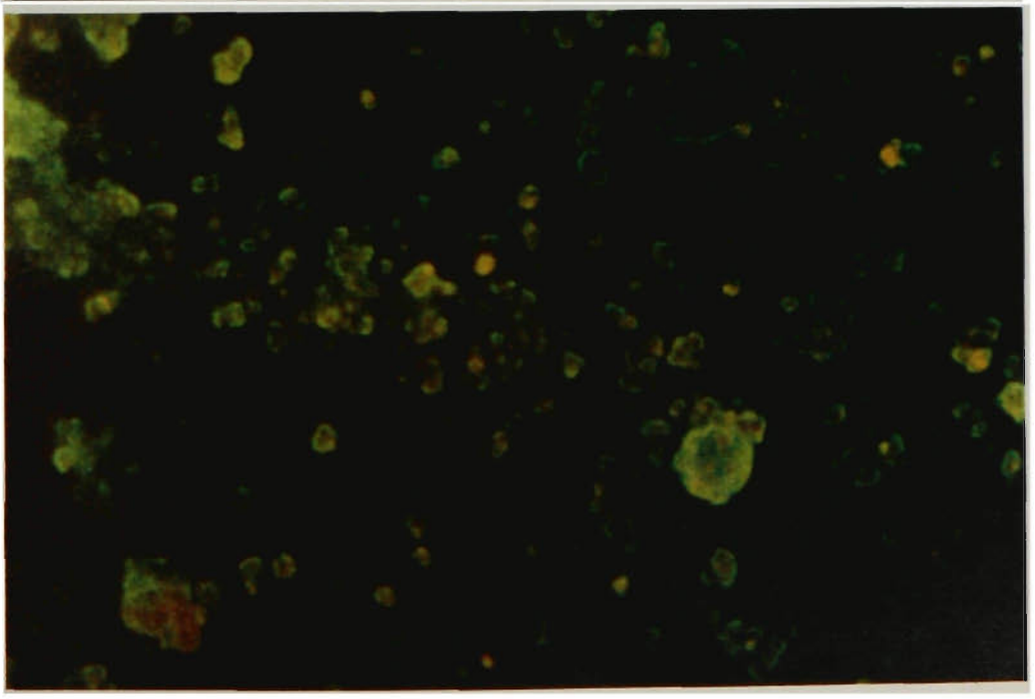
(c): Cells in IGF-I treated cultures.



a



b



c

5.4.4. *Thymidine uptake*

Thymidine uptake in control cultures was found to increase over time. Treatment with insulin, IGF-I, IGF-II or any combination of two of these factors resulted in significantly higher ($p<0.001$, $F=2.67$) levels of incorporated thymidine compared to control cultures (Table 5.1). Thymidine uptake levels in cells treated with insulin, IGF-II and a combination of these factors were significantly lower ($p<0.001$, $F=2.67$) than those observed in IGF-I treated cultures. There were no significant differences between cultures treated with IGF-II, insulin and IGF-I, insulin and IGF-II and those treated with IGF-I and IGF-II at any time.

Table 5.1: Thymidine uptake in cultured cytotrophoblasts.

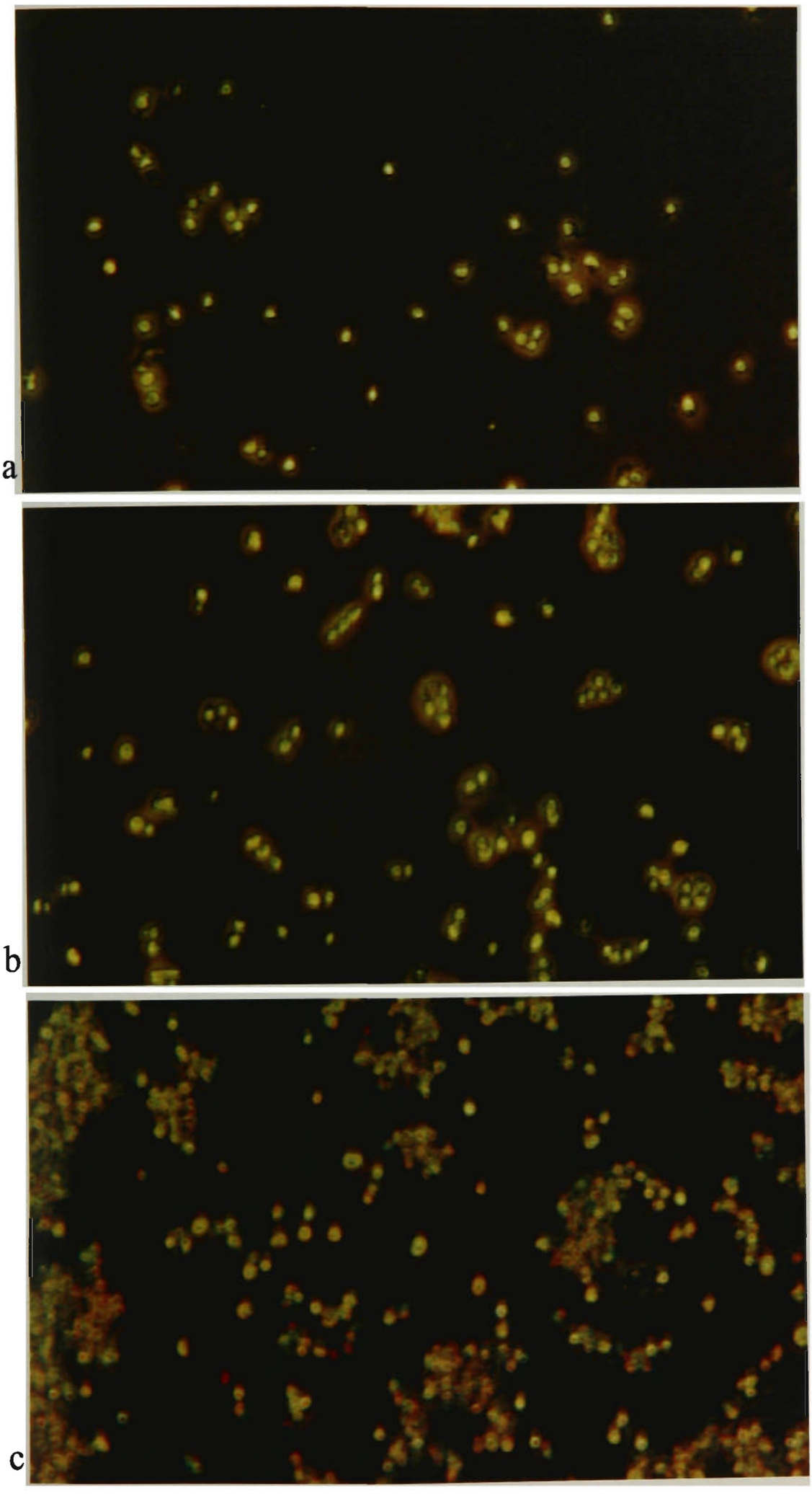
	Thymidine Uptake (cpm)		
Treatment	12h	24h	48h
control (10% FCS)	127.68 ± 45.61	337.31 ± 35.01	435.58 ± 40.23
+ insulin	212.33 ± 29.85*	397.94 ± 30.12*	583.73 ± 55.11*
+ IGF-I	302.12 ± 12.26$^{*\#}$	497.00 ± 14.89$^{*\#}$	753.02 ± 26.53$^{*\#}$
+ IGF-II	255.28 ± 10.15	450.00 ± 12.25*	611.03 ± 25.55
insulin + IGF-I	275.36 ± 14.83$^{\#}$	470.15 ± 19.95$^{\#}$	691.58 ± 29.88$^{\#}$
insulin + IGF-II	270.12 ± 15.29$^{\#}$	468.28 ± 20.05$^{\#}$	655.94 ± 22.39
IGF-I + IGF-II	257.12 ± 10.55	459.84 ± 15.03	639.89 ± 20.46

Thymidine uptake in cells grown in media supplemented with 10% FCS (control) and treated with 0.1nM insulin, 1.0nM IGF-I, 10nM IGF-II and a combination of these factors, over 48h of culture [n=30; mean ± SEM cpm]. (*=significance, $p<0.01$, between treatments; $^{\#}$=significance, $p<0.01$, vs. control and insulin treatment).

Cellular proliferation was confirmed by immunofluorescent staining for PCNA (Figure 5.4). Systematic sampling and photography of cells staining for PCNA demonstrates that a small proportion (18 ± 2%) of cytotrophoblast cultures treated with insulin is actively dividing. In cultures treated with the IGFs, however, the number of cells stained positive for PCNA increases to almost double that of control. It was also noted that the cells centrally located during aggregate formation did not stain for PCNA.

Figure 5.4.: Staining of cells with PCNA.

Photographs of cells at 24h that have been cultured under (a) control conditions (EMEM supplemented with 10% FCS) and treated with (b) 0.1nM insulin or (c) 1.0nM IGF-I. [Mag: X20]


a
b
c

5.4.5. *Immunofluorescent staining*

Prior to culture no multinucleated cells were found after the isolation procedure. Greater than 98% cell viability was obtained in each experiment as assessed by Trypan blue (0.4%) exclusion. Staining for the desmoplakins was used to identify those cells in culture that were differentiating to form giant, multinucleated cells. However, not all cellular aggregates were observed to fuse. As seen in Chapter Four, initially cells stained intensely for desmoplakins I and II. With cellular aggregation, the intensely stained desmoplakins were seen around the border of each individual cell. At 96h, the periphery of the giant fusate cells was intensely stained for the desmoplakins, indicating one large cell containing several nuclei. The staining for desmoplakin then gradually disappeared with time (Chapter Four, Figure 4.3). The proportion of multinucleated cells seen in culture differed between treatments. Insulin treatment increased the number of fusing cells while the presence of IGF-I was found to decrease the number of fusing cells compared to control (Figure 5.3).

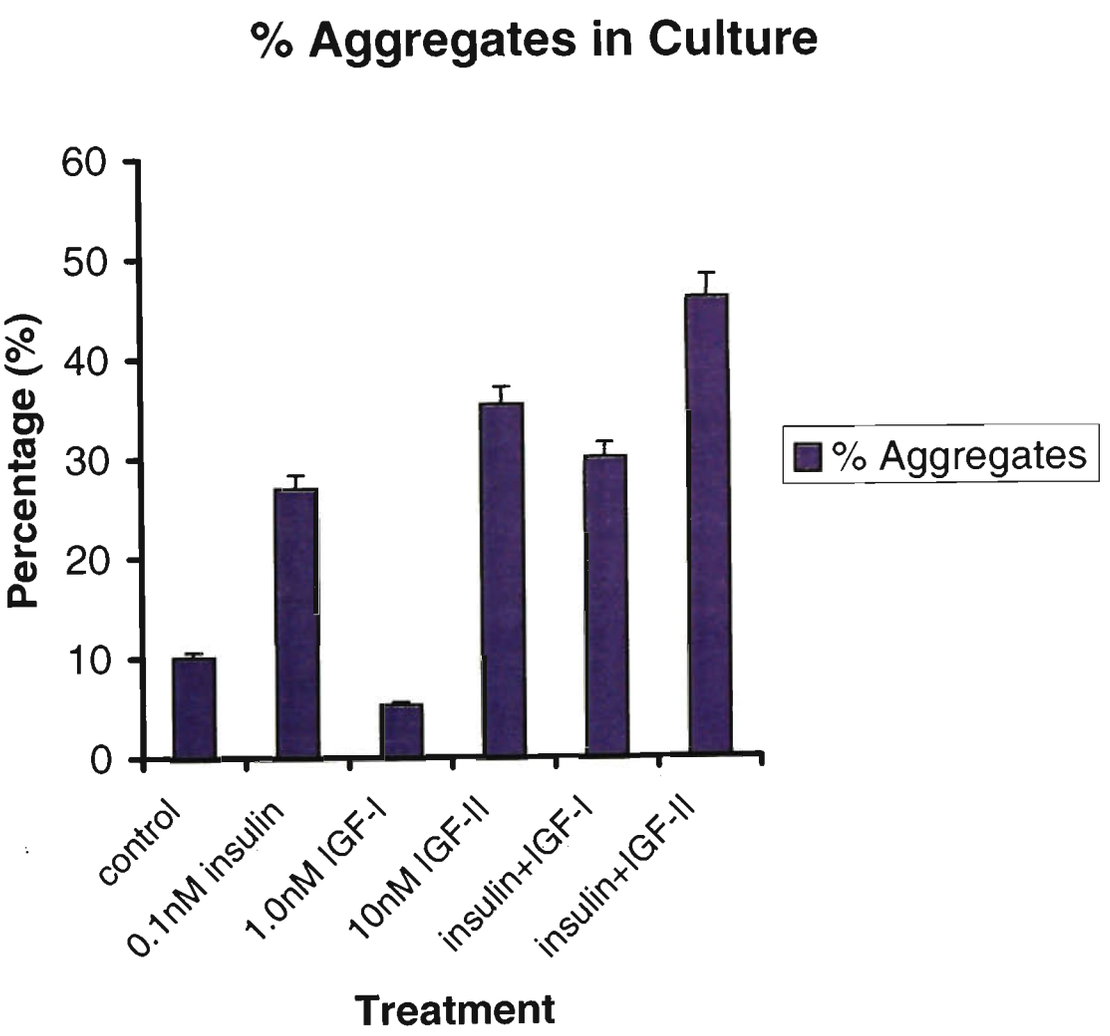
Cellular aggregates were prominent in control cultures (10.01$\pm$8.75%) after 48h incubation. However, in cultures treated with insulin, IGF-II, the combination of insulin with IGF-I and insulin with IGF-II, cell aggregates were observed as early as 24h. By 48h a significant ($p<0.05$) proportion (26.92$\pm$5.14, 35.35$\pm$5.17, 30.05$\pm$6.86 and 46.16$\pm$6.17%, respectively) of the cell population comprised fusing cells (Figure 5.5). The number of cells observed to aggregate in cultures treated with IGF-I alone, however, remained consistently low throughout the culture period.

During the morphological differentiation, the number of nuclei (data not shown) within the cell population did not significantly change. Trypan blue exclusion of cells lifted from cultured showed that minimal to no cell debris was evident in cultures under any treatment. The TUNEL assay stained few cells after 48h in culture. There were no apparent significant differences between control cultures and cultures treated with insulin or the IGFs.

Figure 5.5: Percentage aggregates of trophoblasts in culture.

A comparison of the percentage of cell aggregates at 48h in cultures grown in EMEM supplemented with 10% FCS alone (control) and treated with:

0.1nM insulin,
1.0nM IGF-I,
10nM IGF-II,
0.1nM insulin and 1.0nM IGF-I,
0.1nM insulin and 10nM IGF-II and
1.0nM IGF-I and 10nM IGF-II.


% Aggregates in Culture
Percentage (%)
60
50
40
30
20
10
0
control
0.1nM insulin
1.0nM IGF-I
10nM IGF-II
insulin+IGF-I
insulin+IGF-II
Treatment
% Aggregates

5.4.6. *hCG analysis*

Direct chemiluminescence was used to determine the concentration of hCG in the media of cytotrophoblasts in culture. The concentration of hCG in the medium of control cultures increased significantly ($p<0.001$, $t=23.6$) with time, from initial nondetectable levels to values of 20.0±5.6 mIU/mL at 96 hours. Significant increases ($p<0.001$) in hCG secretion were observed over time in cultures treated with insulin, IGF-II and a combination of these factors (35.0±3.6, 42.0±2.3 and 50.0±4.9 mIU/mL, respectively, at 96 hours). Administration of IGF-I resulted in significantly reduced ($p<0.001$, $t=5.0$) levels of hCG throughout the culture period (Table 5.2).

Table 5.2: hCG concentrations in media.

t(h)	Control	0.1nM insulin	1.0nM IGF-I	10.0nM IGF-II	Insulin IGF-I	Insulin IGF-II	IGF-I IGF-II
24	2.0±0.8	4.0±0.8	3.0±2.3	4.0±1.9	3.2±1.2	6.0±1.8	3.8±1.8
96	20.0±5.6[a]	35.0±3.6[ab]	10.0±3.3[ab]	42.0±2.3[ab]	32.0±1.8[a]	50.0±4.9[ab]	30.0±1.2[a]

Concentrations of hCG (mIU/mL) in media of cytotrophoblasts cultured for 96h and grown in EMEM supplemented with 10% FCS (control) with insulin, IGF-I, IGF-II and a combination of these factors [n=30; mean ± SEM mIU/mL]. ([a]=significance, $p<0.001$, over time; [b]=significance, $p<0.001$, between treatments)

Between treatments there were no significant differences at 24h. However, at 96h after seeding all treatments were significantly higher ($p<0.001$) than control except IGF-I treated cultures which had significantly lower ($p<0.001$, $t=10.08$) levels of hCG compared to control. There were no significant differences at any time between hCG levels of cultures treated with insulin and those with insulin and IGF-I. However, significantly higher ($p<0.01$, $t=25.26$) levels of hCG were found in the medium of cells treated with insulin and IGF-II compared to those of cells treated with insulin or IGF-II alone.

5.5. Discussion

The purpose of this study was to examine and compare the relative proliferative effects of insulin and the IGFs on cytotrophoblasts isolated from human placental tissue. The cells, of 1.048-1.062g/mL cell density, stained positive for cytokeratin 7, a specific trophoblast marker (Blaschitz *et al.,* 1997) and lacked vimentin staining. Cell morphology, size and the absence of the HLA antigen on cell membranes (along with the aforementioned criteria) confirmed that the cells isolated in this study were cytotrophoblasts.

The desmoplakin staining and multinucleated cell formation from cell fusion as well as the hCG concentrations in the medium of the cells grown under control conditions compared favorably with other studies (Hall *et al.,* 1977; Deal & Guyda, 1983; Kliman *et al.,* 1986; Douglas & King, 1990). In the present study, treatment of cells with insulin, IGF-II or both, resulted in a greater proportion cellular aggregation suggesting a potentiative role of these factors in trophoblast differentiation. Interestingly, IGF-I supplementation to trophoblast cultures was found not to stimulate cellular differentiation.

This was reflected in hCG secretion into the medium of cells confirming differentiation. The trends in hCG secretion were found to be similar to those observed by others (Hall *et al.,* 1977; Deal & Guyda, 1983; Kliman *et al.,* 1986). However, the actual hCG concentrations in the medium were found to be significantly lower than those reported elsewhere (Hall *et al.,* 1977; Deal & Guyda, 1983). The difference seen in the present study may be attributed to differences in culture conditions. These include the lower concentrations of FCS used, maintenance of cells in 1mL wells and the medium consisting of minimum essential nutrients which may not have provided sufficient nutrients or the stimulus required for high levels of hCG synthesis and secretion.

This study demonstrates the positive effects of insulin and the IGFs on the proliferative activity of cytotrophoblasts isolated from human placenta. This contrasts with other studies (Deal & Guyda, 1983; Morrish *et al.,* 1997) where cytotrophoblasts isolated from term placentae have demonstrated diminished to no

proliferative activity *in vitro*. This may be explained by differences in the methods adopted in the isolation procedure and the different phenotypes of cytotrophoblasts isolated. For example, other investigators (Morrish *et al.,* 1997) have used the 8-step enzyme digestion process which may select for a particular phenotype of cytotrophoblast.

Other studies (Kliman *et al.,* 1986; Yui *et al.,* 1994) have shown that newly dispersed cytotrophoblasts are mostly in G_0/G_1 phase of the cell cycle with approximately 15% retaining the capacity to enter mitosis (Kliman *et al.,* 1986). In the present study the increase in cell number observed in the cytotrophoblast populations treated with insulin are small (yet significant) compared to that of control cultures. Staining for PCNA demonstrates that a small percentage ($18 \pm 2\%$) of insulin treated cells remain in the cell cycle whilst a greater population of the cells do not proliferate.

