# CHARACTERIZATION OF GROWTH FACTOR RECEPTORS IN HUMAN PLACENTAL CELLS IN CULTURE

by

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Short Title: Growth Factor Receptors in Human Placenta

To My Beloved Father, Mother

Brothers and Sisters

## ABSTRACT

The characteristics and specificity of receptors for insulin, epidermal growth factor (EGF), and the insulin-like growth factors (IGFs), IGF-I and IGF-II, were studied in human early gestation and term placental cell cultures of syncytiotrophoblast origin.

[$^{125}$I]-labeled peptides bound rapidly and reversibly to their cell surface receptors and exhibited a high degree of specificity. Maximal binding occurred at pH 7.5 and binding was proportional to cell protein and ligand concentrations. The placenta was found to be extremely rich in degrading activity for insulin and IGFs, but not for EGF. Bacitracin decreased the degradation of insulin and IGF peptides and was essential for the study of these peptides.

The ontogeny of these growth factor receptors was determined. The percent specific binding of insulin and EGF was significantly increased in term compared to early gestation placentae (insulin $p<0.001$; EGF $p<0.001$). In contrast, specific binding of IGFs was similar at both gestational ages.

Kinetic analysis of displacement data for binding of [$^{125}$I]-insulin and [$^{125}$I]-EGF generated curvilinear Scatchard plots, suggesting receptor heterogeneity. Pretreatment of cells with insulin or EGF resulted in a dose-, and time- dependent decrease in insulin or EGF binding, respectively. The insulin- and EGF- induced loss of binding was due to a decrease in the number of both high and low affinity receptor sites with no significant change in apparent affinity. Cycloheximide blocked EGF- but not insulin- induced down-regulation, and inhibited

recovery of both insulin and EGF receptors.

Preincubation with EGF in the culture medium significantly increased the release of human chorionic gonadotropin (hCG) by mid- term ($p<0.02$) and term ($p<0.001$) placental cells. There was a positive correlation between receptor number and tissue responsiveness to EGF.

Addition of bacitracin, chloroquine or colchicine increased the initial rate of binding of [$^{125}$I]-insulin and [$^{125}$I]-EGF to monolayers at 37 C in a time- and dose-dependent fashion. EGF stimulated ($p<0.05$) [$^{14}$C]-3-O-methyl-glucose uptake in term placental cells but had no effect on amino isobutyric acid uptake.

These studies indicate the feasibility of using human placental cells in culture as a model system to probe the hormonal-cell interaction in the feto-placental unit.

## RESUME

Les caractéristiques et spécificités des récepteurs à l'insuline au facteur de croissance épidermique (EGF), et aux facteurs de croissance insuliniques IGF-I et IGF-II, furent étudiés sur des cultures de cellules du syncytiotrophoblaste provenant de placentae de début de gestation et de placentae à terme.

Les peptides marqués à l'iode 125 [$^{125}$I] se liaient rapidement et réversiblement à leurs récepteurs membranaires et montraient un haut degré de spécificité. Le taux de liaison était maximal à pH 7.5 et était proportionnel à la concentration de proteine cellulaire et de chacun des peptides marqués. Il fut noté que le placenta possédait un pouvoir de dégradation de l'insuline et des IGFs extrêmement important, mais très faible dans le cas de l'EGF. La bacitracine diminuait la dégradation de l'insuline et des IGFs et était indispensable à l'étude de ces peptides.

L'ontogénie des récepteurs aux facteurs de croissance fut déterminée. Le pourcentage spécifique de liaison de l'insuline et de l'EGF était significativement plus élevé pour les placentae à terme, comparativement aux placentae de gestation plus courte (insuline: $p < 0.001$); EGF: $p < 0.001$). Toutefois, la liaison des peptides IGF-I et II ne démontrait aucune différence significative entre les les placentae de différents âges gestationnels.

L'analyse cinétique des courbes de déplacement des peptides [$^{125}$ I]-insuline et [$^{125}$ I]-EGF générait une courbe de Scatchard curviligne indiquant une hétérogénéité du récepteur. L'incubation préalable des cellules avec de l'insuline ou EGF atténuait spécifiquement et significativement la liaison de ces peptides selon la concentration et le temps. Cette diminution était due à une diminution du nombre de récepteurs de haute et de basse affinité sans changement apparent de l'affinité totale. Le cycloheximide bloquait la diminution de récepteurs à l'EGF, mais non celle de l'insuline, et inhibait la récupération des récepteurs EGF et insuline.

La pré-incubation des cellules placentaires avec EGF provoquait une augmentation de la sécrétion de l'hormone chorionique gonadotrope humaine (hCG) pour des placentae du deuxième trimestre de grossesse ($p < 0.02$) et à terme ($p < 0.001$). Il existait une corrélation positive entre le nombre de récepteurs et la réponse cellulaire à l'EGF.

L'addition de bacitracine, chloroquine ou colchicine augmentait le taux initial de liaison de l'[$^{125}$ I]-insuline et de l'[$^{125}$ I]-EGF à une monocouche de cellules incubées à 37$^{o}$C, selon la dose et le temps d'incubation de ces peptides. L'EGF stimulait la captation ($p < 0.05$) du [$^{14}$C]-3-O-Methyl-glucose, mais non celui de l'acide isobutyrique.

Ces études nous montrent la possibilité d'utiliser les cultures de cellules placentaires humaines comme modèle expérimental pour étudier les interactions hormone-cellule dans l'unité foeto-placentaire.

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## PREFACE

This thesis is concerned primarily with growth factor receptors in the human placenta. There are four major parts:

Part one: General introduction

Part two: Growth factor receptors in human placental cells

Part three: General discussion and conclusion

Part four: Contribution to knowledge

Part one begins with a description of the anatomy, growth and functions of the human placenta. This is followed by a general discussion of the interaction between peptide hormones and their cell surface receptors. Additional efforts have been directed at defining steps that occur beyond binding of hormone to its cellular receptor such as internalization, the fate of hormone-receptor complexes inside the cells and the possible biological effects mediated by growth factors.

Specific problems can be encountered in any study of growth factors. For this reason, caution must be exercised in the interpretation of data. Some examples of actual or potential problems relating to different aspects of the hormone-binding studies are presented as part of the general introduction.

Part two is subdivided into five chapters, each of which discusses a particular aspect of growth factor-placental cell interaction.

In Chapter I are presented the results of initial studies comparing [$^{125}$I]-insulin binding in various buffer systems. These studies permitted us to choose an optimal buffer for our subsequent assay systems.

The presence of significant insulin-degrading activity in

placental cell cultures has interfered with previous attempts to study insulin receptor binding and regulation in in vitro systems. In Chapter II we focus on the effective inhibition of insulin degradation by bacitracin, and examine insulin binding characteristics as well as receptor regulation under these new conditions.

In any investigation involving insulin, one should also consider a group of peptide hormones with a similar spectrum of activities, called insulin-like growth factors (IGFs). Chapter III discusses the results of preliminary studies of placental cell IGF receptors.

In Chapter IV we present the results of a study of epidermal growth factor (EGF) receptor binding and regulation, undertaken so as to better understand the mechanisms of action of EGF on the placental unit. Because EGF has been reported to be a tropic modulator of human chorionic gonadotropin (hCG) secretion in a human choriocarcinoma cell line, we have also studied the biological effects of EGF in detail.

In Chapter V we discuss the establishment of a new method that enables us to investigate the binding and metabolic effects of growth factors at 37 C with placental cells in situ.

Part three provides a summary and discussion of the most important observations made during the thesis project. The areas covered include radioreceptor assay methodology, receptor regulation, ontogeny of growth factor receptors and metabolic effects of growth factors. Finally part four outlines the original contributions to scientific knowledge made during this research project.

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# GENERAL INTRODUCTION

## 1. Human Placenta

The human placenta is the multi-functional tissue which acts as an intermediary between the fetus and the mother. There is still a great deal to learn about how the placenta works, but in recent years it has become clear that, rather than serving as a passive filter, the placenta is both active and selective in its transfer of substances essential to the development of the fetus (1,2). Furthermore, the placenta is able to modify molecules of maternal and fetal origin (3,4), and by synthesizing an entire range of hormones, indistinguishable from those manufactured by the pituitary, to regulate many of the body functions of the mother and fetus (5).