What is of interest is the observed spontaneous differentiation of the cells in control cultures, which is enhanced by the addition of *in vivo* factors such as insulin and IGF-II. It has been suggested that EGF is required for complete differentiation of cytotrophoblasts (Morrish *et al.,* 1997). As insulin has been shown to stimulate growth factor release from other cell types (Boni-Schnetzler *et al.,* 1991), it may be possible that insulin may stimulate or enhance the production and secretion of EGF from trophoblast cells to potentiate syncytial formation. It may also be possible that IGF-II may have the same effects, however, this needs to be investigated further.

We have thus shown that insulin potentiates the proliferation of cytotrophoblasts isolated from 'normal' human placentae. This observation supports studies where insulin has been shown to promote proliferation in a number of tissue types (Lin *et al.,* 1992). As insulin and IGF-I share structural homology (Rechler & Nissley, 1985), it is tempting to speculate that the mitogenic activity of insulin may be mediated through the IGF-I receptor, but at a much lower affinity. It may also be possible that insulin may stimulate the secretion of endogenous IGF-I that may act in an autocrine manner to enhance cellular proliferation.

Insulin was also shown to increase the size of the cells from 8-10μm to 10-15μm diameter, which was not observed in cells treated with IGF-I. In a study (Holm *et al.,* 1975) comparing the effects of cell size on rat adipose tissue metabolism it was found that the large fat cells of aged rats were associated with a decrease in insulin effects on glucose metabolism. However, this may be the result of age and/or insulin resistance rather than the size of the cells. It is also worthy noting that the effects in adipocytes may not be reflected in trophoblast cells. The data presented in the current study suggest an increase in metabolic activity in cells treated with insulin and thus, further analysis is required to determine the link between cell size and metabolic activity in trophoblast cells.

In this study, we have shown that IGF-I promotes proliferation in human cytotrophoblasts. The potent mitogenic effects of IGF-I observed in this study also support by findings from other studies. These include the correlation of IGF-I levels to fetal size (Iwashita *et al.,* 1992; Reece *et al.,* 1994; McIntyre *et al.,* 2000) and the increased presence of IGF-I transcripts noted particularly during the proliferative phase of placental growth (Wang *et al.,* 1988).

At higher concentrations, IGF-II was shown to stimulate proliferation in these cells, as well as the accelerated morphological differentiation. Pancreatic β-cell mass is determined by the local production of IGF-II where over-expression of this growth factor has often resulted in nesidioblastosis (Hill *et al.,* 1998). Exogenous IGF-II has also been shown to protect cells from cytokine-induced apoptosis (Hill *et al.,* 1998). Therefore, the increase in cell number observed in the present study may be the result of reduced apoptosis as well as increased proliferation. The differentiative effects of IGF-II have been noted in other cells, namely the fusion myoblasts to form myofibres (Florini *et al.,* 1986).

As discussed in Chapter Two, section 2.5, structural similarities exist between the IGFs, so IGF-II may interact with the IGF-I receptors to potentiate cellular proliferation. Competition for the same receptor site was suggested by the data obtained from cultures grown in the presence of both IGFs as proliferative activity did not increase compared to that of cultures grown with either IGF. However, the increased differentiative response seen with IGF-II administration in this study

suggests that IGF-II may act via a different receptor or that it may mediate a pathway separate to that of IGF-I. It is possible that IGF-II may bind to the 'atypical' insulin receptors which have been reported to be located in the human placenta and have been shown to have unusually high affinities for insulin and IGF-II but not IGF-I (Jonas *et al.,* 1989). Yet, in other human cell types IGF-II has been shown not to require either the IGF-II or IGF-I receptors to enhance proliferation (Conover *et al.,* 1994). So is it possible that another mechanism may be at play in the human placenta?

In some laboratories (Benirschke & Kaufmann, 1995) it is believed that differentiation *in vitro* can only occur in the presence of mononuclear syncytiotrophoblastic elements as purified populations of cytotrophoblasts cultured under 'normal' conditions failed to produce syncytial fusion. It is possible that the isolated cell populations may have, if any, mononuclear syncytial fragments. As previously shown, immunostaining, morphology, density and size indicate that the cells isolated in the present study were cytotrophoblasts. Differences in culture conditions, the cellular environment and/or factors supplemented in the media may have provided the stimulus for cell fusion. The observed differences in the number of cell aggregates within control cultures compared to treated cultures were significant in this study indicating that treatment of the cells with insulin and the IGFs had an effect on the aggregation of the cells.

In this study, when combining IGF-I administration with insulin, cell numbers were significantly lower than in cultures treated with IGF-I alone. It has been shown *in vitro* that prolonged incubation with insulin decreased the binding of IGF-I (DeVroede *et al.,* 1984) which may be the result of insulin downregulating the number of IGF-I receptors (Reddan & Dzeidzic, 1982). The reduced effects of IGF-I in the presence of insulin may also be due to insulin competing with IGF-I for the type 1 receptor.

Thus, in this chapter insulin and the IGFs were shown to enhance the proliferation of cytotrophoblasts isolated from 'normal' placentae with IGF-I proving to be more potent in its effects than those of insulin and IGF-II. Insulin and/or IGF-II

administration potentiated the aggregation and differentiation of cytotrophoblasts whilst IGF-I did not. When combining insulin with IGF-I, proliferative effects were reduced compared to IGF-I treatment alone whilst the combination of IGF-II and insulin accelerated differentiation compared to either insulin or IGF-II treatment. In subsequent chapters, the use of insulin receptor antibody will allow the delineation of the roles of insulin and IGF-I in the proliferation and differentiation processes in cytotrophoblasts.

These results coupled with other studies demonstrating the presence and overexpression of the IGFs during gestation thus strongly suggest that insulin, IGF-I and IGF-II together play an important role in regulating the proliferation and differentiation of cells in the human placenta. Clearly other factors, such as binding proteins, may also impact on the proliferation and differentiation mediated by insulin and the IGFs. Further investigations are required to explore the role of insulin and the IGFs in placental macrosomia in gestational diabetes and to determine the receptors through which these ligands mediate their actions.

CHAPTER SIX

A COMPARATIVE STUDY: THE POSSIBLE GROWTH PROMOTING FUNCTION OF INSULIN ON CYTOTROPHOBLASTS ISOLATED FROM THE PLACENTAE OF 'NORMAL' AND DIABETIC PREGNANCIES

The importance of the 'normal' growth and function of the placenta for optimal fetal growth is exemplified in abnormal conditions such as pregestational diabetes. Pregnancies complicated by IDDM have been associated with placental and fetal macrosomia for which insulin has been implicated. In this chapter, the effects of insulin on the growth of cytotrophoblasts isolated from diabetic pregnancies will be examined and compared to those of 'normal'.

6.1. Introduction

Pre-existing diabetes in pregnant mothers effects the growth and body composition of the fetus and surrounding fetal tissues (Pedersen, 1977). Despite advancements in the methods of maintaining glycemic control in pregnant women, macrosomia remains a significant problem (Berk *et al.,* 1989), particularly in pregnancies complicated by IDDM (Small *et al.,* 1987).

As outlined in Chapter Two, the fetal maldevelopment and macrosomia often associated with pregnancies complicated by IDDM are believed to be linked with increasing maternal glycemic levels and glucose uptake by placental and fetal cells. It is becoming more apparent that increased glucose uptake is not the only factor contributing to placental and fetal malformations in diabetic pregnancies. Other nutrient sources and increased synthesis of several growth factors (such as the IGFs) have been implicated (Eriksson & Swenne, 1993; Styrud *et al.,* 1995). It is also becoming evident that circulating levels of insulin may somehow be involved; either indirectly through the regulation of metabolism, glucose uptake and other cellular processes or directly as a growth stimulant. The role of insulin in the outcomes of pregnancies complicated by IDDM needs further clarification.

6.2. Aims

The aims of this study:

1. To examine and compare the effects of insulin and the IGFs on cultured cytotrophoblasts isolated from human placentae of 'normal' and diabetic mothers.
2. To examine the effects of increasing glucose concentrations on cultured cytotrophoblasts isolated from human placentae of 'normal' and diabetic mothers, with/without exogenous insulin or IGF-I administration.

6.3. Methods and Materials

6.3.1. *Experimental protocol for culture conditions:*

Cells isolated from placentae of 'normal' (n=12) and diabetic -- GDM (n=12) and IDDM (n=10) -- mothers were divided into several treatment groups:

1. i. cells grown in EMEM supplemented with 10% FCS alone (control); with
   ii. the addition of 0.1nM insulin;
   iii. the addition of 1.0nM IGF-I;
   iv. the addition of 10.0nM IGF-II;
   v. any combination of two of these factors; and
2. i. cells grown in EMEM supplemented with 10% FCS alone (control); with
   ii. 5.0mmol/L glucose, and with insulin or IGF-I,
   iii. 7.5mmol/L glucose, and with insulin or IGF-I, and
   iv. 10.0mmol/L glucose, and with insulin or IGF-I.

Clinical information regarding placental tissue and patients, methods adopted and the materials used are as described previously in Chapter Three. All cultures seeded at 0.5 x 10^6 cells/mL contained 0.5μCi/ml ^{3}H-thymidine (sections 3.2.1-3.2.3, 3.2.5, 3.2.7, 3.2.8). Observations and repeated measures were performed in quadruplicate and taken every 2h for 24h and then at 48 and 72h after seeding. At the appointed times, cells were counted (section 3.3) and assayed for thymidine uptake (section 3.4). Slides of cultured cells at the designated times were prepared for immunohistochemical staining for cytokeratins, desmoplakins and PCNA (section 3.5). The proportion of cells staining positive was determined by the systematic counting of cells from 25 random photographs taken of each sample (prepared as per Chapter 3, section 3.5) which were performed in triplicate replicates.

Cells were also assayed for apoptosis using the TUNEL assay (section 3.5.4). The media supernatant was collected after centrifugation (where the cell pellet was discarded) for hCG analysis using direct chemiluminescence techniques (performed in duplicate, section 3.6).

6.3.2. Flow Diagram of Methods

Simple Flow diagram of overall experiment:

Human Placental Tissue (Consent and Collection)
[36-41 weeks -- 'normal', gestational diabetes [GDM and IDDM]

↓

Mechanical and Enzymatic Digestion

↓

Filtration and Centrifugation
(Jones *et al.*, 1989)

↓

Discontinuous Percoll Gradient
[5%, 10%, 20%, 40%, 60%, 80%]
(Kliman *et al.*, 1986)

↓

HLA Purification
(Schmon *et al.*, 1991)

↓

Incubation at 37^0C with 5% CO_2 in air

↓

Plated in 24-well culture dishes flasks
maintained in
EMEM and 10% FCS with:
0.1nM Insulin;
1.0nM IGF-I;
10nM IGF-II;
0.1nM Insulin and 1.0nM IGF-I;
0.1nM Insulin and 10.0nM IGF-II;
1.0nM IGF-I and 10.0nM IGF-II.
Glucose [5.5, 7.5 & 10mmol/L]
Glucose and insulin or IGF-I

Protocol Time (h)	0	12	24	48	72	96
Cell number	**********			*	*	*
Thymidine uptake	**********			*	*	*
Cytochemical stains	*	*	*	*	*	*
hCG analysis	*	*	*	*	*	*

6.3.3. *Statistical and data analysis:*

As described in Chapter Three, section 3.9, all data is expressed as the mean ± SEM. A MANOVA modified for repeated measures was performed on the data collected from cell counts and thymidine uptake analyses using the SPSS statistical package. The level of significance was at $p<0.01$. A Student T test was adopted to analyse the percentage of aggregates at 48h between treatments and the different placentae types, and hCG data between mean values at zero time and at the end of the culture period. The level of significance was at $p<0.05$.

6.4. Results

6.4.1. *Subjects:*

Placenta from each subject was obtained at caesarean section from between 36 and 41 weeks of gestation. For further details on criteria for grouping the placentae refer to Chapter Three, section 3.1. HbA1C results for GDM subjects were within normal range (4.7-6.4 %) and fasting blood glucose levels were found to be higher but not significantly different from that of 'normal'. The weight of the infant and placenta were not significantly different between 'normal' and GDM subjects. For IDDM subject data, however, placental and birth weights were found to be significantly greater ($p<0.01$) compared to that of 'normal' and GDM subjects. Fasting blood glucose and HbA1C levels were also found to be significantly higher ($p<0.01$) than that of 'normal' and GDM subjects (Table 6.1).

Table 6.1. Characteristics of 'normal', GDM and IDDM mothers and their offspring.

Parameters	Subjects 'Normal'	GDM	IDDM
n	12	12	10
Length of gestation (weeks)	39.1 ± 0.7	38.2 ± 0.6	37.0 ± 1.1[a]
Parity	2.0 ± 0.5	2.3 ± 1.0	1.6 ± 0.5
Birth weight (g)	3548.3 ± 301.3	3204.6 ± 435.9	4174.3 ± 259.1[b]
Placental weight (g)	565.0 ± 120.4	642.8 ± 97.5	911.4 ± 49.6[b]
Fetal/placental weight ratio	6.3 ± 1.2	5.0 ± 0.7	4.6 ± 0.3[a]
Fasting blood glucose (M)	4.8 ± 0.6	5.6 ± 0.5	7.7 ± 1.8[c]
HbA_{1C} (%)	5.0 ± 0.8	6.0 ± 0.6	7.4 ± 0.8[a]

Data shows details of placental, fetal and blood glucose levels of mothers and offspring of diabetic and 'normal' pregnancies. [a] *$p<0.01$ compared to 'normal' data only;* [b] *$p<0.01$ compared to 'normal' and GDM data;* [c] *$p<0.05$ compared to 'normal' and GDM data.*

6.4.2. *Confirmation of cell identity*

As shown in previous Chapters Four and Five, the cells isolated and purified corresponded to 1.048-1.062g/mL cell density and comprised small (8-10μm diameter), round, mononuclear cells. The cells isolated from 'normal' and diabetic placentae comprised of cytokeratin 7 positive cells. During culture, >95% of cells observed at peak proliferative time (t=24h) for control and all treated cultures (insulin, the IGFs and glucose) were found to be positive for cytokeratin 7 (Figure 6.1). Over time the population of cells comprising of cytokeratin 7 staining cells was found to be consistently high and these were found interspersed with syncytiotrophoblasts and other aggregating cytotrophoblast cells. No more than 5% of the mononuclear cell population stained for vimentin throughout the culture period.

Figure 6.1. Comparing immunocytochemical staining of cytotrophoblasts from 'normal' and diabetic placentae. [Mag. X20 (b, c); X40 (a, d, e, f)]

The following are cytotrophoblasts isolated from:

(a) GDM placentae and stained with cytokeratin 7, at 24h culture.

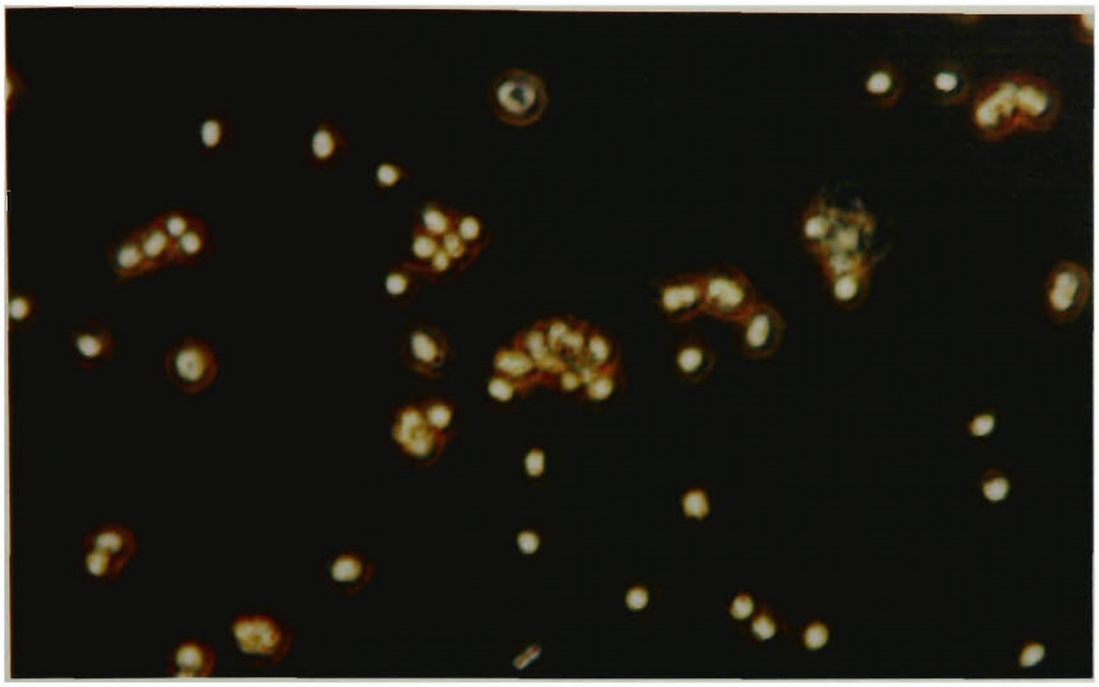
(b) 'Normal' placentae and stained with cytokeratin 7, at 24h culture.

(c) IDDM placentae and stained with cytokeratin 7, at 24h culture.

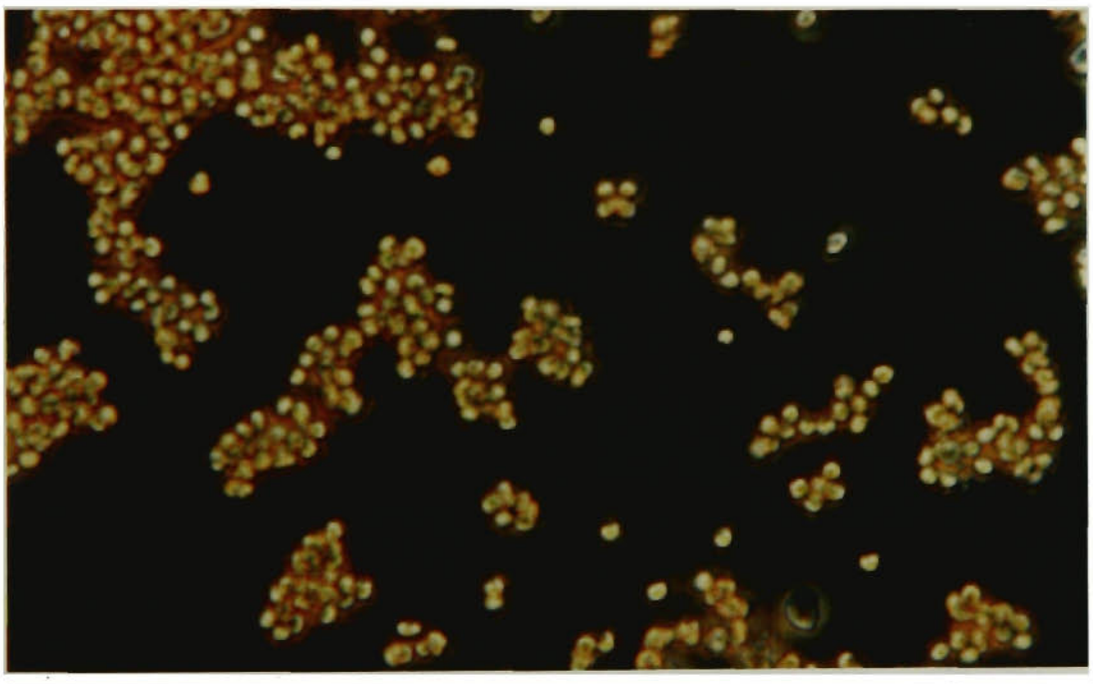
(d) GDM placentae and stained with desmoplakins I and II, at 72h culture.

(e) 'Normal' placentae and stained with desmoplakins I and II, at 72h culture.