### 1.1 Development of the Placenta

Soon after fertilization the single-celled zygote containing genetic information from both parents begins to divide. The fertilized egg initially grows without special maternal provision. However, the phase of high progesterone production that follows ovulation in each menstrual cycle not only facilitates cell division of the zygote in a proper environment but also serves to prepare the uterus for its implantation (2). Following fertilization in the fallopian tube, the ovum undergoes repeated mitosis and reaches the uterus as a solid cell mass or morula, which is rapidly converted into a blastocyst. Surrounding the entire blastocyst is the zona pellucida. When this zone subsequently disappears, the outer cell layer of the blastocyst then proliferates to form the trophoblastic mass which will eventually differentiate into the main structural elements of the

placenta (6,7).

Once the blastocyst is in the uterus it proceeds relatively quickly to provide an environment favorable for its implantation in the uterine wall. Beginning on the 6th or 7th day after fertilization, trophoblast secretes human chorionic gonadotropin (hCG) which stimulates the corpus luteum to produce estrogen and progesterone (8). The estrogen facilitates the implantation of the blastocyst by effecting local permeability changes in the endometrial lining of the uterine wall (9,10).

In the 7th day ovum the peripheral trophoblastic layer of cells secures the fertilized ovum to the uterine wall and forms the beginning placenta. The trophoblast differentiates into a basal layer of large clear mono-nucleated cytotrophoblast cells and a superficial layer of multi-nucleated syncytiotrophoblast (11-14).

As the syncytiotrophoblast penetrates the endometrial lining, the endometrium becomes vacuolated, and a system of small cavities form in which maternal blood from the uterine arteries will eventually empty. In the meantime, the inner cytotrophoblast and the outer syncytiotrophoblast increase considerably in amount, forming a complicated system of projections that penetrate into the pools of maternal blood. These projections, called the primary villi, will serve as interphases between the fetus and the mother (15). Final differentiation of the trophoblast occurs in conjunction with development of the uterine decidual membrane that forms the amniotic sac in which the

fetus will grow (Fig. 1).

1.2 Trophoblast: Early Gestation

In the early gestation placenta the syncytium is of uniform thickness, there are no membranes separating the nuclei, but free remnants of membranes may occasionally be seen (6). The free surface of the syncytiotrophoblast is covered by large microvilli with a central fibrillary core (16). By electron microscopy, the syncytium of the early gestational placenta reveals an abundance of rough endoplasmic reticulum, which forms a syncytial canalicular system. This passes through the full thickness of the cytoplasm and may be of considerable importance for trans-syncytial transfer of nutrients (17). Free ribosomes are abundant in the syncytial cytoplasm and tend to appear in clusters along with numerous granules of glycogen. Mitochondria are moderately numerous and tend to be ovoid or rod-shaped, having very distinct lamellated cristae. The Golgi apparatus is well-developed but often difficult to recognize due to the abundant vacuolated endoplasmic reticulum. In addition, there are large lipid droplets lacking a limiting membrane. Enclosed by a distinct double membrane, small electron-dense secretory granules are also present and are seen close to the Golgi apparatus, tending to increase in size as they approach the syncytial surface. Granular lysosomes, vacuoles and multivesicular bodies are also present.

In the early gestation placenta, the cytotrophoblastic cells are a prominent feature of the trophoblastic layer of the immature villi (14). The cytoplasm is less dense than that of the syncytium and contains fewer subcellular organelles. The

endoplasmic reticulum is poorly developed and does not show vacuolation. Ribosomal clusters, mitochondria and Golgi tend to be larger and less dense than those of the syncytiotrophoblast. Lipid droplets and lysosomal structures are rarely seen and little glycogen is recognizable.

### 1.3 Trophoblast: Term Placenta

The general ultrastructural features of the term placental trophoblast remain essentially unaltered when compared to the immature trophoblast of early gestation (7). The microvilli on the surface of the syncytium of term placenta are fewer in number and tend to be shorter and thicker than those seen in early gestation. The syncytial layer is thinner and within the syncytial cytoplasm the endoplasmic reticulum is less abundant, and secretory granules, lipid droplets, ribosomes and glycogen granules are not as frequently seen. Cytotrophoblastic cells are less numerous than at early gestation but are regularly present (7). In both early and term trophoblast, the syncytio- and cytotrophoblast layers are linked by desmosomes and tight junctions (18).

### 1.4 Placental Transfer

During pregnancy, an important concern is the impact that changes in the maternal nutritional state have on the fetus. Thus, one must examine the placental role in transferring metabolites across the feto-placental barrier (Fig. 2). In the main, these cross in accordance with their concentration gradients, at rates related to their lipid solubility (19). Other factors may also affect rates of transfer, including relative

protein binding and molecular modification by the placenta itself (20,21). The respiratory gases ($O_2$,$CO_2$) and metabolically inert gases ($N_2$) cross the placental barrier by simple diffusion (22,23). The concentrations of calcium,iron, iodine and phosphate in fetal blood are elevated as compared to those found in the maternal circulation, and it has been assumed that these ions are actively transported by the placenta (24-26). Although a large number of studies have been performed relative to water transport across the placenta, to date the exact mechanisms are not known (26). Polypeptides cross the placenta poorly, and for all practical purposes the fetus is dependent upon its own production and secretion of large peptides (27,28). Glucose is transported by the passive process of diffusion (29-31). Amino acids are the exception, as they undergo active transport (32-33). Fetal blood glucose levels correlate well with those of the mother but are 10-20 mg/dl lower, providing a concentration gradient by which glucose diffuses. However, Felig (34) observed that glucose transfer exceeds a rate provided by diffusion alone. Therefore he suggested that glucose transfer at term is promoted by "facilitated diffusion", a process which may involve carrier transport.

In contrast to fetal glucose and amino acids, which are mainly supplied directly from maternal blood, only limited amounts of free fatty acids are thought to cross the placenta (35). Triglycerides from maternal plasma appear to be the major source of fatty acids for the fetus (36), fatty acid synthesis being a minor metabolic process in the placenta. The placental

contribution has been calculated to be about 2% of the total fatty acid requirements in fetal rats (37). There are no comparable human data available. Although albumin, gamma$_2$-globulin, 19S macroglobulin, fibrinogen, transferrin and glycoproteins have been demonstrated to cross the placenta by pinocytosis, the fetus probably synthesizes the vast majority of its structural and enzymatic proteins from amino acids derived from the fetal circulation (26).

Molecular transfer across the placenta is a more complex process than transfer across other cellular membranes, such as the blood/brain barrier (38). Transfer of metabolites may involve passage through several placental cells with or without molecular modification in the process. The marked selectivity of placental transfer has important biological consequences. Whether some disturbances of pregnancy may be due to a breakdown of these discriminatory mechanisms is not clear. An additional unknown is the nature of the specific placental mechanisms which maintain immunological "privilege" for nine months (39).

The most important activity of the trophoblast is to provide materno-fetal communication. It is clear that the pinocytotic vesicles and multivesicular bodies are both concerned with the transfer of carbohydrate and protein across the syncytium (40,41). The function of the microvilli and their role in syncytial activity is rather obscure. It is often suggested that the presence of microvilli facilitates the absorptive area of the syncytial surface. However, the placental microvilli differ markedly from those seen on absorptive epithelia such as the

intestine and renal tubules (14,42). Some investigators have suggested that they are chiefly concerned with trophoblastic excretion rather than absorption (16).

The syncytiotrophoblast is clearly the major site of placental synthesis of steroid and protein hormones (43-47). The non-membrane bound lipid droplets of the syncytium consist of nutritional fat "en route" from maternal to fetal circulation or for use in the production of the steroid hormones. The membrane-bound granules and droplets are probably syncytial hormones that are synthesized in the endoplasmic reticulum and packaged by the Golgi apparatus. Lysosomal structures are particularly enriched in the early term placenta, but their function is uncertain.

## 1.5 Hormone Synthesis

The production of specific hormones which regulate various activities associated with pregnancy is one of the most interesting functions of the placenta.

### 1.5.1 Steroid Hormones

The human placenta is an "incomplete endocrine gland", lacking the enzymes necessary to metabolize progesterone to $C_{19}$ steroids and hence to estrogen (48-50). However, it does have an active aromatase system which depends on provision of $C_{19}$ precursors of maternal or fetal adrenal origin (49,51). The human placenta, utilizing mainly maternal cholesterol derived from the low density lipoprotein fraction, synthesizes pregnenolone and progesterone and secretes them into both the maternal and fetal circulation (52,53). The fetal adrenal utilizes pregnenolone, efficiently converting the placental-derived or its own endogenously-produced pregnenolone to dehydroepiandrosterone

sulfate (DHAS). The fetal adrenal also synthesizes cortisol primarily from placental progesterone (4). Fetal DHAS is 16-hydroxylated in the fetal liver and subsequently converted to estriol in the placenta to account for approximately 80% of total estrogens in the mother during the third trimester (54,55). The placenta also synthesizes estrone and estradiol using fetal and maternal precursors (DHAS) in approximately equal proportion (54).

### 1.5.2 Peptide Hormones

From the earliest days of pregnancy the cells of the trophoblast are responsible for the production of hormones which regulate many activities associated with pregnancy. The first to be synthesized in significant amounts is hCG. hCG is a glycoprotein with a molecular weight of 45,000 to 50,000, about 30% of its weight being due to carbohydrate. hCG is composed of two subnits, alpha and beta, with the beta subunit being the hormone-specific component. The amino acid sequence of the alpha subunit of hCG is identical to the alpha subunit of the other glycoprotein hormones: human lutenizing hormone, follicle-stimulating hormone and thyroid-stimulating hormone (56,57). hCG is detectable in maternal plasma 8 to 10 days after implantation of the fertilized ovum and its level rises rapidly to reach a peak at about the 11th to 12th week of gestation, after which time it gradually declines until term. During the early differentiation stages of the trophoblast this hormone is found coating the cell surface, where it is suggested that it acts as protective layer, preventing rejection of the blastocyst and

facilitating implantation (58). In early pregnancy hCG stimulates estrogen and progesterone production by the corpus luteum until the feto-placental unit can take over (8).

Human placental lactogen (hPL) is also synthesized by the syncytiotrophoblast and is detectable in maternal plasma within one or two weeks after conception and implantation. Levels of hPL rise gradually, with maximal secretion occurring at term (59). The biological function of hPL is largely a matter of speculation but it has been suggested that its action is to decrease maternal utilization of carbohydrate and to increase the supply of carbohydrate as an energy source for protein anabolism by the fetus (60,61). Thus, hPL has two opposing actions: as an insulin antagonist and as promoter of insulin action (62). hPL also has lactogenic effects on the maternal breast and about one-hundredth the growth promoting activity of pituitary growth hormone (63).

Maternal levels of prolactin rise considerably during pregnancy. More interestingly, the concentration of prolactin in amniotic fluid is extremely high, as much as 100 times that attained in plasma (64,65). It is thought that prolactin in the amniotic fluid may participate in the homeostasis of amniotic fluid and may regulate amniotic fluid osmolarity and volume (66).

Human chorionic thyrotropin with thyroid stimulating activity (67,68) and human chorionic adrenocorticotrophic hormone (69,70) have been demonstrated. As yet, only very limited information is available.

## 1.6 Human Placental Cells in Culture

Although the human placenta contains a high concentration of peptide hormone/growth factor receptors (71,72), their exact

function is not known. The choice of human placental cells to study receptor binding and hormone action has both advantages and disadvantages. Among the former is the readiness and low cost with which one can obtain large quantities of cells for study. The disadvantages include the possible contamination with heterogeneous population of cells as well as the fact that the culture environment required for these cells to maintain their integrity could possibly mask the true function of this organ in vivo. However, in the study of correlations of hormone binding and possible biological functions, as well as receptor regulation at the cellular level, the advantages of using these cell cultures clearly outweigh the disadvantages.

To place cells in culture, the tissue is intentionally disrupted into individual cells by collagenase or trypsin digestion. The subsequent fate of such primary cultures varies enormously but most are quite short-lived. Two general hypotheses to explain this phenomenon have been proposed. The first suggests that current techniques of cell culture, especially the cell environment, are not adequate to permit continued proliferation and survival of primary cells (73). The second proposal implies that limited survival is intimately associated with the number of cell divisions undergone and is related in some way to senescence in the whole organ (74,75).

## 2. Peptide Hormone /Growth Factor Receptors

### 2.1 Nature of Membrane Receptors

The term "receptor" can be defined as a chemical structure which provides sites which specifically bind ligands in their target tissue, consequently initiating a biological process necessary to produce a final biological response. The first of the two basic requirements for a receptor is the ability to recognize and to bind a particular ligand. The structural arrangement of hormone receptors within the cell membrane is not yet known. Edelman (76) proposed that the plasma membrane is a fluid structure and suggested "lateral mobility" of surface receptors -- specific glycoproteins at the membrane surface appear to play a major role in the changes related to surface modulation. The forces affecting the motion and distribution of the receptor are complicated as is the structure of the receptor itself. Receptors appear to pass through heterogeneous phases as they traverse the membrane. To date, there are no quantitative estimates of the relative contribution of the various forces acting on such a receptor (Fig. 3), but there is some evidence that external cross linkage with other receptors and internal interactions with the submembranous structures provide major contributions. These structural interactions are probably responsible for the clustering, patching and capping of receptors that is induced by cross-linking agents such as bivalent antibodies.

de Petris (77) suggested that the "patch" and "cap" formation subsequent to binding is due to a cross-linkage force and results in a diffusion controlled nucleation of the

receptors. These cross-linked patches of ligand-receptor complexes are then gathered within minutes to one pole of the cell to undergo endocytosis.

Willingham et al (78) have studied the interaction of $alpha_2$-macroglobulin ($alpha_2M$) with the surface of cultured fibroblasts. They concluded that unoccupied $alpha_2M$-receptors are diffusely distributed on the cell surface. However, when $alpha_2M$-receptor complexes are formed, they very rapidly cluster in coated regions or pits in the plasma membrane and are subsequently internalized in coated vesicles. Since insulin and EGF behaved in similar fashion to $alpha_2M$, they suggested that these polypeptide hormones will follow this same pathway.

## 2.2 Internalization and Intracellular Localization of Peptide Hormones

Polypeptides with their receptors can be taken up into the cells by a process called internalization (79). The clustering of ligand-receptor complexes on membrane surfaces is an important prelude to internalization (80). The most plausible mechanism to explain the internalization is endocytosis (81). This process involves invagination of the plasma membrane, fusion of the neck of the invagination, and pinching off of the membrane-bound vesicle.

An analysis of the morphology of hormone-receptor binding can be performed by combining quantitative electron microscopic autoradiography with direct binding studies. Schlessinger et al (80) studied the binding of fluorescent derivatives of insulin and EGF analogues to 3T3 fibroblasts. These analogues retained substantial binding affinity as determined by radio-receptor

assays. The cells labeled with the fluorescent analogue were visualized with a sensitive video intensification microscopic system. They found that both insulin and EGF initially bound diffusely to the cell surface at 4 C. Within a few minutes at 23 or 37 C the hormone-receptor complexes aggregated into patches, initially mobile in the plane of the membrane, and later became immobilized as a consequence of receptor aggregation or internalization.

It thus seems that receptors exist in naturally occurring groups on the cell surface. Specific domains and the microenvironment of the membrane surface likely play a role in regulating the interaction between various active ligands and their unique receptors. Further studies will be required to define more precisely the functional role of these "caps" and "coated pits".

Gorden et al (82) and Carpentier et al (83) utilized quantitative autoradiography to study the fate of [$^{125}$I]-insulin exposed to cultured human lymphocytes and rat adipocytes. In both cell types, labeled insulin binding occurred initially on the plasma membrane; with increasing time and temperature, there was a progressive translocation of some of the autoradiographic grains across the plasma membrane to a distance of about 15% of the radius of the cell. The process was qualitatively similar in both cells, but quantitatively greater in hepatocytes due to the larger volume occupied by the adipocyte nucleus. The internalized autoradiographic grains were preferentially associated with cellular structures in regions quantitatively rich in lysosomal and Golgi elements (82-84).

Goldfine and Smith (85) reported specific binding of insulin to purified nuclei from rat liver. Vigneri et al (86) incubated isolated rat liver nuclei with labeled insulin and reported that 70% of specific insulin binding was associated with the nuclear membrane. The pH optimum for binding of insulin to nuclear membranes plateaued between 6.5 and 7.25. In contrast to insulin binding sites on the plasma membrane, insulin binding sites on the nuclear membrane did not display negative cooperativity. On the contrary, many other investigators (80,82,84) have not found evidence for insulin binding to nuclei. Jackson (87) indicated that during purification, contamination from the plasma membrane fraction to purified nuclei could occur.

Bergeron et al (88) demonstrated specific binding sites for insulin in Golgi fractions isolated from rat liver. The binding sites in Golgi elements were markedly similar to those of the plasma membrane (89). The similarity of the Golgi element and plasma membrane receptor led them to postulate that the Golgi elements may play a role in the synthesis and shuttling of hormone binding sites to the cell surface. The binding sites in other organelles are not known.