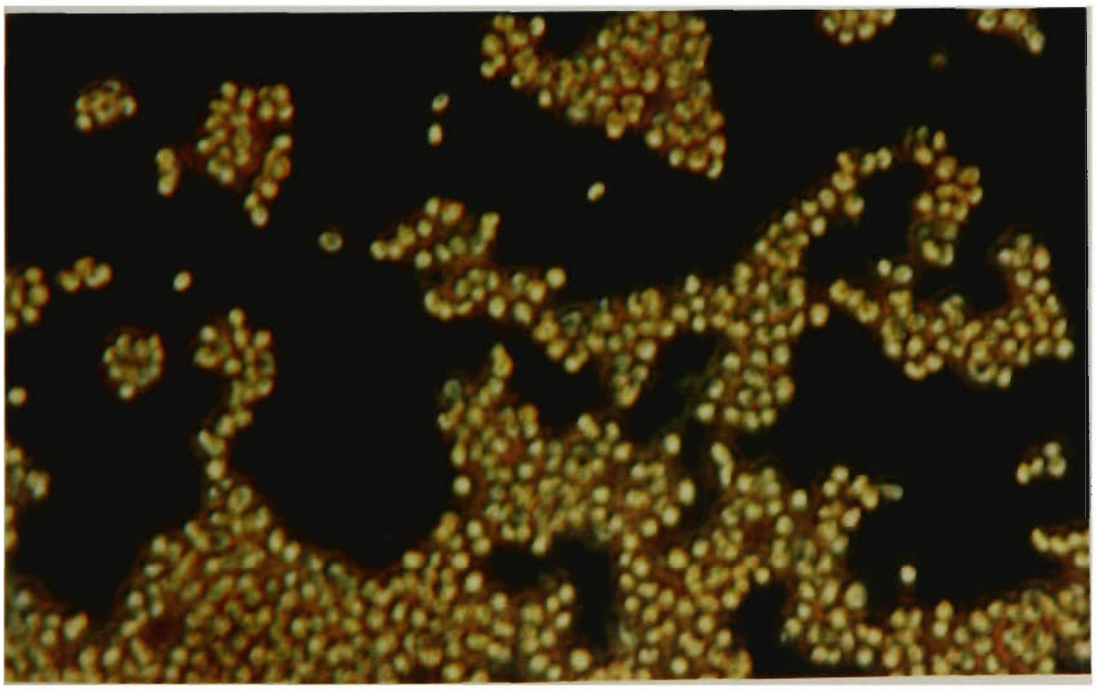
(f) IDDM placentae and stained with desmoplakins I and II, at 72h culture.



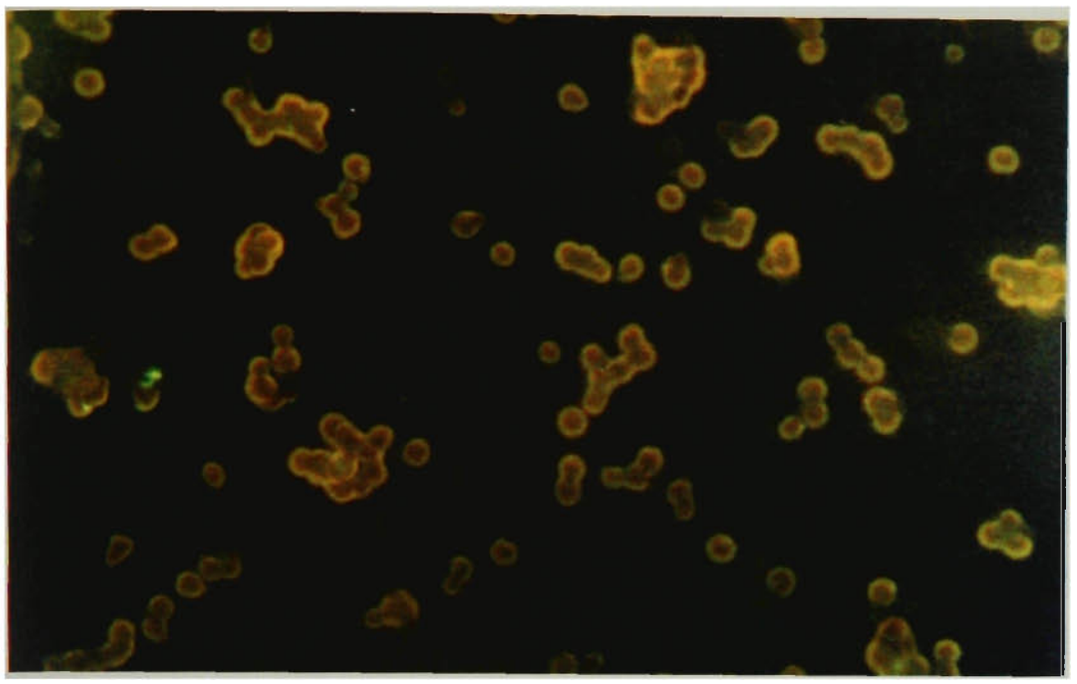
a



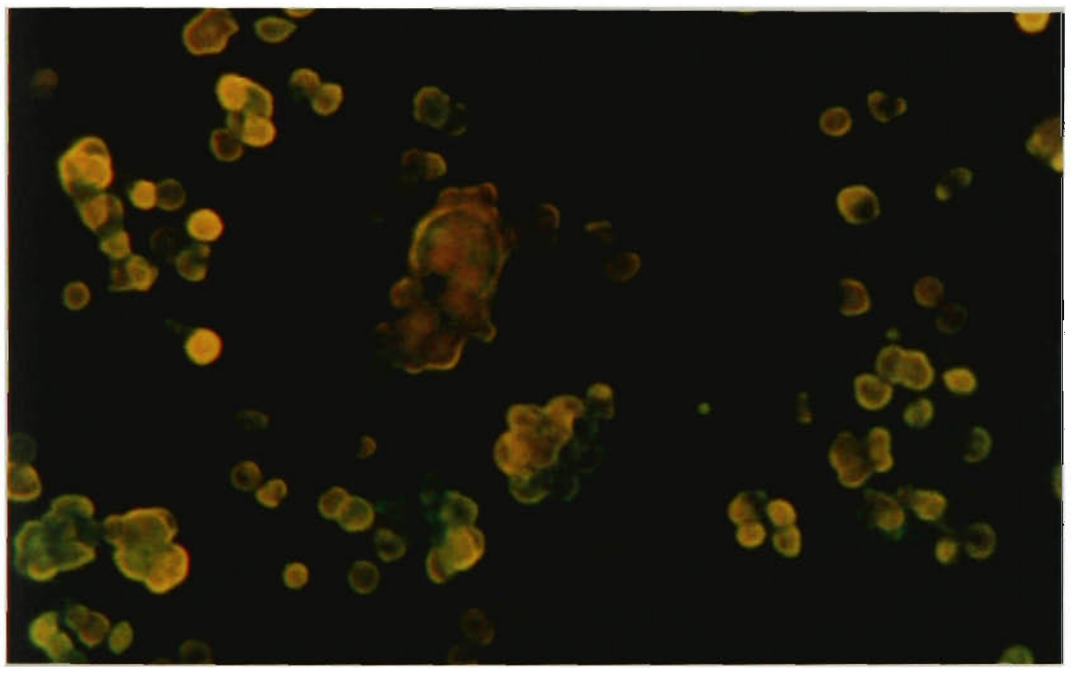
b



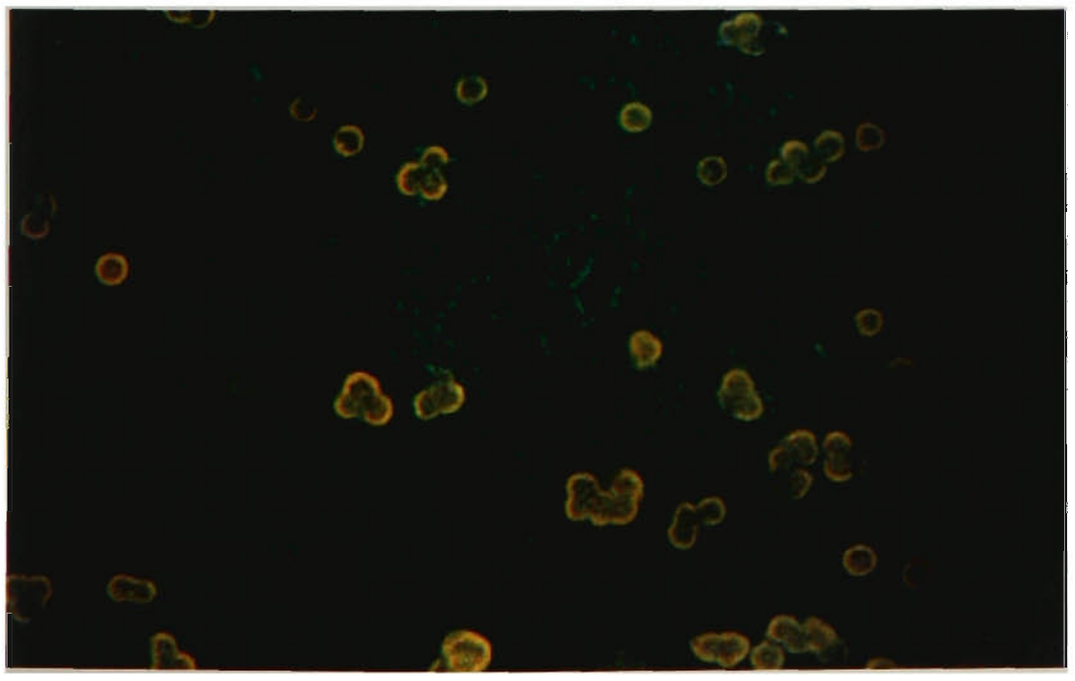
c



a



b



c

6.4.3. *Cell from 'normal' placentae*

As described previously in Chapter Five, cells isolated from placentae of 'normal' mothers, cultured under control conditions, significantly ($p<0.0001$, $F=188.24$) increased in number, peaking ($0.80 \pm 0.01 \times 10^6$ cells/mL) at 24h. The addition of insulin significantly increased ($p<0.05$, $F=4.16$) cell number, throughout the culture period, compared to 'normal' control cultures (Figures 6.2.a. and 6.2.b.). Cell numbers declined after 24h in both control and insulin-treated cultures, however, the decrease was most prominent in cultures treated with insulin. This reduction in cell number coincided with cellular aggregation and differentiation to form syncytia as observed by using immunocytochemical staining for the desmoplakins (Figure 6.1). A few aggregating cells (~10%) were observed to fuse by 96h.

6.4.4. *Cells isolated from placentae of diabetic pregnancies*

At 24h, the cell numbers in control cultures of cells isolated from GDM placentae were found to be significantly lower ($p<0.0001$, $F=31.08$) than that of 'normal' control cultures and did not significantly change over time. GDM cells treated with insulin, were found to be significantly higher ($p<0.0001$, $F=182.73$) in number, from 24h in culture and onwards, compared to GDM control cultures but numbers were significantly lower ($p<0.0001$, $F=419.23$) than those of cells treated with insulin in 'normal' cultures. Cell numbers in insulin treated GDM cultures significantly increased within the first 24h. After 24h, cell number declined, however, this was not significant over time (Figures 6.2.a and 6.2.b).

Cell numbers in both control and insulin-treated cultures of cells isolated from the placentae of IDDM mothers significantly increased ($p<0.0001$, $F=299.24$) up to 24h before regressing gradually in number by 72h. In insulin treated IDDM cultures cell numbers were significantly higher ($p<0.0001$, $F=57.09$) than those of IDDM control cultures (Figures 6.2.a and 6.2.b). These cultures were found to be significantly higher ($p<0.0001$, $F=113.33$) in cell number compared to cultures of cells isolated from placentae of 'normal' and GDM mothers.

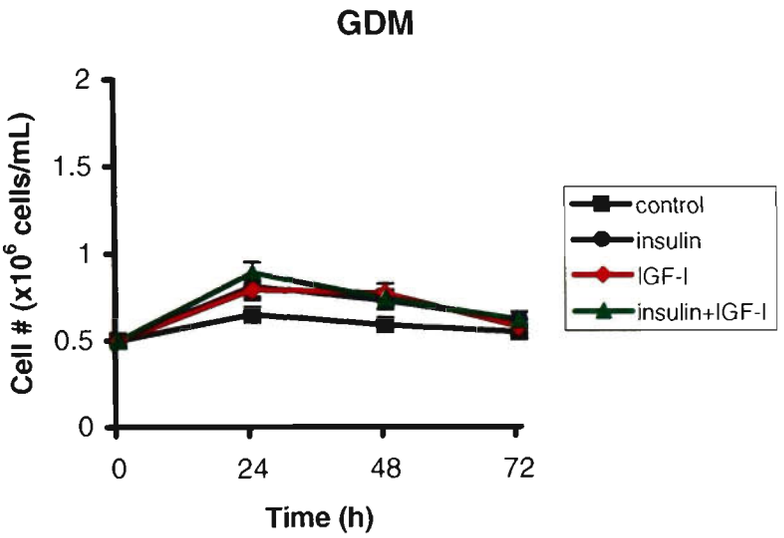
Figure 6.2.a. Comparison of numbers of cells isolated from 'normal' and diabetic placentae: Insulin and IGF-I.

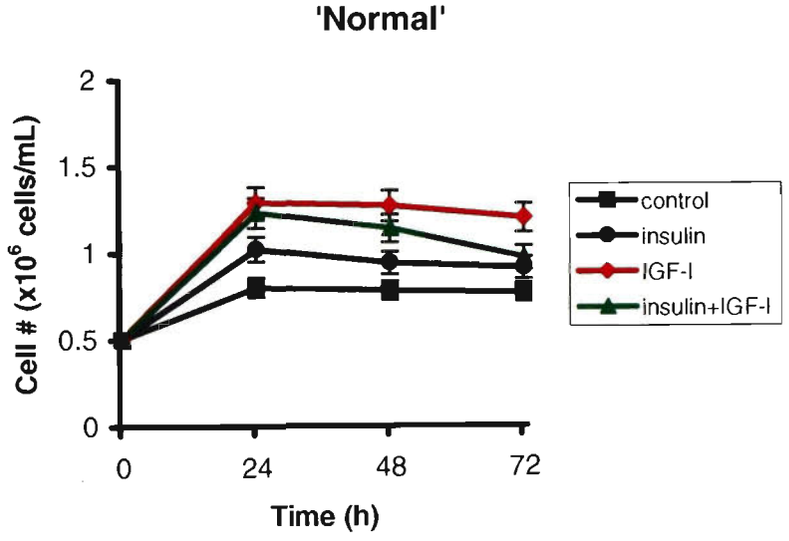
Graphs represent the number of cells grown in EMEM and supplemented with 10%FCS with 0.1nM insulin, 1.0nM IGF-I or both [SEM x 10^6 cells/mL].

(a) GDM placentae (n = 12),

(b) 'Normal' placentae (n = 12), and

(c) IDDM placentae (n = 10).


GDM
Cell # (x10^6 cells/mL)
2
1.5
1
0.5
0
0
24
48
72
Time (h)
control
insulin
IGF-I
insulin+IGF-I


'Normal'
Cell # (x10^6 cells/mL)
2
1.5
1
0.5
0
0
24
48
72
Time (h)
control
insulin
IGF-I
insulin+IGF-I

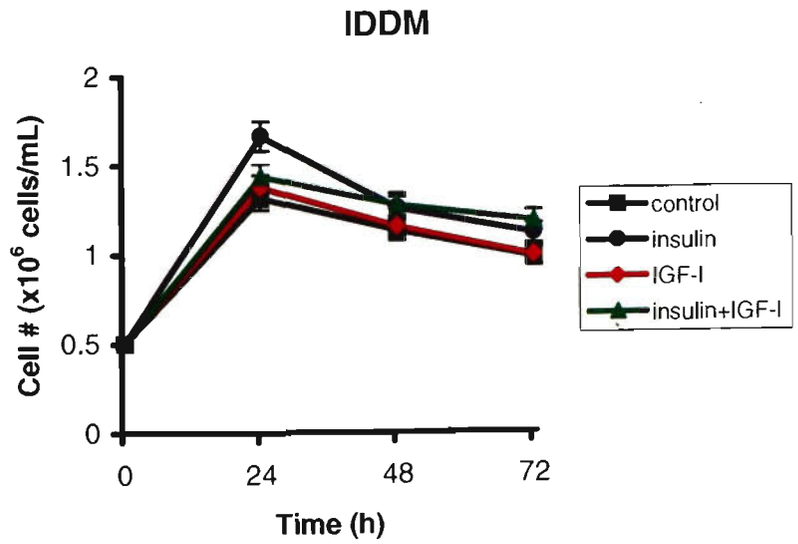

IDDM
Cell # (x10^6 cells/mL)
2
1.5
1
0.5
0
0
24
48
72
Time (h)
control
insulin
IGF-I
insulin+IGF-I

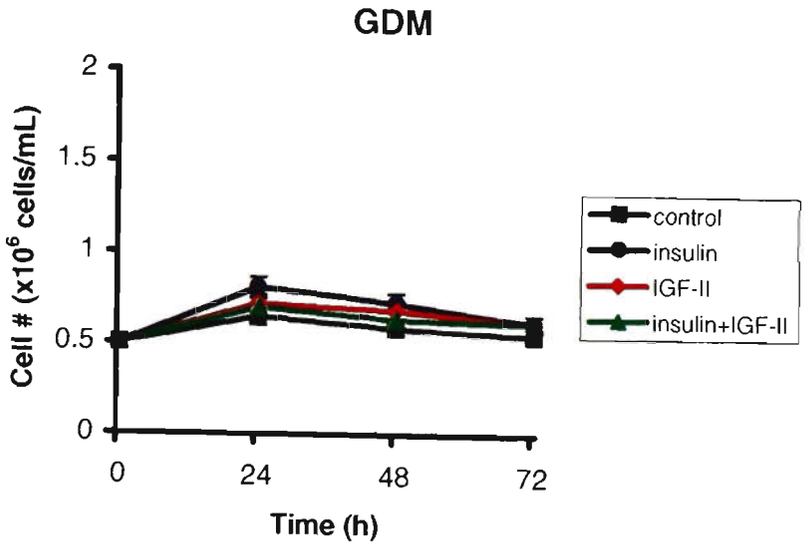
Figure 6.2.b. Comparison of numbers of cells isolated from 'normal' and diabetic placentae: Insulin and IGF-II.

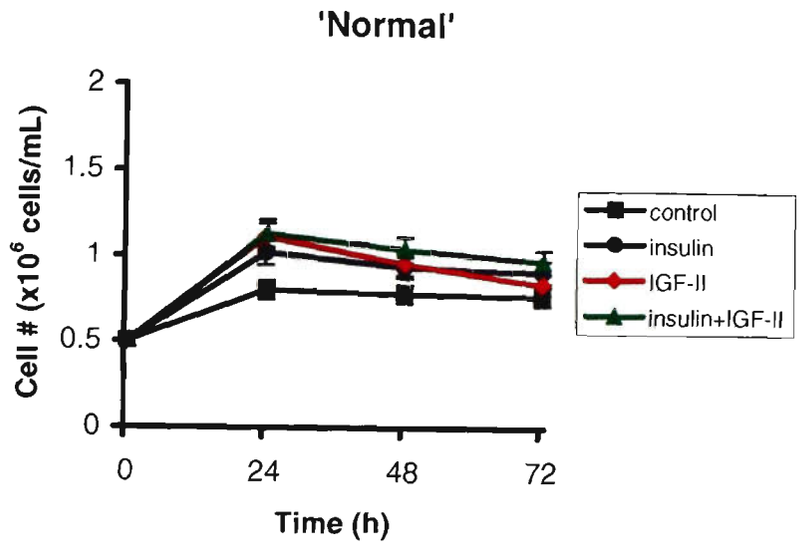
Graphs represent the number of cells grown in EMEM and supplemented with 10%FCS with 0.1nM insulin, 10.0nM IGF-II or both [SEM x 10^6 cells/mL].