The biological role of hormone-receptor internalization remains unsolved. Although receptor patching and capping commonly occur in cells exposed to multivalent ligands such as $alpha_{2M}$, the subsequent process from patching to capping may not apply to the univalent ligands such as polypeptide hormones (78,79).

## 2.3 Hormone-Receptor Binding and Biological Effects of Hormones

There have been a number of attempts to correlate quantitatively the binding of a hormone to its receptor and the

biological effects of the peptide hormone. Frequently, there is a poor correlation between binding to receptor and the magnitude of the hormonal response (90,91). In many tissues, the complete biological response is evoked by occupancy of a small proportion of the available receptors (92). Such observations have led to the recognition that many hormone-responsive tissues are characterized by the presence of "spare" receptors (92). The term "spare" is only used in a purely relative sense, as the degree of excess receptor may differ according to the biological response which is observed. Excess receptors could facilitate the ability of cells to reach the threshold required to evoke a cellular response (93,94). Thus, the presence of a large number of receptors could be essential for occupancy of the much smaller number required for biological response.

In isolated thymocytes, hormone binding and the consequent biological response are obtained only when all receptors are occupied and the binding and response curves are superimposable over the entire range studied (94). Such a close quantitative correlation is rarely observed. There are several well-defined receptor abnormalities which are associated with distinct endocrine dysfunction, such as altered receptor concentration or affinity (95-99); or the presence of anti-receptor antibodies (100).

Prolonged exposure of target tissues to ambient ligand concentrations is frequently followed by loss of sensitivity for the biological effects of the target tissues. Such effects have been particularly well documented for insulin (95,96). It is well known that hyperinsulinemia and insulin resistance are associated

with a decrease in the concentration of insulin receptors per cell (101). An inverse relationship has been found between the plasma insulin concentration and the number of insulin receptors (102), consistent with the concept that it is the hyperinsulinemia which leads to the decrease in insulin receptors. The presence of hormone-induced receptor loss is termed "down-regulation" (101,102). This phenomenon could account for the frequent occurrence of hormone resistance with high circulating levels of hormones (96). The modulation of receptor function in target tissues is most commonly achieved by alteration in receptor concentration with a consequent change in target tissue sensitivity.

A more dramatic form of receptor disorder has been recently recognized to result from the development of circulating antibodies to certain tissue receptors (100,103). Antibodies to the insulin receptor have been detected in some patients with marked insulin resistance, often in association with acanthosis nigricans and systemic immune disease (103). Such antibodies can act as competitive antagonists by decreasing the apparent receptor affinity and desensitizing the target-cell to insulin action.

The extent to which hormonal regulation of tissue receptor concentration, as well as antibodies that affect receptor activation, are responsible for alterations in hormone sensitivity during human endocrine disorders has not been determined. It is likely that desensitization will include a complex group of processes including receptor occupancy, and degradation or processing of hormone-receptor complexes (103).

## 2.4 Mechanism of Target-Cell Activation

The mechanism of target cell activation has not been clarified for polypeptide hormones such as insulin, prolactin, growth hormone and the growth factors. Two general models of hormone action have been proposed (104). The first model suggests that hormone action follows a mechanism similar to that for peptide hormones which activate adenylate cyclase (105,106). In this model, insulin binding to its receptor results in an activation of some membrane-associated messenger for hormone action (107). Hormone-induced peptide messengers may be derived from the receptor itself or from an associated substrate by activation of an intrinsic membrane protease (107). Das et al (108) proposed a transduction mechanism to explain how EGF-receptor interaction can lead to the production of a second messenger which drives the cell through $G_1$ (resting stage) and commits it to enter the S (synthetic period) phase of the cell cycle. They proposed that proteolytic processing of receptors or some other endocytosed membrane protein yields a species which either can serve as second messenger or has the potency to produce one. The generation of membrane-associated peptide messengers may be an important component of the action of several peptide hormones, including insulin and growth factors (107,108).

The second model suggests that the hormone enters the cell and in some form serves as a second messenger itself (109). Regardless of the exact site of localization, some of the internalized hormone is degraded, and this process can be blocked by drugs that are lysosomal protease inhibitors (108,110). This has led to the suggestion that either the hormone or a fragment

of a hormone interacts with other intracellular proteins to produce the final biological response (108). Although intracellular binding sites have been reported (86,88), there is no evidence that these are biologically important in hormone action. In addition, we do not know if receptor-mediated degradation of hormone is a general phenomenon which occurs in all cells that possess specific binding sites for this hormone, or if degradation of receptor bound hormone is a necessary prerequisite for biological activity. While occasionally degradation does correlate with the elicitation of a biological response, more often this is not the case (104,108,111-116) (Table 1).

Recently, insulin (117-119) and EGF (120,121) have been found to stimulate the phosphorylation of a tyrosine residue of their own receptor, suggesting that phosphorylation of protein is an important regulatory mechanism in hormone action. It seems likely that this mechanism could act as a direct signal, could change the rate of internalization, or could modify the activity of a membrane protease which generates a peptide second messenger that is responsible for the effects of certain peptide hormones that are not linked to the adenylate cyclase system.

To date, only a few studies have probed the question of receptor-mediated endocytosis and subsequent internalization of polypeptide hormones. To study the entire sequence of hormone binding and hormone action by morphological and biochemical methods in isolated cells will be a challenge for the future.

## 3. Methodology of Binding Assays and Binding Properties of Hormone Receptors

### 3.1 Methodology Considerations

The basic methodology employed for most direct studies of hormone-receptor interaction utilizes an isotopically labeled hormone incubated with a suitable receptor preparation. After some period of time the hormone-receptor complex is separated from the free hormone by centrifugation, filtration or precipitation and the receptor-bound radioactivity is determined. The simplest method is to label the hormone with a radioisotope. This is equally suitable for quantitative determination and for localization of the bound hormone. The label is attached either to an integral part of the hormone molecule, as with $^{3}H$ or $^{14}C$ labeling of amino acids, or is linked with tyrosyl residue in the hormone molecule, as with $^{131}I$ or $^{125}I$ labeling of peptide hormones and growth factors. Of available radioisotopes, $^{125}I$ is the most useful for labeling peptide hormones because of its long half life. A simple method for iodination using oxidation with chloramine T was first applied to growth hormone (122 ) and was found to be most suitable in generating labeled ligands with high radiospecific activity. In general, it appears that many of the monoiodohormones, such as A14 monoiodoinsulin, have binding affinities and biological potencies indistinguishable from native hormone (123).

Tissue preparations used as receptor source have included plasma membranes and intact cells. The plasma membrane surrounding living cells plays a role in intracellular metabolism by mediating interactions between the cell and its external

environment. Isolation of plasma membranes has been preparative rather than analytical due to the complexities inherent in working with cellular membranes. For instance, despite an appropriate choice of a particular marker of plasma membranes, possible contamination by other subcellular particles may result in the uncertainty of membrane preparations (124). Nevertheless, plasma membranes often possess hormonal responsive receptors and offer an advantage with regard to stability during storage. There is a direct value in using more purified and well-characterized membrane preparations, since a crude fractionation scheme can result in contamination by internal organelles. On the other hand each membrane preparation may give only 10-50% of total yield, and thus, may not be representative of the total plasma membrane (124). To further complicate matters, solid tissues are generally composed of more than one cell type which may affect the final membrane preparation. It is thus important that each membrane preparation utilized for detailed studies be studied for yield, purity and the possible contamination with other organelles.

Intact cell preparations have been obtained from blood, by tissue culture or by enzymatic and/or mechanic disruption of tissues. Cell preparations offer an advantage in that they are metabolically active, thus providing a means for correlation of hormone receptor binding and biological effect (125). However, enzymatic digestion used to isolate cells can affect the concentration or affinity of hormone receptors. Furthermore, hormonal degradation, heterogeneity of cell types, cellular adaptation to the new environment as well as the stage of the cell cycle and possible transformation may all affect receptor

binding (126).

## 3.2 The Cell and its Assay Buffer

Presumably the best assay condition for cells is one providing as nearly as possible the conditions they experience *in vivo*. The number of satisfactory buffers available for use in the physiological range of pH has been small and those most commonly used have been bicarbonate, phosphate, and Tris buffer (127). The function of the assay buffer solution is to maintain the pH and osmotic pressure, and also to provide an adequate concentration of essential inorganic ions.

Phosphate is an essential constituent of normal body fluids, but it has the disadvantage that it precipitates most multivalent cations when present at high concentration (128). Therefore, it is impossible to combine high concentrations both of phosphate and calcium in the same solution, although the picture is complicated by such factors as variations in pH and ionic strength.

The use of Tris buffer was first proposed by Gomori (129), and it has been used extensively *in vitro*, and more recently, *in vivo*. Omachi et al (130) observed volume changes of human erythrocytes in protein-free suspensions containing Tris (300-413 mOsmol/l). They found a swelling of the red blood cells within 10 minutes, the rate of volume change being directly related to the nonionized Tris concentration. *In vivo*, Tris, like most ionizing substances, enters most readily in the uncharged form and its subsequent ionization inside cells leads to a rise in intracellular pH.

Hepes buffer has been shown to be non-toxic to cells and can

be used instead of bicarbonate in which case cells need not be maintained in an atmosphere of 5% $CO_2$ in air (131).

The bicarbonate/$CO_2$ buffer system is capable of permeating all cell membranes easily. This property gives the $HCO_3^-/CO_2$ buffer system the capability, unique among the commonly recognized physiologic buffers, of rapidly influencing intracellular, as well as extra-cellular pH. The most important point about this buffer system is that, under given conditions of temperature and salt concentration, the pH is determined by the ratio of the bicarbonate concentration to the partial pressure of carbon dioxide.

The choice of a buffer system for physiological studies must be based on specific cell requirements. The following are some general criteria: 1. the cells need survive only long enough to display certain basic properties, such as hormone-receptor interaction, 2. the cell should maintain all or most of its normal composition compared to that of normal intracellular fluid, 3. the buffer should be as close as possible in composition to normal extracellular tissue fluids, and 4. the buffer should be identical to one used previously by other workers, so that the preparation as a whole is one for which information exists.

### 3.3 The Binding Properties of Hormone Receptors

Until recently the hormone-receptor complexes have not been isolated, even in a partially purified form, and thus, in most investigations it has been important to define the hormone-receptor interaction by its binding characteristics (132).

### 3.3.1 Hormone Binding Characteristics

The saturability of the hormone-receptor interaction is demonstrated by displacing bound labeled hormone with various amounts of unlabeled hormone. Increase of the hormone concentration accounts in due course for binding of the hormone to all available receptors, so that above a certain concentration limit no further hormone-receptor binding takes place.

The time course of hormone binding to its receptors is dependent on temperature and the concentrations of hormone and receptor. At 22 and 37 C steady states are observed within a few minutes, while at low temperature, a steady state may not be reached for hours (133). The hormone-receptor binding is usually reversible; the bound hormone can be replaced by excess native hormone. This phenomenon, like enzyme-substrate interactions, can be utilized to substantiate the specificity of the hormone-receptor binding on the grounds that only the specifically bound hormone can be displaced by excess native hormone. Bound hormone can also be dissociated by changes in the incubation mixture pH (132) or by dilution (134). Hormone dissociated from the receptor appears to be identical with the native hormone in respect to its physical properties, such as interaction with antibodies or rebinding to other membrane preparations, and in biological activity.

### 3.3.2 Quantitative Analysis of Ligand-Receptor Interactions

The quantitative analysis of hormone receptor interactions has been based on the recognition that receptor occupancy determines the magnitude of pharmacological responses and that

drug-receptor interactions are determined by the law of mass action. A variety of mathematical methods have been applied to the analysis of steady-state hormone-receptor binding data. In most of these analyses, the hormone-receptor interaction is defined as a simple reversible bimolecular equilibrium.

The most widely used method for analysis of receptor binding data has been the Scatchard analysis (135). Data from a competition binding assay can be used to derive receptor concentration and receptor affinity as determined from the Scatchard analysis, which plots bound/free radioactivity as a function of the concentration of bound hormone. A straight line derivation has been thought to represent ligand binding to a single class of homogeneous receptor sites; a curvilinear Scatchard plot represents two or more orders of heterogeneous receptor sites, each with fixed affinity to the corresponding hormones.

To calculate receptor concentration and affinity, it is important to consider the integrity of the tracer employed and to evaluate the extent to which receptors and ligand undergo degradation during the assay procedure (136). In addition, the binding process can be complicated by imcomplete dissociation between hormone and receptor, as well as by sequestration or internalization of the hormone-receptor complexes, and the possibility of unoccupied receptor sites (134,137). Such factors must be taken into account in the analysis of hormone binding sites in order to prevent distortion of the estimates of receptor number and affinity derived from these analyses.

Recently, De Meyts et al (138) have suggested that hormone-

receptor interaction does not appear to follow the law of mass action; when analyzed by the method of Scatchard or other graphical transformations, the binding isotherm appears complex and shows deviations consistent with either heterogeneous receptor sites or negative cooperativity, or both (134).

The association rate constant of the hormone-receptor complex is about $10^4$ - $10^7 M^{-1} S^{-1}$ (132). These rates are significantly lower than would be predicted for a simple diffusion limited process (139). This suggests that most random collisions do not result in a binding reaction and that the reacting molecules have to overcome an activation energy.

## 4. Growth Factors in Development

### 4.1 Growth Factors

The events leading to cell replication *in vitro* and to actual growth *in vivo* are poorly understood. The addition of serum is essential for the growth of cells, and this may be due to the presence of growth factors and other unknown trace but essential elements in the serum. Growth factors are different from nutrients, which can be defined as substances that are used by the cell as metabolic substrate or cofactors. Nutrients such as amino acids, sugars, salts etc. are not sufficient to maintain cell growth. In order to study growth factors, one must maintain cells under conditions such that only growth factors are limiting. Furthermore, for any growth factor to be truly a mitogen, any biological effects of physiological significance must be demonstrated *in vivo*. However, it is extremely difficult to totally deprive an animal of growth factors in order to show that an exogeneous growth factor can stimulate growth.

Although many types of growth factors have been partially isolated and characterized, with variable cellular effects, only a few are available in pure form. These growth factors usually do not produce growth stimulation on their own, but require low amounts of complete serum, suggesting that several factors must act together. Some of the confusion in studying cell growth *in vitro* may be due to ill-defined terms of culture conditions such as "confluent", "stationary" or "quiescent" cultures. In addition, bioassays for growth factors are not specific. For example, human foreskin fibroblasts respond to nanogram concentrations of at least two different and unrelated growth

factors, EGF and fibroblast growth factor (FGF) (140). Thus, DNA synthesis in response to serum is a complex phenomenon with numerous factors interacting in concert.

Although many growth factors are present in serum or plasma, and in other body fluids such as urine, the site of biosynthesis of these factors is obscure. Several growth factors have been isolated from human plasma: the insulin-like growth factors (IGFs): IGF-I (141) and IGF-II (142,143), somatomedin A (Sm A) (144) and somatomedin C (Sm C) (145). Growth factors have also been purified from sources other than blood. EGF was obtained from mouse submaxillary gland (146) and from human urine (147); nerve growth factor (NGF) was prepared from the mouse submaxillary gland (148) and snake venom (149), and FGF from bovine pituitary and brain (150). Growth factors have also been isolated from media "conditioned" by the growth of cultured cells: multiplication stimulating activity (MSA) was isolated from media conditioned by rat liver cells (151) (Table 2).

Somatomedins (Sm) are a family of peptide growth factors that are growth hormone dependent with insulin-like activities (141-145,151,154-158)(Table 3). The Sm family includes Sm A, Sm C, IGF-I, IGF-II and MSA. Sm C and IGF-I are basic peptides that are either very similar or identical, and IGF-II, Sm A as well as MSA are neutral or slightly acidic peptides that are believed to have similar structural relationships (159). Both IGF-I and IGF-II have been purified and have 62% homology with each other and a high degree of homology with human proinsulin (142). Purified Sms have molecular weights around 8,000 daltons, but in human serum

they are bound to carrier proteins of approximately 150,000 and 38,000 molecular weight (160). In the fetus, placental lactogen may be one factor which controls IGF synthesis and release (161). Although the role of IGFs in the growth of the fetus has not been elucidated, it has been shown that fetal tissues are rich in IGF receptors of high affinity (162,163) and fetal cells are stimulated to proliferate in vitro by Sm (164). Human fetal serum has readily detectable concentrations of IGF and the levels in cord blood correlate well with size at birth (165,166).