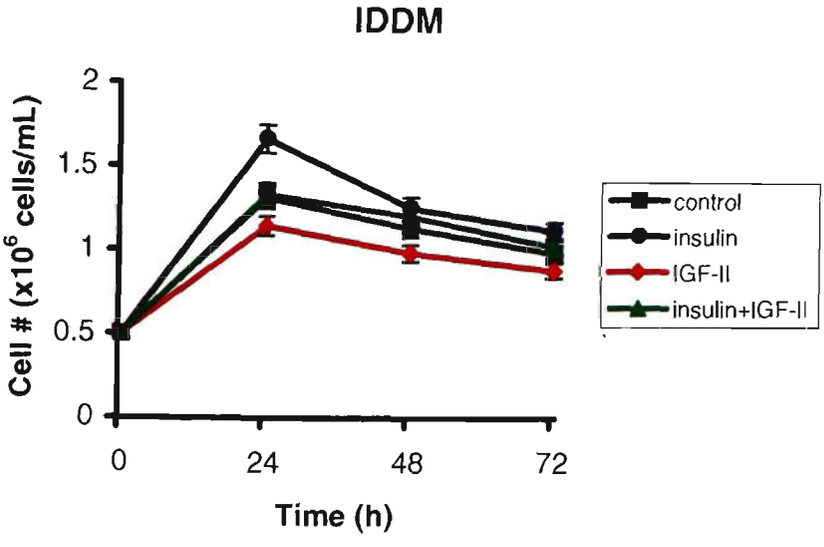
(a) GDM placentae (n = 12),

(b) 'Normal' placentae (n = 12), and

(c) IDDM placentae (n = 10).


GDM
Cell # (x10^6 cells/mL)
2
1.5
1
0.5
0
0
24
48
72
Time (h)
control
insulin
IGF-II
insulin+IGF-II


'Normal'
Cell # (x10^6 cells/mL)
2
1.5
1
0.5
0
0
24
48
72
Time (h)
control
insulin
IGF-II
insulin+IGF-II


IDDM
Cell # (x10^6 cells/mL)
2
1.5
1
0.5
0
0
24
48
72
Time (h)
control
insulin
IGF-II
insulin+IGF-II

6.4.5. *The effects of the IGFs*

The effects of the IGFs on cytotrophoblast proliferation in 'normal' tissue have been described in detail in Chapter Five. Graphs are however presented again in this chapter to provide visual comparisons of the data (Figures 6.2.a and 6.2.b).

In GDM cell cultures treatment with IGF-I or insulin and IGF-I was found to significantly increase ($p<0.01$, $F=182.73$) cell numbers over time compared to GDM control cultures but were not significantly different from those of insulin treated GDM cultures (Figure 6.2.a). IGF-II treatment either alone or in combination with insulin did not significantly differ in cell numbers compared to those of control GDM (Figure 6.2.b). However, all GDM treated cultures were found to be significantly lower ($p<0.001$, $F=61.62$) than those of 'normal'.

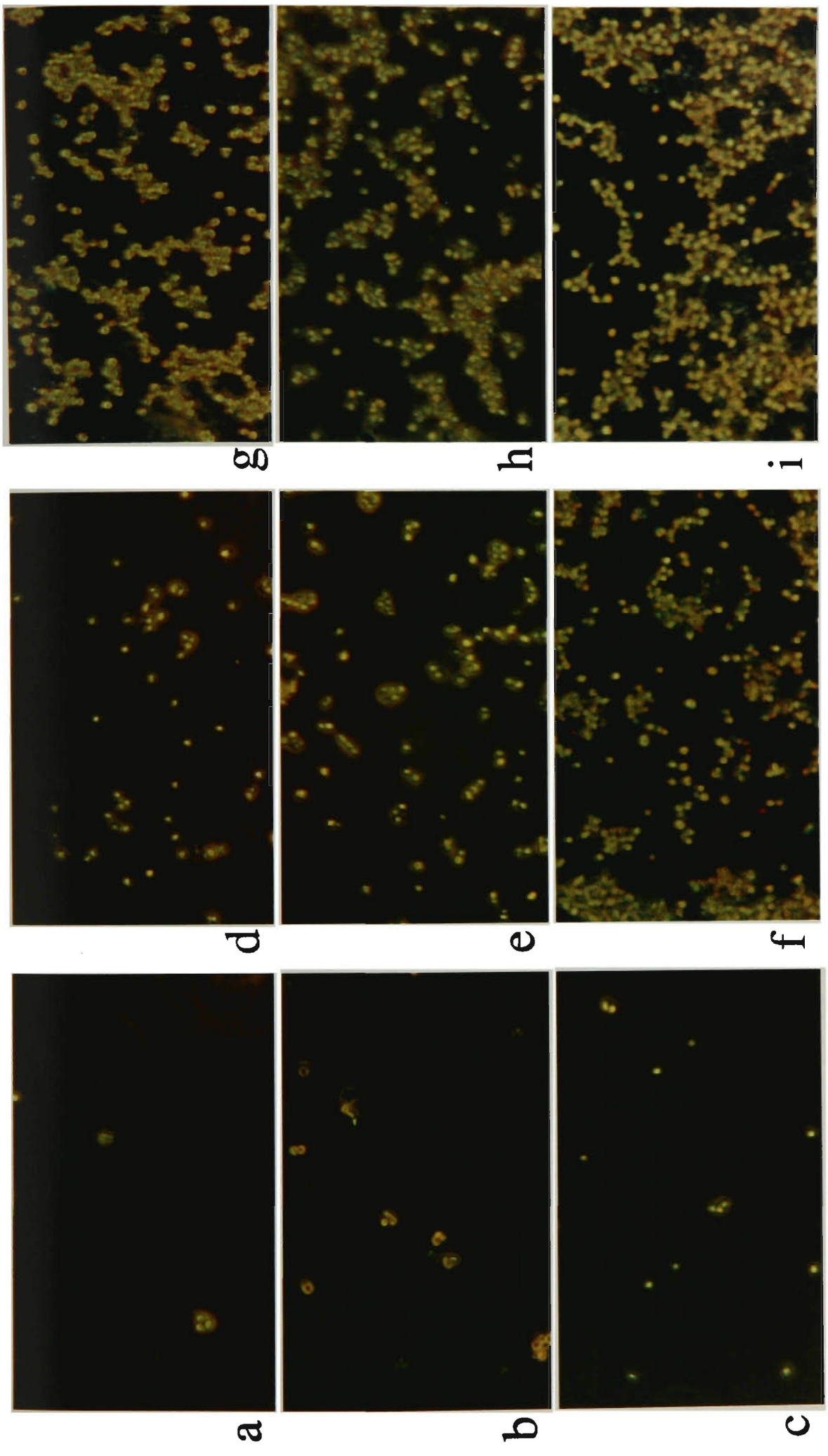
Treatment of IDDM cultures with IGF-I or insulin and IGF-I resulted in significant increases ($p<0.001$, $F=32.97$) in cell numbers over time but were found not to be significantly different from those of IDDM control cultures or IDDM cultures treated with insulin after 48h (Figure 6.2.a). IDDM cultures treated with IGF-II significantly lowered ($p<0.01$, $F=31.66$) cell numbers compared to IDDM control whilst the combination of insulin and IGF-II resulted in cell numbers not being significantly different from IDDM control cultures but significantly higher ($p<0.01$, $F=339.47$) than those of IGF-II treated IDDM cultures (Figure 6.2.b).

6.4.6. *Thymidine uptake*

Thymidine uptake in control cultures of cells from placentae of 'normal' and diabetic mothers was found to increase steadily over time. All cultures treated with the addition of insulin and the IGFs, demonstrated significantly higher ($p<0.05$, $F=4.16$) levels of incorporated thymidine compared to their respective control cultures. Data not shown. Thymidine uptake levels in cells from IDDM placentae were found to be significantly higher ($p<0.01$, $F=297.00$) than those of 'normal' placentae which, in turn, were found to be significantly higher ($p<0.01$, $F=123.81$) than those of GDM placentae.

Figure 6.3. Comparisons of PCNA staining of cells isolated from 'normal' and diabetic placentae (t=24h).

(a) Cells of GDM placentae, control;
(b) Cells of GDM placentae, treated with 0.1nM insulin; and
(c) Cells of GDM placentae, treated with 1.0nM IGF-I;
(d) Cells of 'normal' placentae, control;
(e) Cells of 'normal' placentae, treated with 0.1nM insulin; and
(f) Cells of 'normal' placentae, treated with 1.0nM IGF-I;
(g) Cells of IDDM placentae, control;
(h) Cells of IDDM placentae, treated with 0.1nM insulin; and
(i) Cells of IDDM placentae, treated with 1.0nM IGF-I.
[Mag X20]


a
b
c
d
e
f
g
h
i

Systematic sampling and photography of cells staining for PCNA demonstrates that a small proportion (~20%) of cytotrophoblasts in cultures isolated from 'normal' tissue and treated with insulin was actively dividing. In cultures treated with the IGFs, however, the number of cells stained positive for PCNA increased to almost double that of control (Figure 6.3). It was also noted that the cells that were involved in forming aggregates did not stain for PCNA.

In the cultures of cells isolated from the placentae of diabetic mothers, differences in PCNA staining were noted (Figure 6.3). In cells from GDM tissue very few cells stained positively for PCNA. Treating the cells with insulin or the IGFs did not improve cell proliferation as no differences compared to control GDM cultures were noted. Much of the population comprised non-proliferating cells and cellular debris. In cells isolated from placentae of IDDM mothers, a large proportion of the cells stained positive for PCNA. This was observed in all cultures regardless of ligand treatment (Figure 6.3).

6.4.7. *Immunofluorescent staining for in vitro differentiation*

As observed in the preceding Chapters Four and Five, no multinucleated syncytiotrophoblasts were observed following the isolation procedure and greater than 98% of the cell population obtained in each experiment was viable. Staining for the desmoplakins, as noted in previous chapters, was initially located at the periphery of the cells (Figure 5.3). As fusion began, by 72h, the periphery of these cells was intensely stained for the desmoplakins, indicating one large multinucleated cell. By 96h, the staining for the desmoplakins had faded and gradually disappeared over time.

Cellular aggregates were prominent in control cultures from 'normal' placentae, after 48h incubation. However, in 'normal' cell cultures treated with insulin, cell aggregates were present after 24h, so that by 96h a considerable proportion of the cell population comprised fused cells compared to t=0. The proportion of cells observed to aggregate in cultures of cells isolated from diabetic pregnancies remained consistently low throughout the culture period (Table 6.2). The percentage aggregates was found to be significantly lower ($p<0.05$) in cultures of

cells isolated from IDDM placentae compared to that of GDM cell cultures. Insulin treatment did not significantly change the number of aggregates within the population of cells isolated from either diabetic placentae type (Table 6.2). Not all aggregates in culture fused to form multinucleated cells over the time period observed. TUNEL assay revealed that very few cells in culture were apoptotic throughout the culture period. Data not shown.

Table 6.2. Comparisons of percentage cellular aggregates

	Percentage aggregates in cytotrophoblast cultures		
Treatment	'Normal'	GDM	IDDM
control	20.01 ± 8.75	15.51 ± 2.99	7.16 ± 3.17[bc]
0.1nM insulin	36.92 ± 5.14[a]	18.99 ± 5.51[b]	10.05 ± 3.95[bc]

The percentage of aggregates at 48h in cultures of cells isolated from 'normal' (n=12), GDM (n=12) and IDDM (n=10) placentae and grown in EMEM supplemented with 10% FCS alone (control) and treated with insulin (mean ± SEM %). [a] *$p<0.05$ compared to control cultures;* [b] *$p<0.05$ compared to 'normal' cultures only;* [c] *$p<0.05$ compared to GDM cultures.*

6.4.8. *hCG analysis*

The concentration of hCG in the medium of 'normal' control cultures increased with time, from initial nondetectable levels to peak values of 30.00±4.50mIU/mL at 96 hours. Significant increases ($p<0.05$) in hCG secretion were observed in cultures from 'normal' placentae, treated with insulin, with a peak of 50.00±5.50mIU/mL at 96 hours (Table 6.3).

The media concentration of hCG in cultures of GDM placentae treated with insulin were also found not to be significantly different (25.50 ± 7.50mIU/mL) from that of GDM control and 'normal' control cultures, but were significantly lower ($p<0.05$) than that of 'normal' cultures treated with insulin. In insulin treated IDDM cultures the concentration of hCG in the medium was not significantly different from that of IDDM control nor GDM control cultures. However, this was found to be significantly lower ($p<0.05$) than that of 'normal' cultures and that of insulin treated GDM cell cultures (Table 6.3).

Table 6.3. Comparisons of hCG concentrations in media

	hCG concentrations (mIU/mL)		
Treatments	'Normal'	GDM	IDDM
control	30.00 ± 4.50	20.66 ± 9.33	10.50 ± 5.25[b]
0.1nM insulin	50.00 ± 5.50[a]	25.50 ± 7.50[b]	12.60 ± 6.00[bc]

Concentrations of hCG at 96h in media of cells isolated from 'normal' (n=12), GDM (n=12) and IDDM (n=10) placentae grown in EMEM supplemented with 10% (v/v) FCS (control) and treated with insulin (mean ± SEM mIU/mL). [a] *$p<0.001$ compared to control cultures;* [b] *$p<0.001$ compared to 'normal' cultures;* [c] *$p<0.05$ compared to GDM cultures.*

Cell cultures obtained from either diabetic type placentae and treated with the IGFs was found not to significantly increase hCG concentrations compared to their respective controls. However, again these concentrations were found to be significantly lower than those of all 'normal' cultures. Data not shown.

6.4.9. *Preliminary investigations into the effects of glucose*

The effects of increasing glucose concentrations, to replicate hyperglycaemic conditions *in vitro,* were examined. Three glucose concentrations (dry glucose added to the medium) were used: 5mmol/L (normal physiological concentration, control), 7.5mmol/L (as can sometimes be seen in diabetic pregnancies) and 10mmol/L.

Cultures grown in 5mmol/L glucose were control cultures and have been described in detail previously in sections 6.4.3 and 6.4.4. Increasing the glucose concentration to 7.5mmol/L did not significantly change cell numbers compared to cultures grown with 5mmol/L glucose in all GDM treated cultures and 'normal' control cultures (Figure 6.4). In 'normal' cultures treated with insulin the increase in glucose concentration was found to enhance cell number compared to cultures grown in 5mmol/L glucose but not significantly.

Figure 6.4. The number of cytotrophoblasts cultured with varying glucose concentrations: Insulin and IGF-I (n=4).

The following cultures were grown with 5mmol/L (green line), 7.5mmol/L (red line) and 10mmol/L (blue line) glucose in the medium. Cells were isolated from:

(a) GDM placentae: control,

(b) GDM placentae: treated with 0.1M insulin,

(c) GDM placentae: treated with 1.0M IGF-I,

(d) 'Normal' placentae: control,

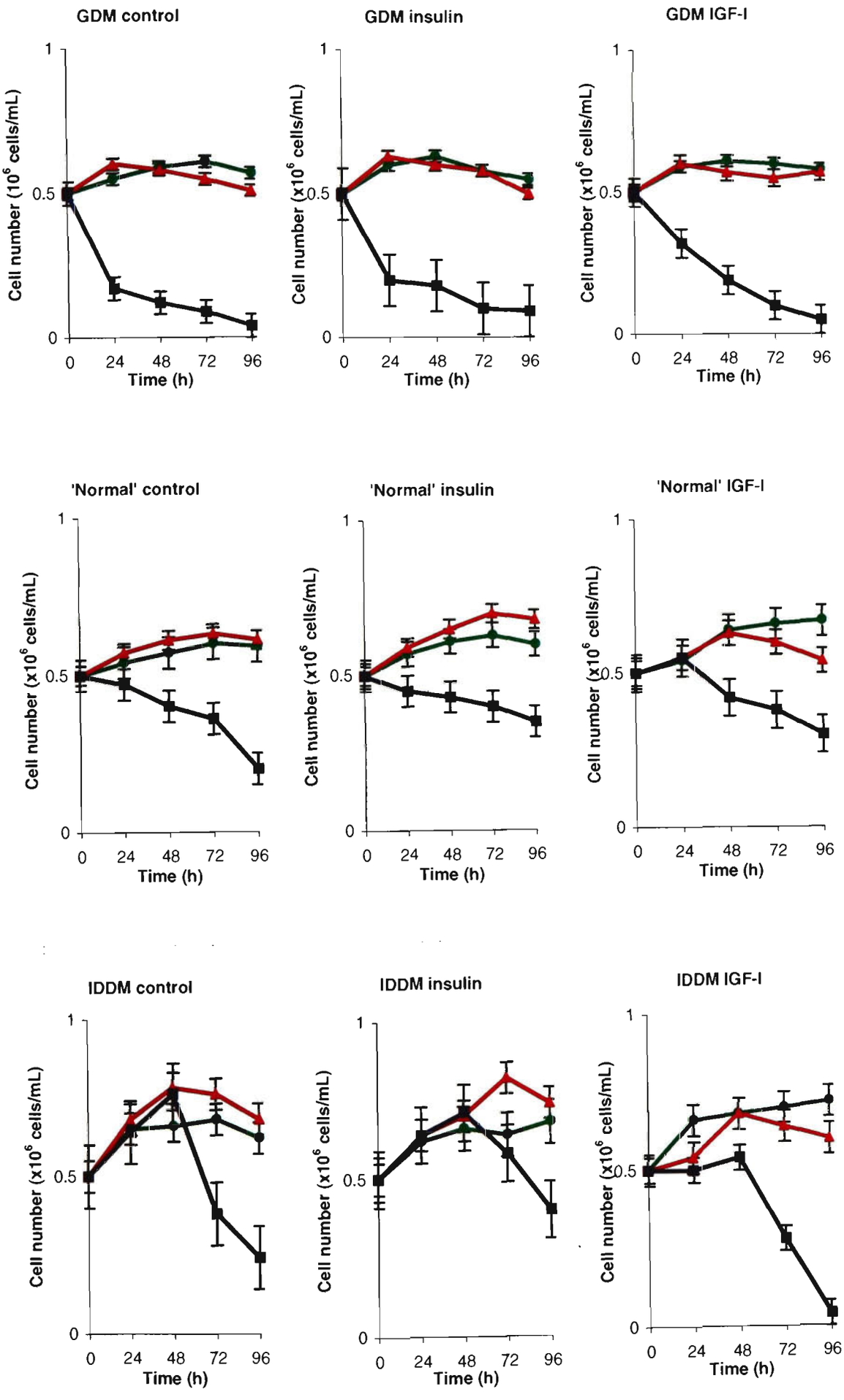
(e) 'Normal' placentae: treated with 0.1M insulin,

(f) 'Normal' placentae: treated with 1.0M IGF-I,

(g) IDDM placentae: control,

(h) IDDM placentae: treated with 0.1M insulin, and

(i) IDDM placentae: treated with 1.0M IGF-I.


GDM control
GDM insulin
GDM IGF-I
'Normal' control
'Normal' insulin
'Normal' IGF-I
IDDM control
IDDM insulin
IDDM IGF-I
Cell number (x10^6 cells/mL)
Time (h)
0
24
48
72
96
0.5
1

However, when cultures grown in 7.5mmol/L glucose were treated with IGF-I, cell numbers and viability were observed to decrease rapidly compared to those of 5mmol/L glucose cultures (Figure 6.4 and Table 6.4). TUNEL assay revealed that the decrease in cell number was associated with apoptosis and when assessing viability with Trypan blue exclusion, cellular debris was evident (data not shown).

In IDDM cultures grown with 7.5mmol/L glucose, no significant differences were noted in cell numbers compared to those grown in 5mmol/L glucose (Figure 6.4).

Table 6.4. Percentage viability of cells grown in varying glucose concentrations

	Percentage viability (%)								
	5mmol/L glucose			7.5mmol/L glucose			10mmol/L glucose		
	C	Ins	IGF-I	C	Ins	IGF-I	C	Ins	IGF-I
'N'	>99	>99	>99	>99	>99	>99	42	77	55
GDM	>99	>99	>99	>90	>90	88	24	45	16
IDDM	>99	>99	>99	>99	>99	88	38	62	17

Percentage viability of cells from 'normal' ('N', n=4), GDM (n=4) and IDDM (n=4) placentae, grown with 5mmol/L, 7.2mmol/L and 10mmol/L glucose and treated with 0.1M insulin (ins) or 1.0M IGF-I. (C = control)

The excessively high levels of glucose (10mmol/L) were found to have detrimental effects on the numbers of cells and their morphology over time in culture. This was particularly noted in cells of GDM and 'Normal' placentae, with no significant differences noted when insulin or IGF-I was administered to the cultures. In the case of cells from IDDM placentae, cell numbers were observed to initially increase to 48h followed by rapid decreases in the numbers to the end of culture.

6.5. Discussion:

One of the aims of this study was to examine and compare the relative proliferative effects of insulin on cells isolated from human placental tissue of 'normal' and diabetic pregnancies. The diabetic placentae obtained in this study were divided into two groups: GDM and IDDM. This subdivision represented different glycemic conditions of the fetus and placenta (as evidence by fasting blood glucose levels) allowing some investigation into the effect of such an environment on cellular processes such as proliferation and differentiation and the activity and concentration of insulin binding sites compared to 'normal'.

As discussed in the preceding chapter (Chapter Five), the cells isolated from both 'normal' and diabetic placentae were identified as cytotrophoblasts. This was determined by cell size, morphology and immunohistochemical staining. Greater than 98% of the cell population isolated stained positive for cytokeratin 7. Less than 5% of the cells stained for vimentin and this was found to remain consistent throughout the culture period. Staining for the desmoplakins revealed the differentiating process of these cells over time *in vitro*. Here single, mononuclear cells (cytotrophoblasts) were observed to aggregate to form clusters of cells which eventually fused together to form giant multinucleated cells (syncytiotrophoblasts).

The increases in hCG concentration over time found in this study were similar to those observed by others (Hall *et al.,* 1977; Deal & Guyda, 1983, Kliman *et al.,* 1986) as previously discussed in Chapter Five. In this study insulin treatment was found to enhance the differentiation of the cells isolated from 'normal' placentae as a greater proportion of the cell population comprised cellular aggregates and larger-sized fused cells by the end of culture compared to 'normal'. However, in GDM and IDDM cell cultures, the proportion of aggregating cells remained significantly lower than 'normal', particularly in cells of IDDM placentae irrespective of insulin treatment. This was reflected in the concentrations of hCG in the media which was also found to be significantly lower than 'normal' despite treatment with insulin. This result suggests that cellular aggregation and

differentiation are affected in gestational diabetes and that the role of insulin in these processes is modified.

It has been shown in this study that insulin potentiates the proliferation of cells isolated from human placentae and that the relative proliferative effects of insulin differs between 'normal', GDM and IDDM placentae. This confirms the anatomical differences observed between such placentae as discussed in Chapter One, section 1.4, where diabetic placentae are shown to have greater numbers of cytotrophoblast cells (Jones & Fox, 1976) and increased numbers of trophoblastic sprouts (Kaufmann *et al.,* 1987) compared to the placentae of 'normal' pregnancies. In the present study, cells of GDM placentae were shown to be less prolific compared to 'normal' whilst those of IDDM placentae were found to be more active in proliferation that 'normal'. This result indicates that the modifications to insulin activity and those of proliferation of placental trophoblasts between diabetic types are different. That is, in GDM placentae activity may be inhibited whilst in IDDM placentae proliferative activity may be potentiated.

The observation of increased proliferative effects also support other studies where insulin has been shown to promote proliferation in a number of tissue types (Lin *et al.,* 1992) via the synthesis and release of growth factors that may act in an autocrine manner. In rat hepatocytes, insulin administration stimulates IGF-I production (Boni-Schnetzler *et al.,* 1991). Hence, it may be that in cytotrophoblasts, insulin may increase IGF-I expression and synthesis which, in turn, may result in increased proliferation of these cells.

The sustained hyperglycaemia often associated with diabetes is believed to contribute to the macrosomic effects observed in such pregnancies. In the present study, increased glucose levels did result in increased proliferative effects, particularly noted in cells of 'normal' and IDDM placentae. Diabetic placentae have been shown to possess increased numbers of GLUT1 glucose transporters (Gaither *et al.,* 1999) which may explain the increased effects on proliferation seen in cells of IDDM placentae in the present study. The increases seen in cells of 'normal' placentae may be a result of upregulation of these GLUT1 transporters.

Sustained hyperglycaemia has been shown to down regulate both GLUT1 mRNA expression and glucose uptake into trophoblast cells (Hahn *et al.,* 1998). Perfusion studies have also demonstrated the reduced uptake and metabolism of glucose in placentae of diet controlled gestation diabetic women (Osmond *et al.*, 2000). It is possible that in cases of glucose flux or short term hyperglycaemic exposure, as in the present study, the initial response of the cells to high levels of glucose may be an increase in glucose uptake.

Insulin administration produced an additive effect on glucose treated cultures suggesting that insulin may increase glucose uptake for cellular activity and metabolism. It has been shown in adipocytes that glucose upregulates insulin binding sites (Eriksson *et al.*, 1992). In our study, glucose may increase the growth promoting effects of insulin by increasing insulin receptor numbers in cytotrophoblasts. IGF-I, however, did not improve the growth rates of cells like insulin indicating that increased glucose levels may modulate IGF-I activity in cytotrophoblasts or that IGF-I in placenta may not function to increase glucose uptake. Very high glucose concentrations (representative of poor glycemic control seen in poorly controlled diabetes) did prove to be detrimental to the growth of cells from all placental types. However, in cells from IDDM placentae, an initial increase in cell numbers was observed followed by a decline in numbers.

In the experiments of this study, insulin was administered at the beginning of culture. Normally, *in vivo* insulin concentrations are homeostatically maintained continuously while in diabetic cases hyperinsulinaemia may occur as a consequence of therapeutic administration. It is possible that the role of the ligands in potentiating glucose uptake, cell proliferation and metabolism in the *in vitro* studies may have limited effectiveness which may be influenced by normal degradative processes associated with both cells and ligands. This suggests that the readministration of the ligands periodically during culture may result in sustained metabolic and/or proliferative activity. However, this requires further investigation. It may also be possible that the metabolic products (assumed to be highly concentrated due to the initial high concentrations of glucose) may have detrimental effects on the cells resulting in the rapid decline in numbers. By changing the medium more often, the observations in terms of cell number may

have produced different outcomes (i.e., less detrimental effects). Again, this needs to be investigated further.

In this study, data show the effects of increased glucose are enhanced in the presence of insulin. This data confirms the theory that insulin may act to increase metabolic activity, particularly with increased nutrient supply, resulting in increased cellular activity such as proliferation. Preincubation of placental brush border membranes with insulin resulted in an increase in glucose influx into the membrane vesicles (Brunette *et al.,* 1990). The *in vivo* environment of diabetic pregnancies is associated with a potent combination of high glucose levels and increased levels of growth factors such as insulin and IGF-I (Roth *et al.,* 1996). However, it should be noted that regardless of insulin treatment, cytotrophoblasts do have a glucose threshold that when exceeded results in detrimental effects to the cells. This is confirmed by another study on the effects of increased glucose levels on placental cells (Nelson & Curran, 1989). It is also worthy of noting that the detrimental effects of increased glucose may be the result of increased osmolality as a result of increased glucose in the medium.

In this study, insulin and the IGFs were shown to enhance the proliferation of cells isolated from 'normal' and diabetic placenta. Increased nutrient supply enhanced proliferative activity of cytotrophoblasts, which is potentiated by the addition of insulin. IGF-I, however, did not improve cell number or viability under hyperglycaemic conditions. The data in this study does not provide conclusive evidence as to whether the increased proliferation observed in cultures supplemented with glucose and growth factor is the result of increased metabolic activity, increased nutrient supply or a direct effect of the growth factors themselves. To begin to answer this query, it becomes essential to establish through which receptors, and their underlying mechanisms, insulin and the IGFs are acting to promote the growth of these cells. The following chapter will proceed to attempt to do this.

CHAPTER SEVEN

MODE OF ACTION:
A BINDING SITE FOR INSULIN

The previous chapter showed that further investigation was essential in understanding the effects of insulin and the IGFs on cytotrophoblast growth. In this chapter, preliminary investigations into the receptors through which these ligands act, and an understanding of their underlying mechanisms will be undertaken.

7.1. Introduction

As was reviewed earlier in Chapter Two, studies on whole placental tissue have located insulin receptors to syncytiotrophoblasts, endothelial cells, fibroblasts and macrophages (Desoye *et al.,* 1994). Minimal levels of insulin receptor have been reported on cytotrophoblasts of early placental tissue (Jones *et al.*, 1993). However, the presence of binding sites for insulin on cytotrophoblasts isolated from term placenta has yet to be determined.

The physiological significance of the insulin receptor in the placenta requires further clarification. Insulin has been shown to be involved in a number of growth promoting effects in several different tissue types (O'Brien & Granner, 1991; Lin *et al.,* 1992; Desoye & Shafrir, 1994) as discussed in Chapter Two, section 2.2. Insulin binding studies have also shown a correlation between the number of insulin receptors in the human placenta and the weight of the fetus (Potau *et al.,* 1981). However, the role of the insulin receptor in the proliferation of cytotrophoblast cells has not been comprehensively studied and requires further examination.

As previously discussed in Chapter Two, section 2.10, alterations in insulin receptor mediated responses have been implicated in the outcomes of gestational diabetes. These have included possible receptor mutations (Baron *et al.,* 1991; Takata *et al.,* 1991) and differences in receptor numbers and binding (Desoye *et al.*, 1992).

The mechanisms for the effects of insulin on the proliferation and differentiation of cytotrophoblasts remain unclear. From the previous chapter it becomes apparent that a need arises for investigation into which receptors insulin mediates its responses. By comparing this information with that obtained from diabetic placentae the role of insulin in placental and fetal growth, and its contribution to fetal macrosomia, may be better understood.

7.2. Aims

The aims of this study:

1. To examine the presence of specific binding sites for insulin on the cell membranes of cytotrophoblasts isolated from 'normal' and diabetic placentae.
2. To examine and compare insulin binding in cells obtained from placentae of 'normal' and diabetic pregnancies.
3. To examine whether the increased proliferative effects of insulin are mediated via the insulin receptor.

7.3. Methods and Materials

7.3.1. Experimental protocol

Cells were isolated from 'normal' and diabetic placentae and purified as described in Chapter Three, section 3.2. The cells were then plated at 0.5 x 10^6 cells/mL in 24-well culture plates coated with collagen. Cells were divided into the following groups:

1. Cells isolated from 'normal' (n=4) and diabetic (n=4) placentae were maintained in humidified atmosphere containing 5%CO_2 in air at 37^0C in EMEM supplemented with 10% vol/vol FCS alone ($control_1$) and treated with
   a. 0.1nM insulin;
   b. 0.1nM insulin with the presence of anti-insulin antibody (1:50 dilution);
   c. 1.0nM IGF-I;
   d. 1.0nM IGF-I with the presence of anti-insulin antibody (1:50 dilution).

Cell morphology was examined and cell counts (section 3.3.) were taken every 2h for 24h and then at 48, 72 and 96h after seeding.

The antibody against the insulin receptor was kindly donated by Dr Aiden McElduff of the Royal North Shore Hospital, NSW, Australia. The antibody was received in the form of serum from a patient with severe type B insulin resistance, a pathological condition found to be associated with the spontaneous production of autoantibodies against the insulin receptor (Van Obberghen & Kahn, 1981; Van Obberghen *et al.*, 1982; Rodriguez *et al.*, 1992). These antibodies are believed to

mimic insulin in its effects to down-regulate the insulin receptor (Taylor & Marcus-Samuels, 1984).

2. For receptor binding analysis, the cells isolated from 'normal' (n=4) and diabetic (n=4) placentae were grown in 75mL culture flasks. Following 24h incubation, the cells were harvested and washed twice in EMEM alone. The cells were kept on ice in EMEM alone in 2mL eppendorf tubes until assayed. Cells were assayed within 2h of harvesting (section 3.8). For assay, 50mL aliquots (≡2 x 10^5 cells/mL) were used. Receptor analysis included
   a. Saturation studies where cells were divided into 4 groups:
      i. Cells alone in EMEM;
      ii. Cells blocked in excessive amounts of insulin (2.5mg/mL);
      iii. Cells blocked with excessive amounts of IGF-I (200μg/mL); and
      iv. Cells blocked with excessive amounts of IGF-II (200μg/mL).
   b. Competitive studies where cells were blocked with increasing concentrations of unlabelled insulin.

For saturation studies using blocking agents, established methods recommend using 100X the concentration of the radiolabelled agonist. As the IGFs (blocking agents) were of limited supply, the concentrations used were determined through trial and error. It was found that 200μg each was sufficient to significantly reduce Bmax. Increasing the concentration of these agents did not significantly alter the data and the data was found to be replicable.

7.3.2. *Statistical and data analysis:*

All data is expressed as the mean ± SEM. A MANOVA modified for repeated measures was performed on the data collected from cell counts and thymidine uptake analyses using the SPSS statistical package. The level of significance was at $p<0.01$. See Chapter Three, section 3.9 for detailed description of statistics used.

Insulin binding data was analysed by non-linear regression functions, which described the dependent, observed variable (bound insulin, cpm) as a function of the independent variable (concentrations of unlabelled insulin). This was done using a curve-fit program in Quattro Pro analysis package. Non-cooperative models were adopted which assumed either one class of binding sites or two classes of independent, non-interacting binding sites. Non specific binding was calculated in all receptor-binding experiments and subtracted from the obtained data. Here, cells were blocked with excess (100-fold of radiolabelled ligand) of insulin. It was assumed that this would result in blocking all insulin and IGF receptors. Saturation experiments were then performed where any binding of radioactive ligand was assumed to be non-specific (Chapter Three, section 3.9).

7.4. Results

7.4.1. Receptor Binding Analysis

The Scatchard plot was found to be non-linear. Therefore, the data was calculated using non-linear regression analysis and the data fitted the assumption of a two binding site model better than that for a single binding site. The receptor binding data show that differences exist in both the maximum binding of the receptor and the number of insulin binding sites between cells isolated from 'normal' and diabetic placentae (Table 7.1).

There were significant ($p<0.01$) differences between 'normal' and IDDM diabetic placentae in terms of maximum binding but not between 'normal' and GDM. However, the maximum binding of GDM placentae was found to be significantly less ($p<0.01$) than that for IDDM placentae. Data show no significant differences in receptor affinities between diabetic and 'normal' placental cell membranes, although IDDM placentae had binding affinities that were relatively higher than 'normal' whilst those obtained from GDM were relatively lower.

Table 7.1. Characteristic parameters for insulin binding to cytotrophoblast membranes: Competition assay

	Subject groups		
Parameters	'Normal'	GDM	IDDM
R^2	0.998	0.976	0.988
Bmax ($x10^{-9}$ M)	4.40 ± 0.97	3.80 ± 0.56	6.06 ± 0.63[ab]
K_D ($x10^{-6}$ M)	2.41 ± 1.12	1.99 ± 1.25	3.30 ± 1.20
NSB (cpm)	766 ± 245	780 ± 210	901 ± 486

Comparison of maximum binding (Bmax) and receptor affinities (K_D) of cells isolated from placentae of 'normal' (n=4), GDM (n=4) and IDDM (n=4) mothers [mean ± SEM].[a] $p<0.01$ compared to 'normal'; [b] $p<0.01$ compared to GDM. NSB = non-specific binding; R^2 = regression analysis.

Receptor binding data from saturation binding curves (Figure 7.2 and Table 7.2) show differences in maximum binding between 'normal', GDM and IDDM placental cells, with maximum binding in cells from IDDM tissue being significantly higher ($p<0.01$) than that for 'normal' and GDM placental cells. The maximum binding data was altered when the cells were blocked with either IGF-I or IGF-II. Receptor affinity remained unchanged (Table 7.2). When examining the characteristic parameters for insulin binding on cell membranes of 'normal' placentae, blocking with IGF-II did not produce any significant changes to either maximum binding or the affinity of the insulin receptor (Table 7.2.a).

Figure 7.1. Mean binding curves: Competition assay

Competitive binding assay for the insulin receptor. Cytotrophoblasts from 'normal' (n=4), GDM (n=4) and IDDM (n=4) placentae were assayed for radiolabelled insulin bound to membranes that were previously blocked with increasing concentrations of unlabelled insulin (mean $\pm$ SEM).