EGF receptors appear very early in fetal tissues (167,168), and evidence for an effect of EGF on fetal cell proliferation and growth of the lung and palate has been obtained (169,170). However, there has been no demonstration of a normal physiological role for EGF either in the adult or the embryo. Messmer and Holley (171) introduced rabbit antibodies to EGF into pregnant female mice and did not find any detectable effect on fetal development even though the EGF antibodies were able to bind to EGF and to prevent EGF from binding to cells. This might be interpreted as strong evidence against a role for EGF in fetal development. On the other hand, the active fetal form of EGF may not be neutralized by the antibodies because it may be produced and/or removed as needed only in restricted locations (171). In addition to its role as a mitogen, EGF was reported to stimulate the transport of small metabolites into cells (172), which could be of enormous nutritional importance to rapidly growing cells. Furthermore, EGF caused cell surface and shape changes in some cell types (173-176) which may be related to its ability to phosphorylate certain cellular proteins (120). However, increased

phosphorylation stimulated by EGF does not seem to be correlated with mitogenic effect (177).

It is well recognized that during postnatal life insulin stimulates cytoplasmic growth by promoting protein, glycogen and fat synthesis (178-180). Insulin also serves as a coordinator of inter-organ metabolic process (181). Because of its anabolic properties, insulin has been recognized as one of several possible growth factors during fetal life (153). Plasma membranes from human fetal livers between 15 and 18 weeks of age bind less than one-fourth as much insulin as do membranes from 26 to 31 week old fetuses (182). A positive correlation between infant birth weight at birth and binding of insulin to monocytes from normal infants has been reported (183). However, the exact mechanism of action of insulin and its interaction with other growth factors during fetal life is not clearly understood.

NGF may have a clear developmental role that has been demonstrated by the introduction of exogeneous NGF to embryos *in vivo* (184). In addition, NGF is concerned with the growth, development and maintenance of sympathetic and some sensory neurons (185). The mechanism of its action is not yet known, but it could involve the formation of microtubules and internalization (186,187). FGF is a basic polypeptide synthesized in the pituitary (152). It is more specific for mesoderm- derived cells, whereas EGF is more specific for endoderm- or ectoderm-derived cells (152). The role of FGF *in vivo* is not known.

## 4.2 Role of Growth Factors in Fetal Development

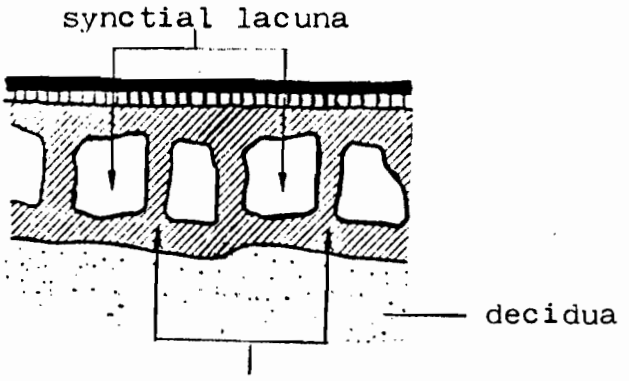
Studies aimed at the elucidation of the role of growth

factors in fetal development have been generally productive for each growth factor. Although a great deal is known about the *in vivo* effects of insulin, studies on growth factors such as EGF and IGF during fetal development are complicated by the production of placental factors that could interact with maternal as well as fetal tissues (171). They are also complicated by the possibility that factors may be transmitted to the fetus by the placenta from the maternal circulation at levels below the sensitivity of standard assays, or by temporarily restricted and/or localized interactions between cell types that may never be suspected from *in vitro* results. In addition, there may also be complex interactions between developing tissues. Furthermore, it is likely that the control of growth occurs as a delicate balance of growth factor production, cell receptor affinity, receptor regulation, and differential recognition for related growth factors. In addition, the nutritional supply and ensuing metabolic activity will be important in cell growth and it should be borne in mind that increased intracellular transport of low molecular weight nutrients is an observed effect *in vitro* of all the known growth factors (152).

In view of the above considerations, studies of placental monolayer cultures may provide readily available and relatively simple methods to assess growth factor binding and biological activity. Furthermore, these studies may give clues to the possible *in vivo* effects of growth factors.

Fig. 1 Diagrammatic representation of the early development of the placenta

A). 9-13 days




B). 13-21 days

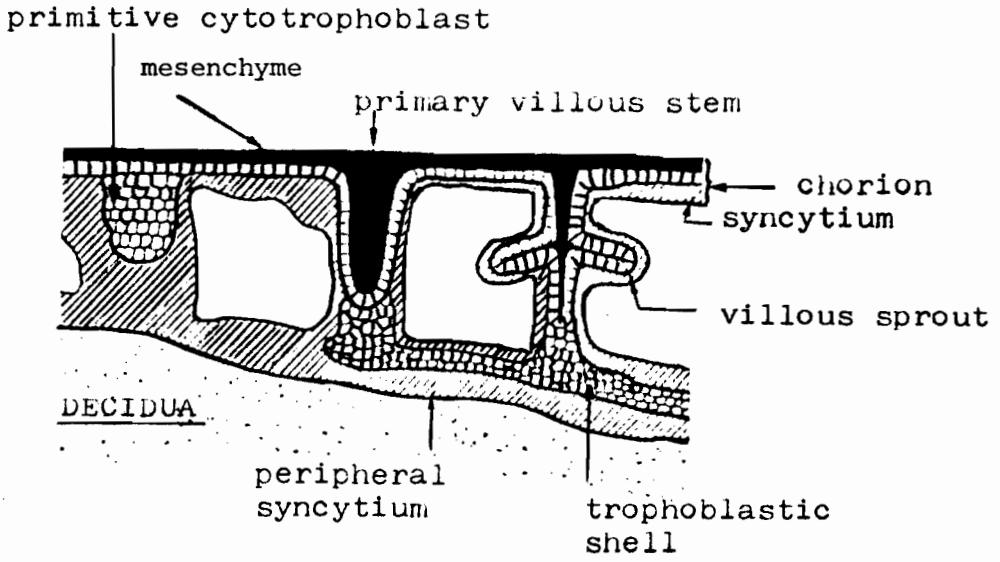


Fig. 2 Transplacental Transport Mechanisms of Various Substances

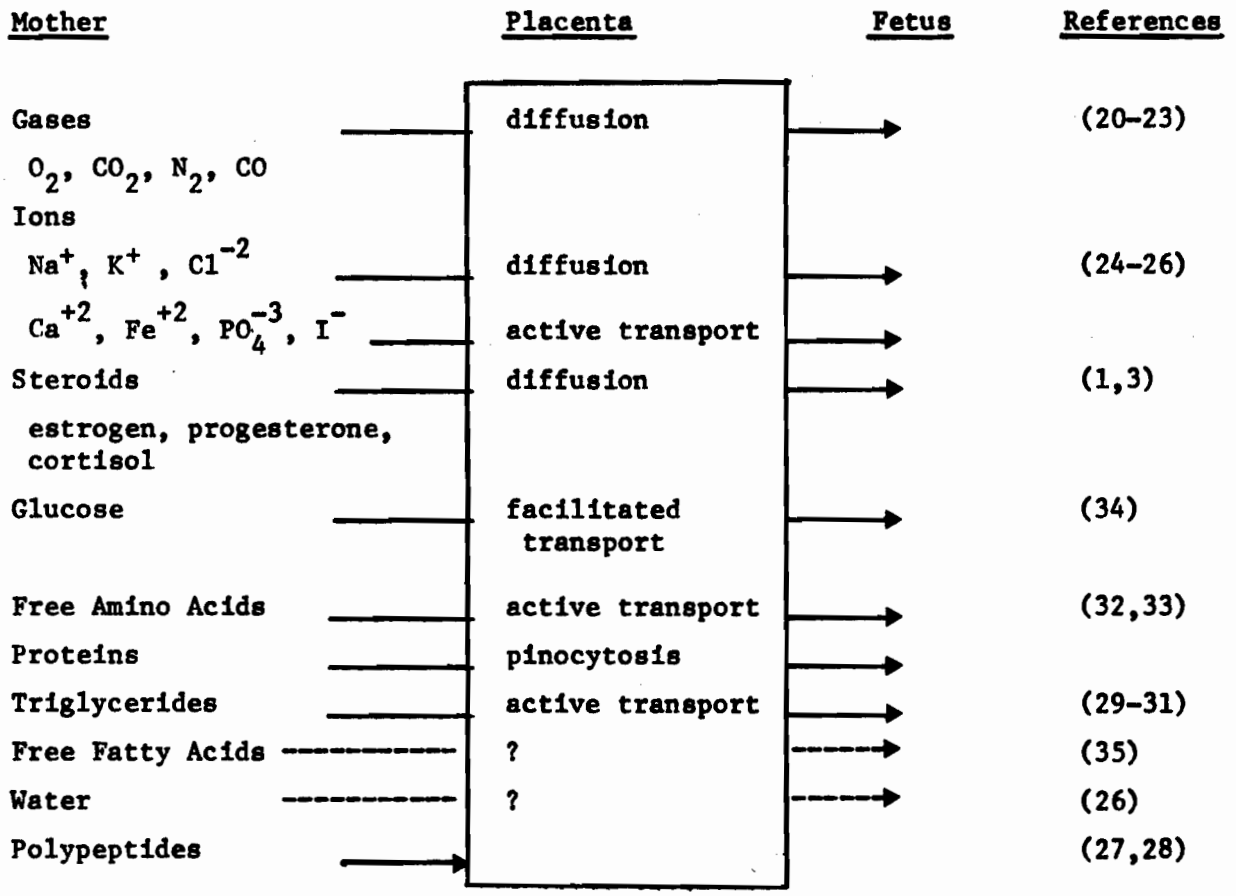


Fig. 3 Schematic illustration of surface receptor--specific glycoproteins at the membrane surface appear to play a major role in the changes related to surface modulation (76).