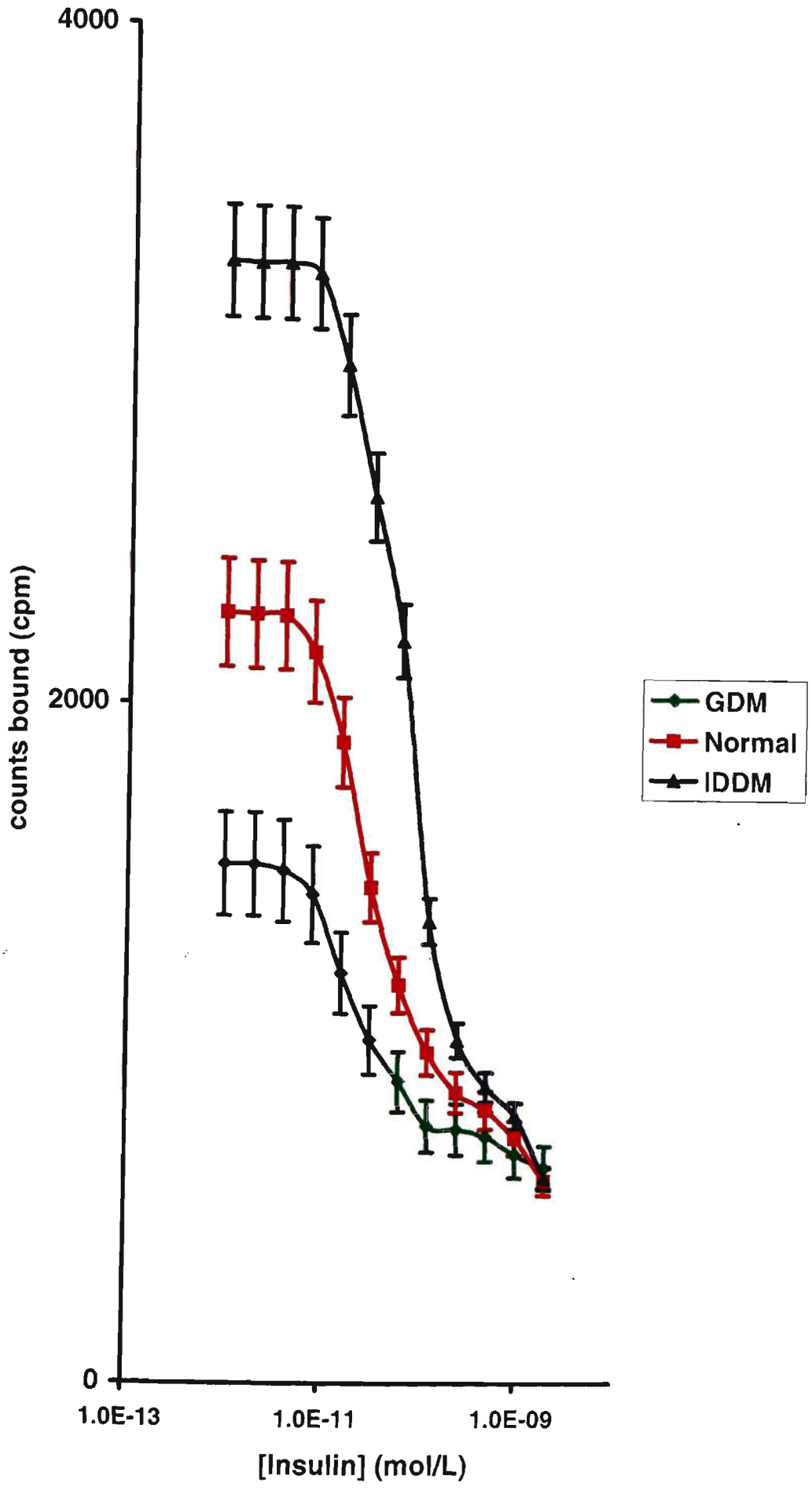

4000
2000
0
counts bound (cpm)
1.0E-13
1.0E-11
1.0E-09
[Insulin] (mol/L)
GDM
Normal
IDDM

Table 7.2. Characteristic parameters for insulin binding to cytotrophoblast membranes: Saturation assay

a. 'Normal'

	Treatment groups		
Parameters	control	IGF-I block	IGF-II block
R^2	0.932	0.903	0.977
Bmax ($x10^{-9}$ M)	3.40 ± 1.53	1.40 ± 0.15[a]	3.20 ± 1.85
K_D ($x10^{-6}$ M)	1.42 ± 0.72	2.09 ± 0.85	2.29 ± 0.06
NSB (cpm)	550 ± 245	541 ± 210	601 ± 486

b. GDM

	Treatment groups		
Parameters	control	IGF-I block	IGF-II block
R^2	0.976	0.942	0.857
Bmax ($x10^{-9}$ M)	3.10 ± 1.86	2.70 ± 1.78	3.00 ± 1.34
K_D ($x10^{-6}$ M)	1.10 ± 0.55	1.90 ± 0.60	1.90 ± 0.20
NSB (cpm)	532 ± 117	527 ± 89	477 ± 123

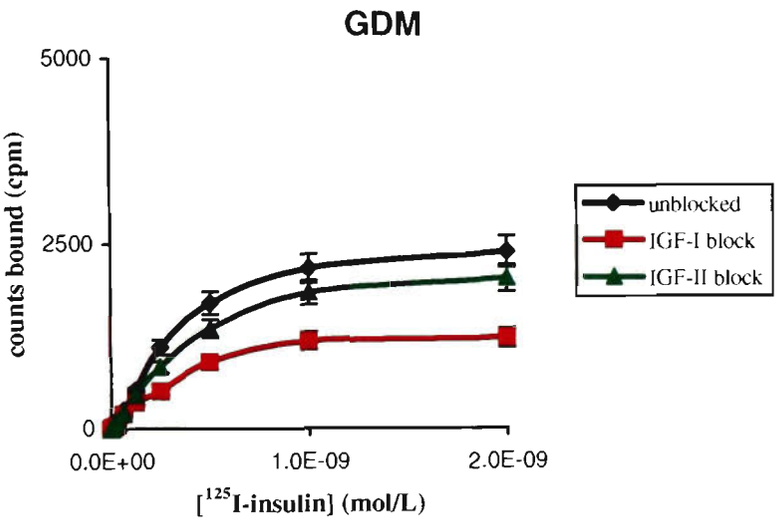
c. IDDM

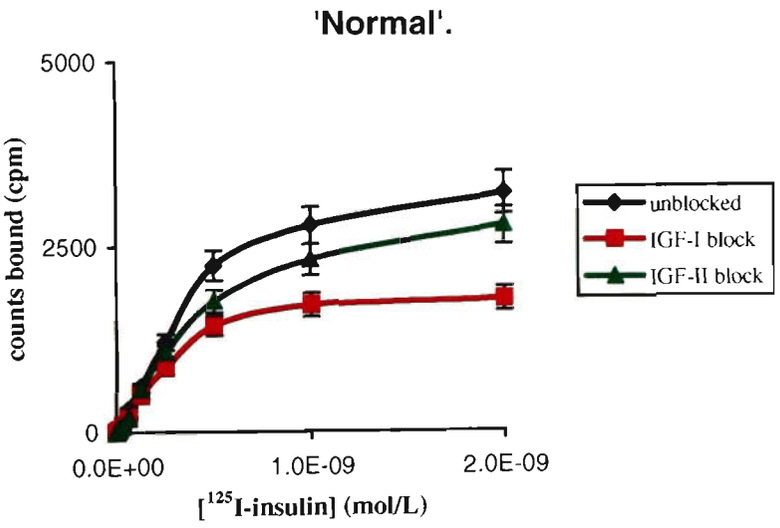
	Treatment groups		
Parameters	control	IGF-I block	IGF-II block
R^2	0.962	0.928	0.972
Bmax ($x10^{-9}$ M)	7.0 ± 0.50	4.8 ± 1.30[a]	8.4 ± 0.7[ab]
K_D ($x10^{-6}$ M)	2.20 ± 0.10	2.30 ± 0.15	2.40 ± 0.11
NSB (cpm)	1120 ± 324	1007 ± 230	901 ± 286

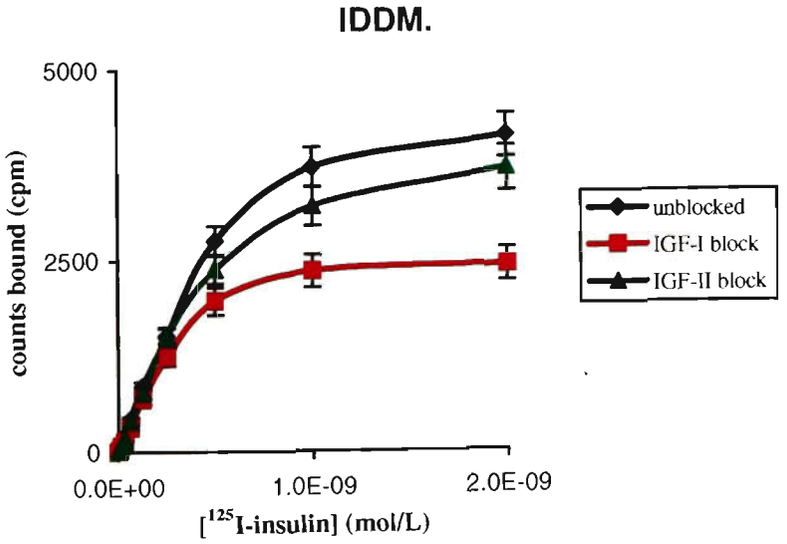
Maximum binding (Bmax) and receptor affinities (K_D) of cytotrophoblasts from placentae of 'normal' (n=4), GDM (n=4) and IDDM (n=4) mothers and blocked with 200μg/mL IGF-I or 200μg/mL IGF-II [mean ± SEM]. [a] *$p<0.01$ compared to control data;* [b] *$p<0.01$ compared to IGF-I block data. (R^2 = regression analysis, NSB = non-specific binding)*

Figure 7.2. Mean binding curves: Saturation assay

Saturation binding curves for insulin binding on cytotrophoblasts isolated from (a) GDM (n=4), (b) 'normal' (n=4) and (c) IDDM (n=4) placental tissue and blocked with excess amounts of insulin, IGF-I or IGF-II.


GDM
5000
2500
0
counts bound (cpm)
0.0E+00
1.0E-09
2.0E-09
[125I-insulin] (mol/L)
unblocked
IGF-I block
IGF-II block


'Normal'.
5000
2500
0
counts bound (cpm)
0.0E+00
1.0E-09
2.0E-09
[125I-insulin] (mol/L)
unblocked
IGF-I block
IGF-II block


IDDM.
5000
2500
0
counts bound (cpm)
0.0E+00
1.0E-09
2.0E-09
[125I-insulin] (mol/L)
unblocked
IGF-I block
IGF-II block

However, when blocking the cell membranes with IGF-I maximum binding decreased significantly ($p<0.01$) to less than half that of control, with receptor affinity remaining unchanged. In cells of GDM placentae, blocking with either IGFs did not significantly change the binding affinity of the insulin receptor or the maximum binding (Table 7.2.b).

Cells from IDDM placentae were blocked with IGF-I resulting in a significantly reduced ($p<0.01$) maximum binding compared to control. Again, the receptor affinity was unaltered. When blocking the receptors on cell membranes with IGF-II, maximum binding for insulin increased significantly ($p<0.01$) compared to control and IGF-I blocked cells with receptor affinity remaining unchanged (Table 7.2.c).

7.4.2. Proliferative Activity and the Insulin Receptor

To assess whether the proliferative effects potentiated by insulin were mediated via the insulin receptor, cells were maintained in media containing anti-insulin receptor antibody-rich sera.

In all treated cultures ('normal', GDM and IDDM) grown without antibody, growth curves are as described previously in Chapter Six, sections 6.4.3, 6.4.4 and 6.4.5. In the present study, the presence of the anti-insulin receptor antibody was shown to affect the growth of cytotrophoblasts. In control GDM cultures with antibody no significant effect on the reduction of cell numbers and differentiation was noted compared to those cultures where the antibody was absent. Treatment with IGF-I did not produce any significant effects in the presence of the antibody compared to when cultured without antibody (Figure 7.3).

In cells isolated from placentae of IDDM mothers a significant decrease in cell numbers was noted particularly in insulin ($p<0.0001$, $F=198.92$; throughout the culture period) and IGF-I treatment ($p<0.0001$, $F=323.62$; after 48h) when antibody was present compared to when no antibody was present (Figure 7.3).

Figure 7.3. Cell numbers of cytotrophoblasts grown in the presence of antibody.

Cytotrophoblasts isolated from GDM (n = 4), 'Normal' (n = 4) and IDDM (n = 4) and grown in EMEM supplemented with 10%FCS (control) and treated with 0.1nM insulin or 1.0nM IGF-I with/without the presence of anti-insulin receptor antibody (1:50 dilution)

(a) GDM: Cell number in control cultures and those treated with insulin and IGF-I.

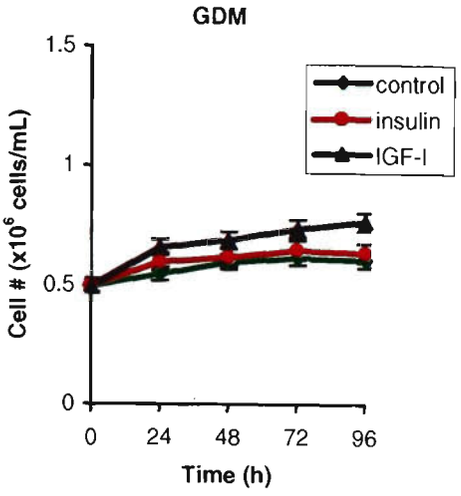
(b) GDM: Cell number in control cultures and those treated with insulin and IGF-I in the presence of anti-insulin receptor antibody.

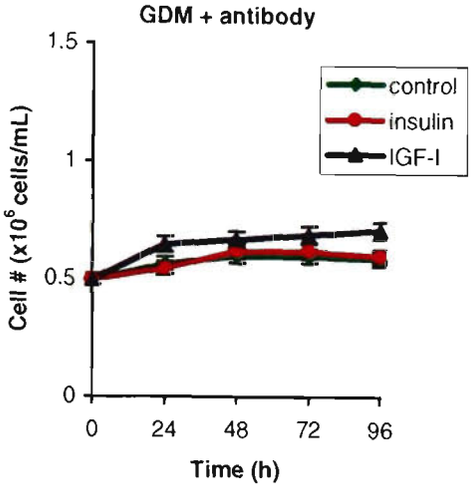
(c) 'Normal': Cell number in control cultures and those treated with insulin and IGF-I.

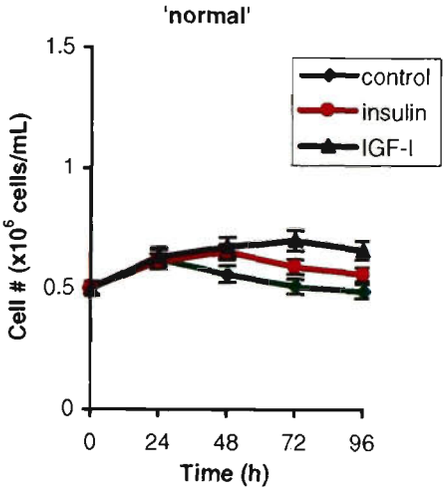
(d) 'Normal': Cell number in control cultures and those treated with insulin and IGF-I in the presence of anti-insulin receptor antibody.

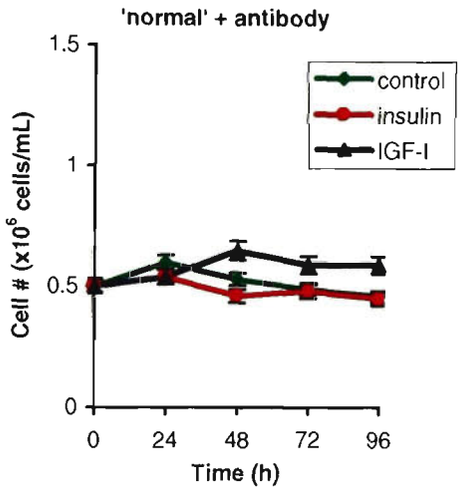
(e) IDDM: Cell number in control cultures and those treated with insulin and IGF-I.

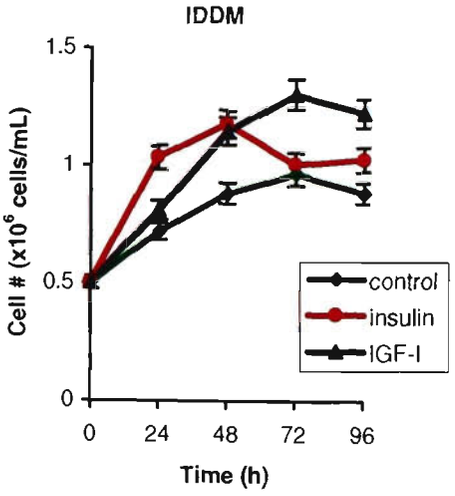
(f) IDDM: Cell number in control cultures and those treated with insulin and IGF-I in the presence of anti-insulin receptor antibody.

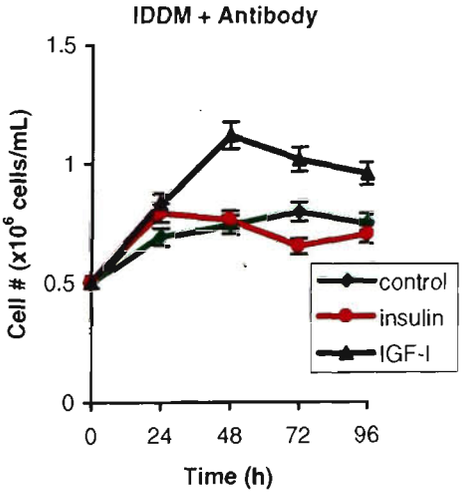

GDM
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
96
Time (h)
control
insulin
IGF-I


GDM + antibody
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
96
Time (h)
control
insulin
IGF-I


'normal'
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
96
Time (h)
control
insulin
IGF-I


'normal' + antibody
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
96
Time (h)
control
insulin
IGF-I


IDDM
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
96
Time (h)
control
insulin
IGF-I


IDDM + Antibody
Cell # (x10^6 cells/mL)
1.5
1
0.5
0
0
24
48
72
96
Time (h)
control
insulin
IGF-I

In all treated 'normal' cultures grown with antibody, cell numbers were found to be significantly lower than those of cultures grown without the presence of the antibody, particularly after 48h of culture. Very few cells in any treatment were observed to fuse together and differentiate to form giant multinucleated syncytiotrophoblast cells in the presence of antibody (data not shown).

7.5. Discussion

In the present study, insulin-binding sites on placental cell membranes isolated from 'normal' and diabetic placentae were examined. To date, insulin binding studies have not been performed in isolated cells. As explained in Chapter Six, the diabetic placentae, GDM and IDDM, represented different glycemic environments of the fetus and placenta. This allowed for investigation into the effect of such environs on the activity and concentration of insulin binding sites compared to 'normal'. The cells isolated from 'normal' and diabetic placentae were found to be cytotrophoblasts as determined by cell size and density, morphology, immunocytochemical staining and hCG profile as assessed in the previous two chapters (Chapter Five and Six).

We have shown that the isolated cytotrophoblast cells possess insulin binding sites on their membranes. This confirms work by Jones and colleagues (1993) who had immunohistochemically localised insulin receptors to extracellular cytotrophoblasts in early placenta. Much attention has been focussed on syncytial insulin receptors and the potential role they may play in placental metabolism and fetal nutrient supply. However, anatomical evidence implicates maternal diabetes and, particularly insulin in influencing cytotrophoblast proliferation (Jones and Fox, 1976; Kaufmann *et al.*, 1987). The presence of specific insulin binding sites on cytotrophoblast membranes, shown in the present study, suggests the ability of insulin to communicate with these cells.

Alterations in insulin receptor mediated responses have been implicated in maternal diabetic pregnancies. Suggestions have included changes in receptor

structure (Takata *et al.,* 1991; Baron *et al.,* 1991) to differences in the number of insulin receptors (Desoye *et al.,* 1992). In the present study, differences were found in the number of binding sites between 'normal' and diabetic placentae. No significant differences were observed in the affinities of the binding sites on cytotrophoblasts of 'normal' and diabetic placentae. In IDDM placentae, cells were found to possess greater numbers of insulin binding sites compared to cells of 'normal' placentae whilst those of GDM were lower. This data confirms that of Desoye and colleagues (1992) and may explain the significant differences observed in proliferative activity in the cells from IDDM and GDM placentae compared to 'normal' as seen in Chapter Six.

The present study also demonstrated the specific nature of the binding sites for insulin and how the IGFs may alter binding site availability. Despite the presence of excess concentrations of IGF-I or IGF-II, insulin still bound to the cytotrophoblasts demonstrating the affinity of these binding sites for insulin. Saturation assays demonstrated that in 'normal' and diabetic placentae, the number of available binding sites was reduced by almost a third when blocked with IGF-I. Such data suggests that insulin may also bind to the IGF-I receptor, at a lower affinity, and that some of the mitogenic activity of insulin may possibly be mediated through this receptor. The binding data reflected the biological response data, seen previously in Chapters Five and Six where cells grown with both insulin and IGF-I were found to be less prolific compared to cells treated with IGF-I but more so than those cells treated with insulin.

Interestingly, in IDDM placentae only, the number of available insulin binding sites increased with IGF-II administration suggesting that IGF-II may upregulate insulin receptors in cytotrophoblasts. However, this result was not indicative of the biological response data seen in Chapter Six, where cells grown in the presence of insulin and IGF-II were found to be less proliferative than those treated with insulin. These results combined suggest that the function of IGF-II in placenta may not be to contribute to the proliferation of these cells but to serve another function such as decreasing apoptosis, as suggested by Hill and colleagues (1998) or increasing metabolism. IGF-II binding may initiate an intracellular pathway that is separate to that of insulin, thereby producing a different response.

In this study, experiments involving anti-insulin receptor antibody revealed that in GDM and 'normal' placentae, minimal proliferative effects were observed in the presence of antibody. However, in IDDM placentae, the antibody was shown to significantly reduce proliferation of cells particularly those treated with insulin. This observation suggested that the modulating effects of insulin on cell proliferation were mediated through the insulin binding sites. The anti-insulin receptor antibody experiments confirmed the receptor binding data showing that the cells possess specific insulin binding sites and that the receptor numbers vary between placentae type. The presence of anti-insulin receptor antibody in cultures was also found to reduce cell numbers in IGF-I treated cultures suggesting that some of the proliferative effects (as observed in Chapters Five and Six) may be mediated via the insulin receptor. It may also be possible that the antibody may bind to the type 1 receptor (possibly at lower affinity) resulting in lower available numbers of this receptor leading to the reduced effects observed.