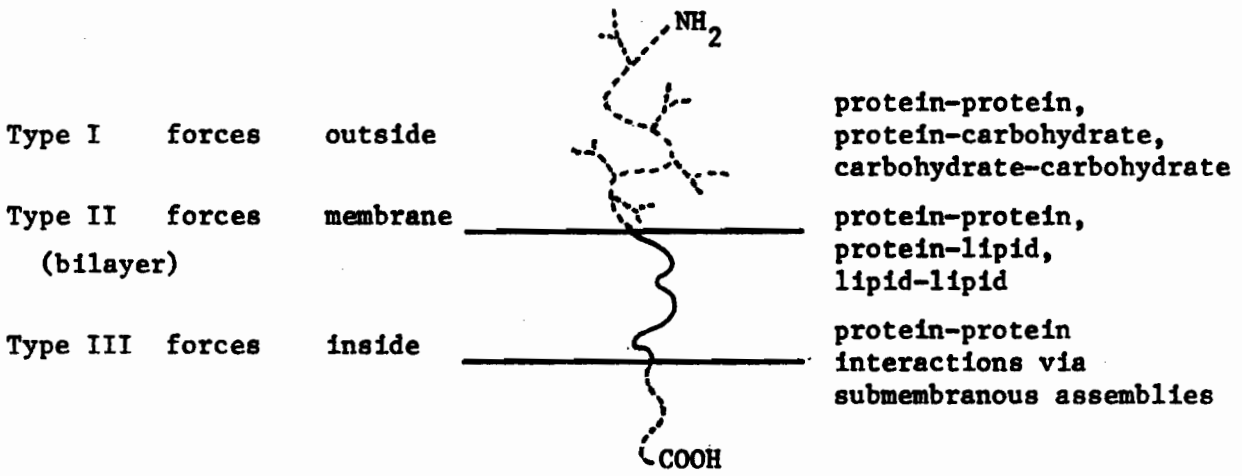


Table 1 Internalization, Degradation and Hormone Action

| Hormone | Cell Type | Biological Effect | Inhibitor | Effect of Inhibitor on | | | References |
|---|---|---|---|---|---|---|---|
| | | | | internalization | degradation | biological response | |
| Insulin | | | | | | | |
| | adipocyte | glucose transport | DNP*, KCN, $N_3$ | + | + | + | (111) |
| | | phosphodiesterase | chloroquine ] diabucaine ] tetracaine ] | - | + | + | (112) |
| | | glucose oxidation | chloroquine ] methylamine ] bacitracin ] | + | - | - | (104) |
| | | phosphodiesterase | antibody to glutathione-transhydrogenase | - | + | + | (113) |
| | hepatocyte | AIB transport | methylamine | + | + | - | (114) |
| | | glycogen synthetase | chloroquine ] bacitracin ] | - | + | + | (115) |
| | fibroblast | DNA synthesis | methylamine | + | + | - | (104) |
| EGF | | | | | | | |
| | fibroblast | DNA synthesis | methylamine | + | + | - | (116) |
| | | | nacodazole | + | + | + | |
| | | | leupeptin | + | + | - | |
| | granulosa | DNA synthesis | leupeptin | + | + | - | (108) |

* DNP: 2,4 dintrophenol

Table 2 Growth Factors

| Growth Factors | Source | Biological Action | Reference |
|---|---|---|---|
| Insulin | pancreas | anabolic action and mitogenic effect | (153) |
| EGF | mouse submaxillary gland | early eye opening in mice | (146) |
| | human urine | mitogenic effect | (147) |
| NGF | mouse submaxillary gland | sympathetic nerve cell growth | (148) |
| | snake venom | | (149) |
| FGF | bovine pituitary | mitogenic effect | (150) |
| Ovarian GF | bovine pituitary | mitogenic effect | (152) |
| FGF | bovine brain | mitogenic effect | (150) |
| Somatomedins: | | | |
| IGF-I | human plasma | insulin-like and growth hormone dependent for all Sms | (141) |
| IGF-II | human plasma | idem | (142,143) |
| Sm A | human plasma | idem | (144) |
| Sm C | human plasma | idem | (145) |
| MSA | liver cell culture medium | idem | (151) |

Table 3 Insulin-Like Growth Factors and Biological Effects

| Sm/IGF | Source | M.W. (estimated) | P.I. | Plasma Protein Carrier | GH dependent | Insulin-like metabolic activity | Sulfation effect | Growth stimulation | References |
|---|---|---|---|---|---|---|---|---|---|
| A. IGF-I like | | | | | | | | | |
| 1. IGF-I | human plasma | 7649 | 8.2-8.4 | + | + | + | + | + | (141) |
| 2. Sm C | humam plasma | 8567 | 8.1-9.5 | + | + | + | + | + | (145) |
| 3. Basic Sm | human plasma | NA * | 8.6 | + | + | + | + | + | (155) |
| 4. Rat Sm | serum of rats with pituitary tumor | NA | basic | + | + | + | + | + | (156) |
| B. IGF-II like | | | | | | | | | |
| 1. IGF-II | human plasma | 7471 | neutral | + | + | + | + | + | (142) |
| 2. MSA | call serum and liver cell culture | 7484 | 5.5-7.5 | ? | + | + | + | + | (151) |
| C. Other | | | | | | | | | |
| 1. Sm A | human plasma | 7000 | 7.1-7.5 | + | + | + | + | + | (144) |
| 2. ILAs | human plasma | 9400 | 6.4-6.7 | + | + | + | + | + | (143) (154) |

Partial characteristics and biologic effects of some purified Sms/IGFs. On the basis of their chemical and biological properties, at least two classes of Sms/IGFs are recognized: A. IGF-I like are basic peptides which demonstrate a greater GH-dependence, B. IGF-II are neutral peptides which exhibit strong insulin-like activity. Both IGF-I and II have been sequenced, and have 62% homology with each other as well as a high degree of homology with human proinsulin (157, 158).

* NA: not available

## PART TWO: GROWTH FACTOR RECEPTORS IN HUMAN PLACENTAL CELLS

## CHAPTER I Insulin Binding to Human Placental Cells in Culture: Effect of Buffer

### I.1 Introduction

A physiological buffer is a solution of inorganic salts in which an isolated organ or cultured cells can survive for a short term and continue at least some normal functions. Since many buffers are empirical, we examined the most acceptable buffer for the cell suspensions used in our binding assays.

The osmotic properties of various cell types have been studied by Roti Roti et al (188,189) who suggested that, with changes in the tonicity of the suspending medium, erythrocytes of several species all behave roughly like simple osmometers. On the contrary, mouse lymphoma cells (L5178Y) and several other cultured mammalian cells do not behave as simple osmometers when the osmolarity of their medium is reduced, and seem to adapt to hypotonic conditions (188). In general, these cells are tolerant of a broad range of osmolarity.

Short-term human placental monolayer cultures have been established in this laboratory. Deal et al (190) have characterized the insulin receptors of term and choriocarcinoma derived cells (JEG-3) with respect to their specific binding and specificity by using hypotonic assay buffer (Tris-$H_2O$). In this chapter, we have compared [$^{125}$I]-insulin binding in various buffer systems and studied the nature of the adverse effects of deviation from the optimum.