Control cultures of 'normal' and diabetic placentae were not affected by the presence of the antibody. This result suggests that insulin may not exclusively control the proliferation and differentiation process of cytotrophoblasts *in vitro*. Other factors (endogenous or present in the serum) may also be involved as shown by others, including EGF (Morrish *et al.*, 1997), or TGFα and IGF-II (Lala *et al.*, 1998), which have been shown to potentiate proliferation and differentiation of trophoblasts in culture.

The variation in results seen between 'normal' and diabetic and between GDM and IDDM placentae may be explained by structural and/or functional alterations to the insulin binding sites. These alterations may be a result of long-term exposure to imbalances in nutrients and growth factors found in the fetal milieu in IDDM pregnancies, which may not necessarily be seen in well-controlled GDM pregnancies. It is also possible that the sera containing the antibody has provided cultures with other growth factors and cytokines that may have an effect on the biological response of the cells, such as growth promotion, thereby confounding results. However, there is evidence to suggest that in the presence of antibody, the effects of insulin on cell proliferation were reduced, confirming the growth promoting role of insulin on fetal tissues.

Thus, in this study we have established the presence of specific insulin binding sites on the membranes of cytotrophoblast cells of 'normal' and diabetic placentae, which may be facilitated by IGF-II binding. Cells of IDDM placentae had greater numbers of these binding sites compared to 'normal' whilst GDM cells had fewer binding sites, but the affinities were the same. Although the binding assays suggested that some of the proliferative effects of insulin might be mediated via the IGF-I receptor, antibody experiments showed that blocking of insulin binding sites resulted in reduced cellular proliferation. This confirms the growth promoting function of insulin on cytotrophoblasts and may explain the contribution of insulin to the macrosomic effects particularly noted in IDDM pregnancies.

CHAPTER EIGHT

A PRELIMINARY INVESTIGATION: CO-CULTURE OF AMNION, CHORION AND CYTOTROPHOBLASTS

Trophoblasts are within close proximity to other fetal tissues and maternal elements, which interact with one another to support and maintain fetal growth and function in utero. The previous chapters explored the effects of insulin and the IGFs on trophoblast cell growth. This chapter is a pilot study into the examination of some of the relationships between different cell types in utero and how they may affect cytotrophoblast growth.

8.1. Introduction

As reviewed in the literature in Chapter One, the trophoblasts of the placenta are found located adjacent to fetal membranes (amnion and chorion) and maternal elements (decidua and maternal blood). These tissues comprise several cell types that interact with one another to sustain optimum growth and development of the fetus *in utero*. The amnion and chorion are of particular interest as these tissues have been shown to be highly active in metabolism and secretory function (Schmidt, 1992). They have also been shown to secrete a wide array of factors such as binding proteins, cytokines and hormones.

Co-culture techniques have recently been employed to investigate the possible interactions between neighbouring cells. So far such studies have examined the effects of abnormal fetal tissues on proximate tissues (Smarason *et al.*, 1993), the morphological interaction of neighbouring tissues (Babawale *et al.*, 1996) and the paracrine regulation of trophoblast function by other cells (Cervar *et al.*, 1999).

These studies indicate that the tissues found adjacent to the placenta may impact on the growth and function of placental cells. The purpose of this study is to examine some of the relationships between fetal tissues and placenta and to investigate the effects of amnion and chorion on the proliferation and differentiation of trophoblast cells.

8.2. Aims

The aims of this study:

1. To examine the effects of insulin and IGF-I on the growth of cells isolated from amnion and chorion of 'normal' pregnancy.
2. To successfully co-culture cytotrophoblasts with amnionic and chorionic cells obtained from 'normal' pregnancy.
3. To preliminarily investigate the effects of amnionic and chorionic cells on cytotrophoblast proliferation and differentiation when cells are co-cultured.

8.3. Methods & Materials

8.3.1. Experimental protocol

Cytotrophoblasts and cells of the amnion (n=4) and chorion (n=4) were isolated and purified as described previously in Chapter Three, sections 3.2.1 to 3.2.4 and 3.2.6. Cells were seeded at the same density of $0.5x10^6$ cells/mL in EMEM supplemented with 10%FCS (control) and treated with 0.1nM insulin or 1.0nM IGF-I. Cultures were divided into several groups:

1. Amnionic cells;
2. Chorionic cells;
3. Cytotrophoblasts;
4. Co-culture of amnionic cells and cytotrophoblasts; and
5. Co-culture of chorionic cells and cytotrophoblasts

Co-cultures had the cells grown together within the same well of the culture plate using translucent cell culture inserts (Falcon, Becton Dickinson Labware) of high pore density with 0.45μm diameter pores. For descriptive details of co-culture methods see Chapter Three, section 3.2.6. Cells were harvested at various time intervals, lifted with trypsin treatment and morphologically examined, counted using a cytometer and assessed for percentage viability as determined by Trypan blue exclusion.

8.4. Results

The viability of the cells after isolation and purification was found to be >98% for all cell types. The high percentage viability was maintained throughout the culture period. Data not shown.

8.4.1. Characterisation and growth of cells isolated from amnion and chorion

The amnion consisted of spindle shaped, epithelial cells, which became more ovoid in shape over the period of culture (Figure 8.1). Cells adhered loosely to the collagen matrix and cells were observed to increase exponentially over time

(Figure 8.2). After 72h, cells were observed to almost outgrow the culture well and appeared as one intact sheet of amnion. Distinguishing individual cells became difficult. No significant differences in cell numbers were seen between cultures treated with insulin or IGF-I.

Cells of the chorion laeve, however, readily attached to the collagen matrix within the first 6h of culture. The cells formed a single monolayer that reached confluence by 5 days of culture. Some of the chorionic cells displayed cytoplasmic variations whilst others became elongated with time. Most of the cells isolated from the chorion laeve had numerous cytoplasmic secretory granules and were spindle shaped (Figure 8.1). Again the cells formed a thin sheet in culture making single cells difficult to distinguish. Cells numbers were observed to increase significantly ($p<0.01$; $F=13.10$) over time to $4.0x10^6$ cells/mL at 96h after seeding. Unlike the amnion cultures, insulin and IGF-I was found to significantly increase ($p<0.01$; $F=84.97$) the number of cells compared to that of control cultures (Figure 8.2).

The morphological and immunocytochemical characterisations of cytotrophoblasts isolated from 'normal' placentae have been described in detail in the preceding Chapters Four and Five. The growth curves in terms of cell number and thymidine uptake for cytotrophoblasts grown in control conditions and treated with insulin and IGF-I have also been described previously in detail in Chapter Five and will not be repeated in this chapter. However, the graphs for these results will be represented here to aid in comparisons of the data (Figure 8.2).

Figure 8.1. Photographs of amnionic and chorionic cells in culture

Cells of the (a) amnion and (b) chorion of 'normal' placentae were isolated and cultured in EMEM supplemented with 10%FCS (t=48h).
[Mag: X40]

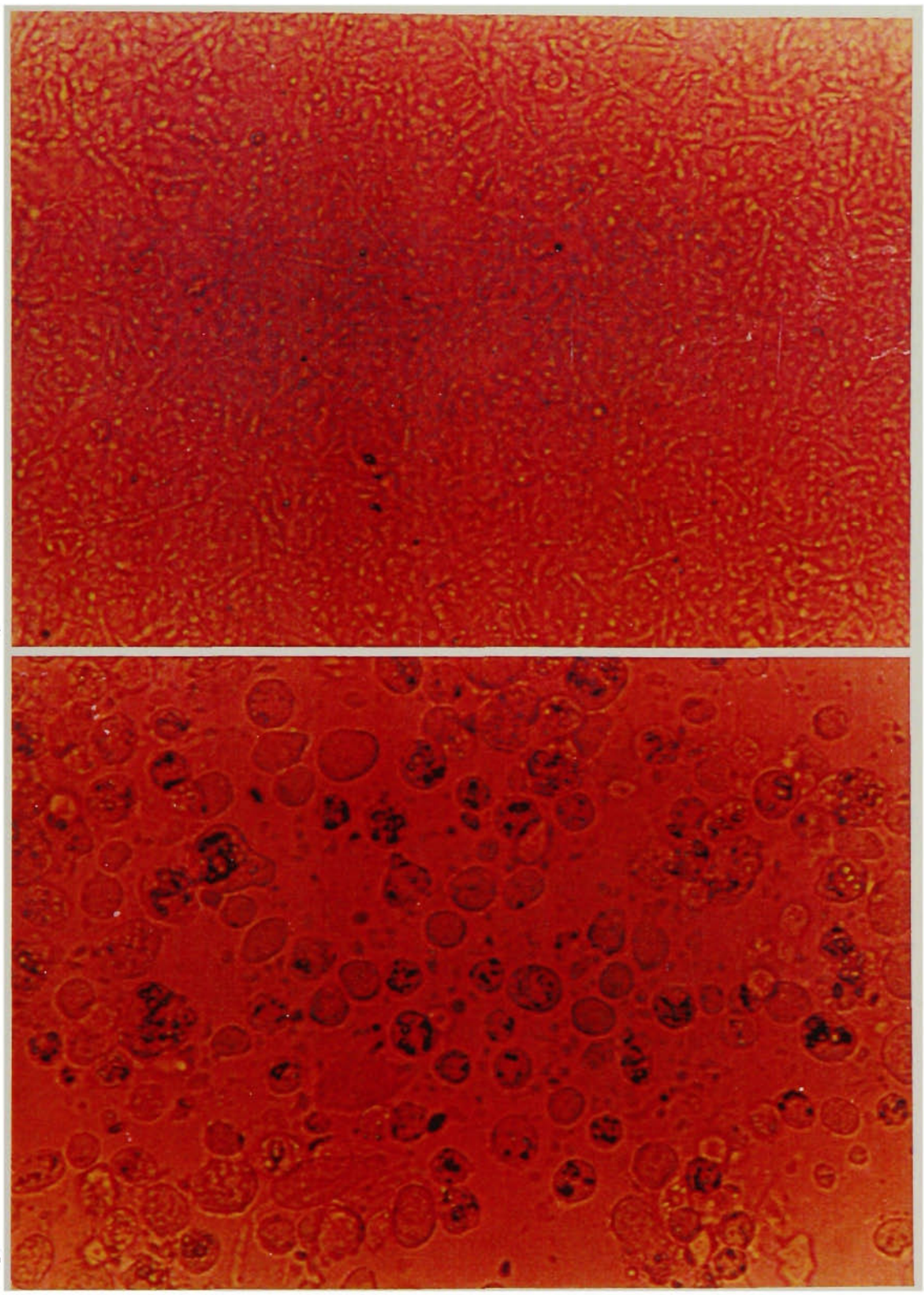

a
b

Figure 8.2. Number of cells grown in co-culture

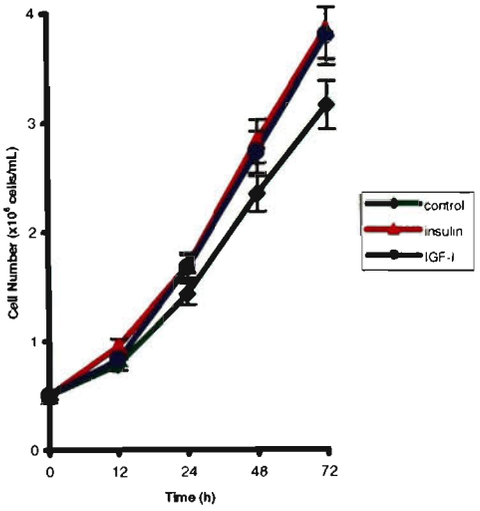
(i) Number of cells in culture isolated from the chorion laeve and grown in EMEM supplemented with 10%FCS (n=4).

(ii) Number of cells in culture isolated from the amnion and grown in EMEM supplemented with 10%FCS (n=4).

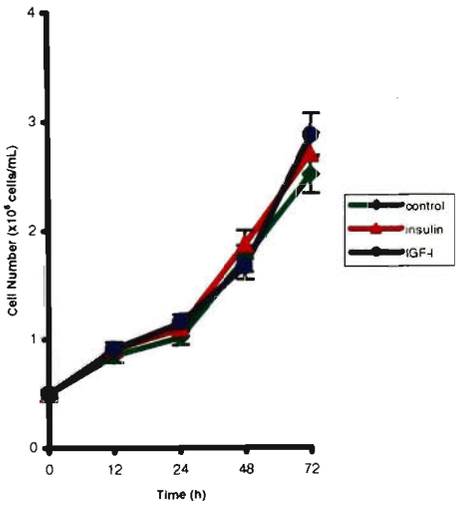
(iii) Number of cytotrophoblast cells grown in co-culture with chorion in EMEM supplemented with 10%FCS and treated with
0.1nM insulin and
1.0nM IGF-I.

(iv) Number of cytotrophoblast cells grown in co-culture with amnion in EMEM supplemented with 10%FCS and treated with
0.1nM insulin and
1.0nM IGF-I.

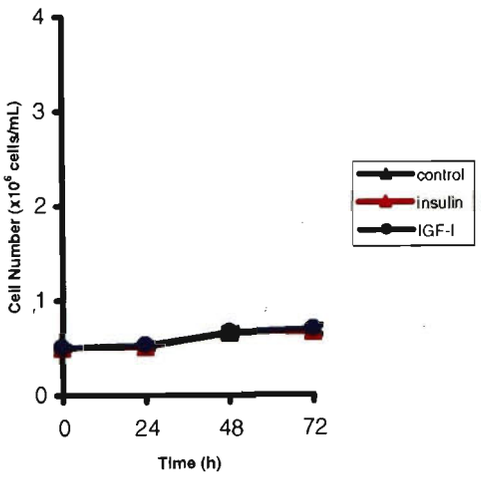
Number of Chorionic Cells



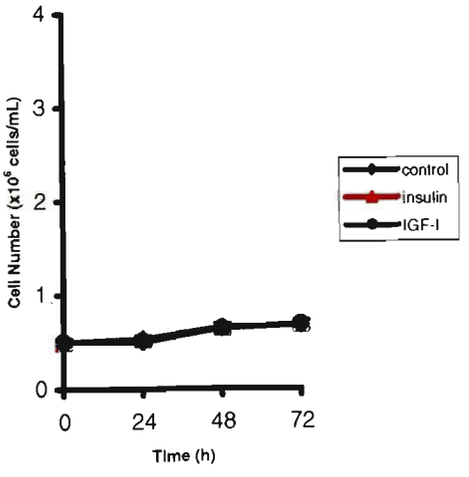
Number of Amnion Cells



Chorion Co-culture: Trophoblast Number



Amnion Co-culture Trophoblast Number



8.4.2. Co-culture of cells: amnion, chorion and cytotrophoblasts

In co-cultures, cells of the amnion and chorion were observed to increase in number over time as described previously in section 8.4.1. Cells of the amnion and chorion were prolific and seen to increase in cell number exponentially. The growth curves of the cytotrophoblasts co-cultured with either amnion or chorion were found to be significantly different from those of cytotrophoblasts cultured in monolayer (as recorded earlier in Chapter Five). Refer to Table 8.1.

Cytotrophoblasts co-cultured with either amnion or chorion cells were observe to increase in cell number over time from $0.5x10^6$ cells/mL to almost $0.6x10^6$ cells/mL by 72h of culture. These cell numbers were found to be significantly lower than those of cytotrophoblasts cultured alone (monolayer). Treatment of co-cultures with insulin or IGF-I did not significantly change cell numbers. However, numbers of cytotrophoblasts in all treatments co-cultured with amnion or chorion were found to be significantly lower than those of the monolayer cultures (Table 8.1).

With co-culture cytotrophoblasts were observed to aggregate with one another by 72h of culture. Any further morphological differentiation (ie, fusion of cells) was not observed within the culture period. Data not shown.

Table 8.1. Number of cytotrophoblasts grown in monolayer and in co-culture.

	Number of cytotrophoblasts ($x10^6$ cells/mL)								
	Monolayer			Amnion co-culture			Chorion co-culture		
t(h)	C	Ins	IGF-I	C	Ins	IGF-I	C	Ins	IGF-I
0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
24	0.80±0.01	1.02±0.02	1.29±0.03	0.51±0.02	0.51±0.01	0.53±0.02	0.50±0.01	0.53±0.02	0.51±0.03
48	0.78±0.02	0.94±0.02	1.27±0.05	0.64±0.02	0.65±0.01	0.64±0.02	0.65±0.02	0.67±0.02	0.64±0.02
72	0.77±0.01	0.91±0.02	1.20±0.10	0.67±0.02	0.68±0.02	0.68±0.03	0.68±0.02	0.66±0.03	0.69±0.04

A comparison of the number of cytotrophoblasts grown in
monolayer culture;
co-culture with amnionic cells; and
co-culture with chorionic cells.
Cells were cultured for 72h in EMEM supplemented with 10%FCS and treated with 0.1nM insulin or 1.0nM IGF-I.
(n=4; SEM x 10^6cells/mL; C = control, Ins = insulin treated, IGF-I = IGF-I treated).

8.5. Discussion

This chapter was a pilot study into co-culture techniques. The experiment was a preliminary investigation into the effects of co-culture of cytotrophoblasts with cells of the amnion and chorion and to examine any differences in cytotrophoblast proliferation and differentiation processes between treatments, as observed in monolayer, single cell type culture, in the presence of different cell types. The advantage of co-culture techniques is to provide an insight into cellular interactions of adjacent tissues. This does not directly mimic *in vivo* but supplies information involved in the regulation of neighbouring cellular processes.

The morphological characteristics of the amniotic cells and cells of the chorion laeve isolated in this study compared favorably with that of other studies (Acker *et al*, 1982; Gibb *et al*, 1986). The amnion cells were initially spindle shaped which then became more ovoid over time. Cells of the chorion laeve were also spindle shaped but possessed many secretory granules within the cytoplasm.

The amnion and chorion cells were found to be highly proliferative in culture with cell numbers observed to increase almost exponentially over time. The cells formed tightly packed sheets of tissue in culture with individual cells becoming increasingly difficult to distinguish over time. Cell proliferative activity did not appear to be inhibited by cell contact or lack of well floor space in the culture dishes as cells were seen to overgrow one another and proceed to fill the entire well.

In the present study, co-culture with amnion and chorion cells proved to be inhibitory to cytotrophoblast proliferative activity. This inhibition of cellular proliferation may be due to many factors. As reviewed in the literature, the amnion and chorion comprise several cell types and are very active in secretory function, producing a variety of factors that may interact with the surrounding cells to modify activity (Schmidt, 1992). These factors include protein hormones, cytokines and binding proteins, some of which are known regulators of cell proliferation (Lysiak *et al.*, 1995a; Han *et al.*, 1996; Cervar & Desoye, 1998; Hill

et al., 1998) that may affect only the villous trophoblasts. Therefore, in this study, it may be that the cells of the amnion and chorion secrete these factors into the media, which then act to retard proliferative activity in the neighbouring cytotrophoblast cells.

Amnion and chorion are also highly metabolically active (Schmidt, 1992). It is possible that the reduced rate of cytotrophoblast growth may be due to lack of nutrient supply (resulting largely from the fibroblast-like cells of the amnion or chorion) for which the different cell types compete. The increase in metabolite products in the medium may be inhibitory to cytotrophoblast growth. The set up of the co-culture may also have resulted in inefficient gaseous exchange as CO_2 availability to the cytotrophoblasts may have been reduced. Or it is possible that for the number of cells seeded within the one well, the amount of medium provided may not have been sufficient for optimum growth of the different cell types.

The administration of IGF-I did not have any effect on the proliferation of co-cultured cytotrophoblasts. This may be explained by the production and release of IGF binding proteins (Povoa *et al.*, 1984; Baxter & Martin, 1989; Han *et al.,* 1996) into the growth medium, thereby inhibiting the effects of IGF-I. Other factors such as TGFβ, also secreted by fetal tissues, have been shown to have inhibitory effects on cell proliferation (Lysiak *et al.,* 1995a). Again, the lack of proliferative activity may also be due to nutrient competition, lack of CO_2, or insufficient media provisions.

Insulin administration in co-culture did not produce any significant differences in cytotrophoblast proliferation and differentiation *in vitro* compared to that of control co-cultures. Again, this may be due to a number of inhibitory factors or metabolic products or simply a result of the co-culture technique adopted as discussed earlier. However, studies have demonstrated an increased likelihood of gestational diabetes with increasing levels of insulin in amniotic fluid (Weiss *et al.*, 1988; Star *et al.*, 1997). Yet, amniotic fluid concentrations of insulin alone proved to be an inefficient predictor of macrosomia (Carpenter *et al.*, 1996). This

(along with the work performed in the present study) demonstrates the complexity of the varying factors involved in gestational diabetes and its resultant effects on fetal outcome. These factors include the different nutrients available and their rates of uptake by fetal and placental cells, the many growth factors present in the fetal milieu and their varying inhibitory/trophic functions and effects on cellular processes and the interactions of all these growth factors and nutrients and the effects of the proximal tissues surrounding the placenta and fetus. It becomes necessary, therefore, to incorporate some co-culture techniques into more studies particularly when examining the effects of growth factors on the placenta/fetus as the surrounding tissues may modify the effects observed in single cell type cultures. It also allows for a better insight into the complex interactions that occur during pregnancy.

The present study shows that the adjacent fetal tissues impact on the proliferation and differentiation of cytotrophoblasts and may mediate insulin and IGF-I activity on cytotrophoblast cells. This demonstrates the complex interactions of many factors involved in maintaining the growth and development of the fetus *in vivo*.

CHAPTER NINE

SUMMARY AND CONCLUSIONS

9.1. The story so far...

Pregnancies of women with pregestational diabetes are at greater risk of fetal growth anomalies and perinatal mortality than those with gestational diabetes. Pregnancies of women with pregestational diabetes has been associated with imbalances in insulin and the IGFs, which have been shown to correlate with fetal weight, length and age as previously cited in the literature review (Chapter Two, sections 2.5.1, 2.10). However, their role in fetal growth and development has yet to be clarified. This study undertook the examination of the effects of insulin and the IGFs on cell proliferation and differentiation *in vitro* of trophoblasts isolated from human placentae at term to better understand the role these factors play in fetal growth and development. From this study, greater insight was gained from the findings that in turn will necessitate the requirement for further investigation for future directions outlined herein.

Investigations in Chapter Four resulted in the isolation and maintenance of a pure population of trophoblasts from placental tissue (34-41 weeks) in monolayer culture. This provided a model for the examination of the effects of insulin and the IGFs on cellular proliferation and differentiation. The small, round cells isolated were of 1.048-1.062g/mL cell density and stained positive for cytokeratin 7, a specific trophoblast marker. These cells lacked vimentin staining. Cell morphology, size and the absence of the HLA antigen on cell membranes (together with the aforementioned criteria) confirmed that the cells isolated were cytotrophoblasts. It is generally accepted that placentae of 37+ weeks are classified as 'term'. In this study, placentae harvested at 34-41 weeks were used and showed no significant differences in the effects of insulin and the IGFs on proliferation and differentiation on the cells isolated.

Further study in Chapter Five demonstrated the positive effect of insulin on the mitogenic activity of human term placental cells in short term culture. The IGFs were also found to increase the proliferative activity of cytotrophoblasts *in vitro*. IGF-I treatment was shown to significantly increase cell numbers compared to insulin and IGF-II treatment of cytotrophoblasts. In this study, physiological

concentrations of insulin and the IGFs were administered (sections 5.4.2, 5.4.3, 5.4.4).

Insulin was also found to potentiate the differentiation of cytotrophoblasts from 'normal' placental tissue as evidenced by the increase in the number of cells aggregating and fusing to form multinucleated cells in culture (Chapter Five, section 5.4.2). The effect of insulin on cellular differentiation was further potentiated by the addition of IGF-I or IGF-II. In the presence of IGF-I alone, aggregation and differentiation of the cells was found to be minimal (section 5.4.3). This was confirmed by hCG assay where significant increases in hCG secretion over time were noted in insulin treated cultures and cultures treated with both insulin and the IGFs (section 5.4.6). The significant proportion of cells observed to differentiate into multinucleated cells might explain the observed decline in numbers over time as the occurrence of apoptosis was found to be insignificant. The desmoplakin staining and multinucleated cell formation through cell fusion (differentiation) and hCG concentrations in medium, of cells isolated from 'normal' tissue and grown in control conditions, compared favorably with other studies (section 5.4.5).

As a result of experiments undertaken in Chapter Six (section 6.4.4) insulin was found to have a positive effect on the proliferation of cytotrophoblasts obtained from the placentae of diabetic mothers as well. The proliferative activity of cells from IDDM placentae was found to be significantly pronounced whilst that of GDM was significantly reduced compared to that of 'normal'. The effects of the IGFs as described for 'normal' placentae were similar in the cells isolated from diabetic tissue but at increased levels. These differences in the effects of insulin noted between 'normal' and diabetic placenta are believed to be the result of differences in insulin receptor numbers (Chapter Seven) which will be discussed further.

The cellular differentiation observed in cultures of cells from 'normal' placentae was not reflected in the cultures of cells isolated from the placentae of diabetic women. The relative percentages of aggregates and the numbers of fused cells in these cultures were found to be significantly reduced compared to both insulin

treated and control 'normal' cell cultures (Chapter Six, section 6.4.7). In cultures of cells isolated from GDM and IDDM placentae, insulin treatment had no significant effect on hCG synthesis compared to that of control cultures and was found to be significantly lower than that of 'normal' cell cultures (section 6.4.8). As previously discussed (Chapter Two, sections 2.4, 2.10) alterations in the activity of the insulin receptor have been implicated in diabetes which may explain the modified ability of insulin to potentiate aggregation in cytotrophoblasts from diabetic placentae.

Preliminary glucose studies in Chapter Six (section 6.4.9) demonstrated that increasing glucose concentrations, within the physiological range, resulted in increased proliferative activity. This effect was further enhanced by the addition of insulin or IGF-I *in vitro*, particularly in cytotrophoblasts isolated from the placentae of 'normal' and IDDM women. This may be the result of upregulation of insulin receptors. Glucose levels reflective of poorly controlled glycemia (as seen in poorly controlled diabetes), proved to be detrimental to cell survival *in vitro* regardless of insulin or IGF-I administration.

The presence of insulin binding sites on the membranes of cells isolated from human term placenta was demonstrated in Chapter Seven, with significantly greater receptor numbers found on membranes of cells from IDDM tissue compared to that of 'normal' and GDM (section 7.4.1). This binding data reflected that of other studies and more importantly confirmed the presence of insulin receptors on cytotrophoblasts as previously noted in Chapter Two (sections 2.3, 2.10). Anatomical and histological evidence has implicated maternal diabetes and particularly insulin in influencing cytotrophoblast proliferation (Chapter One, section 1.4). The presence of insulin binding sites on cytotrophoblast membranes, as shown in the present study, suggests the ability of insulin to communicate with these cells.

Further investigation in Chapter Seven also showed the specific nature of the binding sites for insulin and how the IGFs may alter binding site availability. Despite the presence of excess concentrations of IGF-I or IGF-II, insulin still bound to the insulin receptor demonstrating the high affinity of the binding sites

for insulin. However, IGF-I was shown to reduce the number of available binding sites for insulin by almost a third (section 7.4.1). Due to structural homology, cross-reactivity between insulin and IGF-I is likely suggesting that insulin may bind to the type 1 IGF-I receptor, at a much lower affinity, and may therefore mediate direct proliferative effects. Experiments involving the anti-insulin receptor antibody (section 7.4.2) confirmed that the growth promoting effects of insulin were mediated via the insulin receptor.

The results obtained from experiments in Chapter Seven indicate that IGF-II does not affect the number of insulin receptors nor their affinity in cytotrophoblasts of term placentae of 'normal' or GDM pregnancies. Interestingly, in cytotrophoblasts isolated from the placentae of IDDM pregnancies, the number of available insulin binding sites increased in the presence of IGF-II.

Finally, the fetal membranes and placenta are physically closely associated with one another (Chapter One, section 1.8). The different tissues comprise different cell types that are believed to interact with one another to maintain the optimum growth and development of the fetus. In preliminary investigations in Chapter Eight on co-culture, results showed that the potentiating effects of insulin and the IGFs on cytotrophoblast proliferation and differentiation were not so pronounced in the presence of cells isolated from amnion or chorion. These results indicate that paracrine interaction between tissue types occur and that the presence of amnion or chorion modifies the proliferation and differentiation of cytotrophoblasts *in vitro*.

Thus, in the present study, insulin has been shown to potentiate the proliferation of cytotrophoblasts isolated from placentae of 'normal' and diabetic pregnancies. From the findings presented, this process is most probably mediated via specific insulin binding sites (found in greater number in IDDM placentae than 'normal' or GDM) located on the membranes of trophoblasts. This study has also demonstrated the complex interlinking of several factors. These include the increased availability of insulin and the IGFs and glucose along with altered insulin receptor number (and possible structural changes) and increased cell numbers observed in diabetic pregnancies. Whether the effects of insulin on the

growth of placental and fetal tissues are the result of increased metabolism or a direct stimulation of insulin has yet to be determined. Future investigation may look into the relationship of the insulin receptor with GLUT transporters in the human placenta. The findings of this study, however, give further insight to assist in the treatment of the effects of maternal diabetes on the maldevelopment of placental and fetal tissues during pregnancy.

9.2 Future directions

This study exclusively examined cytotrophoblasts obtained from term placentae and the effects of insulin and the IGFs on proliferation and differentiation of these cells. The human placenta is a complex organ comprising several different cell types that contribute to optimal fetal growth and development. To obtain a complete picture of the effects and interactions occurring within the placenta further investigations should examine the effects of insulin and the IGFs on other cell types of the placenta including endothelial cells, stromal cells, macrophages and the like. To gain further insight into the effects of insulin and the IGFs throughout gestation, further investigations should also aim to elucidate the role or interaction of these factors on cells isolated from early placental tissue. These studies would provide information on the stage at which cells are responsive to insulin and the IGFs and whether receptors to these ligands can be induced. Such information would further our insight and may lead to therapy adopted earlier in pregnancy.

Insulin and the IGFs, in this study, were found to potentiate proliferation of cytotrophoblasts isolated from term placenta of 'normal' and diabetic pregnancies. Future studies should examine the effects of insulin and the IGFs on other cellular functions of the isolated placental cells to further clarify the role of these factors in placental and fetal growth and development. Further investigations should also examine the effects of insulin and the IGFs on cells obtained from tissue of other diabetic classifications such as NIDDM and GDM treated with insulin. It would also be recommended to look into any interactions with other growth factors in maintaining placental growth and function, such as EGF and the TGFβ family.

From this study, glucose was found to increase the proliferation of cytotrophoblasts *in vitro*, which was further enhanced in the presence of insulin. Examination into the effects of glucose and insulin on other cellular processes of trophoblasts as well as investigation of the effects of other nutrients, such as lipids and amino acids, on cytotrophoblast proliferation and differentiation should be undertaken in future studies. It would also be recommended to investigate the metabolic effects of trophoblasts treated with insulin, IGF-I and/or IGF-II. Such studies should aim to determine whether the growth promoting effect of insulin is the result of increased metabolism or a direct effect of insulin.

Receptor binding analysis revealed the presence of high affinity insulin receptors on cytotrophoblast membranes where the availability of receptors were altered in the presence of the IGFs. Further investigation is warranted to extend receptor studies to include IGF-I and IGF-II binding assays. Future studies should also examine the effects of long term hyperglycaemic conditions on the availability of insulin receptors to further understand the relationship of glucose and insulin in gestational diabetes and their role in macrosomia.

As studies have suggested structural alterations in the insulin receptor as an outcome of the effects of diabetes on gestational tissues, then future studies should include the investigation of such alterations (eg, receptor splicing) and the investigation of 'atypical' insulin receptors, the presence of which has been suggested by others. Signalling pathways for the receptors for insulin and the IGFs (as well as the atypical insulin receptor) and the proto-oncogenes involved should also be examined to determine whether the diabetic state has altered intrinsic receptor activity and function. The information gained from such studies would improve the knowledge of the underlying mechanisms involved in diabetes and therefore improve therapeutic techniques. The antibody experiment would be repeated using immunopurified sera.

Co-culture experiments in this study have revealed modifying interactions between cytotrophoblasts and cells of gestational tissues surrounding the placenta, demonstrating paracrine and/or autocrine regulation of the effects of insulin and the IGFs on cytotrophoblast proliferation. Future studies should examine the

effects of these paracrine factors, particularly inhibitory factors and binding proteins on the cellular effects of insulin and the IGFs *in vitro*. As many of these factors are largely produced by the decidua, it would be highly recommended to perform co-culture studies with decidual tissue as well. This would provide a more complete picture of the complex interactions that occur between the different gestational tissues during pregnancy.

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APPENDIX ONE

CONSENT FORMS FOR TISSUE COLLECTION

The following are the explanation and consent forms provided to the pregnant mothers by the maternity staff of each hospital (Mercy Hospital and Royal Women's Hospital) prior to delivery. These forms provided information regarding the study and the need to collect placental tissue and a form of consent was then signed when these mothers agreed to partake in the study. All information obtained was confidential.

Mercy Hospital For Women

Explanation and Consent Form

Title: Paracrine Interactions in the Human Placenta

Investigators: Dr. M. Towstoless and Dr R. Fairclough

We seek healthy pregnant volunteers who are willing to provide us with samples of the placenta (afterbirth) and attached membranes prior to their normal disposal.

These tissues (placenta and attached membranes) are usually removed from the womb at the time of birth by your doctor and disposed of through the Hospital's system. The collection of these tissue samples for this research project will not interfere with the delivery of your baby nor cause problems with any future pregnancies you may desire.

A wide range of chemical compounds (such as hormones and related substances) are thought to play a vital role in the growth, development and maintenance of the fetus, the placenta (afterbirth) and attached membranes, and in the initiation of labor. These compounds are produced by the placenta and attached membranes and often exert their effects locally on neighboring cells.

In this study we seek to understand the role of these compounds in normal pregnancy and labor.

Mercy Hospital For Women

Consent Form: Paracrine Interactions in the Human Placenta

Would you feel comfortable about disclosing whether you smoked cigarettes during your pregnancy? Yes/No

Have you smoked cigarettes while you have been pregnant? Yes/No

Did you smoke throughout your pregnancy? Yes/No

How many days/weeks did you smoke?

How many cigarettes/day (on average) did you smoke?

I.....agree to participate in the research study entitled "Paracrine Interactions in the Human Placenta".

I have read and understood the attached information sheet and .....has discussed the study with me so I believe that I am fully informed on the matter.

I also understand that I am free to withdraw from the study at any time and that my treatment at the hospital will not be affected if I do.

.....	.....	.....
Patient's name (print)	Patient's signature	Date
.....	.....	.....
Witnesses name (print)	Witnesses signature	Date

Weight (placenta) =
Weight (infant) =
Weeks gestation =
Parity =

Dept of Perinatal Medicine RWH Patient/Delivery Data

Delivery date:	Delivery time:
Gestation:	Infant sex:
Gravidity:	Infant weight:
Parity:	Apgars: @1min @5min
Antenatal Course - Clinical condition	Antenatal medications

Labor Onset:	Spontaneous		
	Augmented		ARM
	Induced		Oxytocin
	No labor		Prostaglandins
Labor length	Stage 1:	Stage 2:	Stage 3:
Membrane rupture:	SRM	date:	
	ARM	time:	
Delivery mode:	NVD OVD	CS (not in labor)	CS (in labor)

Drugs in labor:	Medication	Administration time
	Nitrous Oxide	
	Pethidine	
	Phenergan	
	Maxalon	
	Epidural Anaesthetic	
	Spinal Anaesthetic	
	General Aneasthetic	
	Pudendal Anaesthetic	
	Oxytocin	
	Synometrine	
	Ergometrine	
	Other	

Placental weight:

Diabetes Study Data Sheet

Maternal age:

Maternal height:

Date	Period of Gestation	Weight (booking, 32+36 weeks)	GCT (2h BSL >8.0 GDM)	GTT

Insulin

(booking for IDDM or commencement of insulin for GDM +32 and 36 weeks)

Date & doses	BSL

HbA1C results

Date and gestational age	Result (Normal 4.7-6.4)

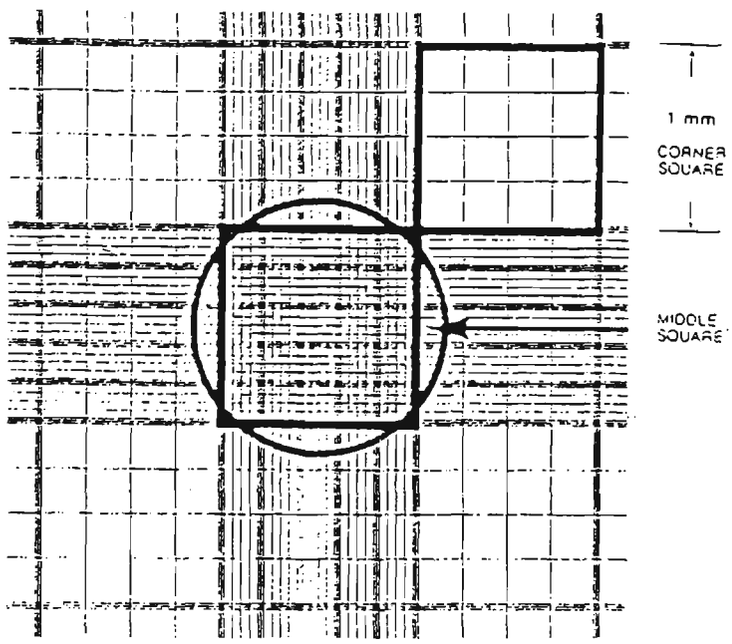
Notes/comments

APPENDIX TWO

CELL COUNTING CHAMBER

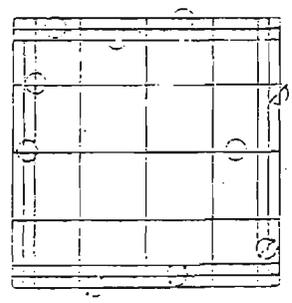
The following is a diagrammatic representation of the grid found on the cytometer used for counting of the cells.

DIAGRAM II
STANDARD HEMOCYTOMETER CHAMBER



The circle indicates the approximate area covered at 100 × microscope magnification (10 × ocular and 10 × objective). Include cells on top and left touching middle line ( ). Do not count cells touching middle line at bottom and right ( ). Count 4 corner squares and middle square in both chambers (one chamber represented here).

DIAGRAM III
CORNER SQUARE (ENLARGEMENT)



Count cells on top and left touching middle line ( ). Do not count cells touching middle line at bottom and right ( ).

APPENDIX THREE

CO-CULTURE SET-UP AND INSERTS

The following demonstrates the set up for co-culture experiments. One cell type was maintained within the well of the plate whilst the other cell type was maintained within the insert. Enough media was provided to cover both cell types within the one well. Refer to Chapter Three for details of co-culture set-up.

DIAGRAM A
THE FALCON CELL CULTURE INSERT SYSTEM
FALCON INSERTS USED WITH A FALCON COMPANION TISSUE CULTURE PLATE