## I.2 Materials and Methods

### Placental Monolayer Culture Preparation and Materials

Normal human term (38-41 wk; N=6) placentae were obtained at the time of caesarean section. The method for preparing placental monolayers has been previously described in detail (190,191). The placenta was placed in cold bicarbonate buffer ($Ca^{+2}$- and $Mg^{+2}$-free Earle's Balanced Salt Solution) and aseptically processed within 1 h after delivery. After extensive rinsing with cold bicarbonate buffer and gauze filtration of the tissue mince, about 20 g of placental mince was placed in a sterilized 100 ml bottle with 75 ml 0.25% trypsin (ICN; Montreal, PQ) and 500 U deoxyribonuclease (DNAase)(1 mg/ml) (Sigma; St Louis, MO). The trypsin digestion was undertaken under a hood (37 C) with occasional agitation for 15 min. The supernatant was collected in centrifuge tubes containing 2 ml fetal bovine serum (FBS) (Grand Island Biologicals Co., Grand Island, NY) to inactivate trypsin, and the cells were harvested by centrifugation at 70 x g for 10 min. This procedure was repeated 3 to 4 times. The cell crops of each trypsin digestion were monitored with an inverted phase contrast microscope (model 42410) (Nikon; Tokyo, Japan). Mono- or multi-nucleated type cells began to appear after the second or third trypsinization. The size of these cell crops was heterogeneous and varied with each preparation. After the 4th or 5th trypsinization, the amount of trypsin was reduced to about 40 ml, and the period of exposure was shortened to about 10 min without DNAase.

The cell crops were resuspended in 10 ml of freshly prepared ammonium chloride-phosphate buffer (0.83% $NH_4Cl$ in phosphate

buffer saline, pH 7.2) at 4 C for 10 min with occasional hand agitation. Two ml of FBS was then carefully placed onto the bottom of each centrifuge tube and the cells were again pelleted by centrifugation. The cells were then pooled, repelleted and resuspended in Ham's F-10 medium (Ham's F-10 supplemented with 10% FBS and antibiotics: 200 IU/ml penicillin G sodium (Glaxo Lab; Montreal, PQ), 5 ug/ml amphotericin B (Squibb and Sons; Montreal, PQ) and 40 ug/ml gentamicin (Schering Inc; Montreal, PQ). Cell crops were plated in a test culture dish and allowed to sit for at least 5 min; the final cell concentration plated was determined by the degree of confluency achieved in the test dish. Cells were plated confluently in 60 x 15 mm or 100 x 20 mm culture dishes and maintained at 37 C in a humidified atmosphere with 95% air and 5% $CO_2$. The following day the medium was removed by aspiration and the cultures were gently rinsed twice with fresh Ham's F-10 medium to remove any remaining debris and red blood cells. The cells received medium changes daily.

Placental cells are sensitive to prolonged exposure to trypsin. Thus, the faster the whole procedure can be carried out, the better the results that will be achieved. Furthermore, a successful placental monolayer preparation is dependent on the batch of FBS used. It is important to test several lots of FBS before utilizing each new supply in order to monitor cell morphology, viability and levels of steroid and peptide hormone released by these cells.

Chemicals and Hormones

Bacitracin, N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic

acid (Hepes) and purified bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO), trasylol from Miles Pharmaceuticals (Rexdale, ONT), Tris (Hydroxymethyl) Aminomethane (THAM), sodium bicarbonate, sodium carbonate, calcium chloride, trichloroacetic acid (TCA), potassium chloride and magnesium sulfate from Fisher (Fair Lawn, NJ), glutaraldehyde and cacodylate buffer from J.B. EM Service Inc., (Dorval, PQ) dextrose from Anachemia (Toronto, ONT) and 100 x 16 mm heparinized vacutainers (143 units of sodium heparin) from Becton Dickinson (Mississauga, ONT).

Binding Studies

On day 3, the cells were washed with Tris washing buffer (25 mM Tris, 10 mM $MgCl_2$, 0.12 M NaCl; pH 7.5) and then gently removed from the culture dishes with a rubber policeman. Cell suspensions were pelleted at 4 C for 6 min at 70 x g. Cell protein was determined by the method of Lowry et al (192), using purified BSA as standard. Aliquots of cell suspensions were dissolved with 0.6 N NaOH by boiling 20 min in a water bath, and the cells were incubated with Lowry color reagents for 45 min, then read at 660 mu in a spectrometer (Beckman; Palo Alto, CA).

The effect of different buffers on [$^{125}$I]-insulin binding was studied with the following buffers: Tris-$H_2O$ (25 mM Tris, 10 mM $MgCl_2$) (190), Tris-NaCl (25 mM-Tris, 10 mM $MgCl_2$, 0.12 M NaCl), Hepes buffer (0.1 M Hepes, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose) (128), phosphate buffered saline (PBS) (0.05 M phosphate buffer, 0.12 M NaCl) (131), Buffer G (50 mM Hepes, 50 mM Tris, 10 mM $MgCl_2$, 2 mM EDTA, 10 mM dextrose, 10 mM $CaCl_2$, 50 mM NaCl, 5 mM KCl) (193), and Krebs-Ringer Bicarbonate buffer

(KRB) (131). To each was added 0.1% purified BSA at pH 7.5, 100 ug cell protein, and 5-6 x $10^4$ cpm [$^{125}$I]-insulin in the presence or absence of unlabeled insulin (50 ug/ml) in a final volume of 0.5 ml in 12 x 75 polystyrene tubes (Simport Ltd., Montreal, PQ). Incubations were carried out with constant shaking at 4 C. Binding assays were terminated after 20 h incubation by the addition of 3 ml cold washing buffer with 0.1% crude BSA (Fraction V, ICN Chemicals, Montreal, PQ) and centrifuged at 2000 x g for 30 min at 4 C.

Percent specific binding was calculated from the difference between radioactivity in the presence (50 ug/ml) or absence of native insulin. Cell viability was over 85% as assessed by trypan blue exclusion.

Morphology Studies

After 72 h of plating, the cells were washed with o.1 M Hepes assay buffer and cell suspensions prepared as described for binding studies. Aliquots of cells were fixed before the binding assay as the control or after 20 h incubation with Hepes assay buffer with constant shaking at 4 C. Cell suspensions were pelleted at 4 C for 6 min at 70 x g. The supernatant was discarded and the cell pellets were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1.5 h at room temperature. The glutaraldehyde was then decanted and cells were rinsed with 0.1 M cacodylate buffer. The cell pellet was post-fixed in 1% osmium at pH 7.4 for 1 h, rinsed with distilled water three times, embedded in 2% agar, dehydrated in graded alcohol and embedded in Epon. Electron microscopy was performed with the

technical supervision of Mr. H. Leung (Montreal Children's Hospital-Research Institute).

Iodination of Insulin

Insulin labeled with $Na^{125}I$ (New England Nuclear Corp., Boston, MA) was prepared by a modification of the chloramine T method (122) to specific activities of 150-180 uCi/ug. The insulin was iodinated immediately before each binding assay and showed greater than 95% TCA precipitation.

Insulin Degradation

Degradation of insulin by cultured placental cells was determined by examing the ability of [$^{125}I$]-insulin in the incubation medium to be precipitated by 10% TCA. The percent [$^{125}I$]-insulin which remained intact was then determined by the following formula:

(% intact of incubated insulin/ % intact of unexposed insulin) x 100

I.3 Results

Determination of Optimal Assay Buffer

Whole blood (20 ml) was obtained with 100 x 16 mm heparinized vacutainers from two normal non-obese adult males, centrifuged and an aliquot of red blood cells was resuspended at room temperature in 2 ml of the following buffers: Tris-$H_2O$, Tris-NaCl, Hepes buffer or Ham's F-10 placental medium enriched with 10% FBS. The erythrocytes lysed immediately in Tris-$H_2O$ buffer. No hemolysis, on the other hand, was observed during the 90 min observation period for the remaining buffers and the erythrocytes remained intact after storage in these buffers or conditioned medium at 4 C overnight.

Term placental monolayers prepared as described above were maintained in 3 ml of sterilized Tris-$H_2O$ buffer, Hepes buffer or Ham's F-10 medium with 10% FBS at 37 C in 60 x 15 mm culture dishes and observed under a contrast microscope. In the presence of hypotonic buffer (Tris-$H_2O$), cells underwent changes in volume. They first swelled and then shrank, reaching a new steady state after 1.5 h, with their volume close to normal. On return to Hepes assay buffer or Ham's F-10 medium, the cells remained the same volume. Thus the change of cell volume in hypotonic buffer is immediate and readily reversible. When cells were maintained in Hepes assay buffer or Ham's F-10 medium with 10% FBS, cell volume remained unchanged.

Effects of Buffers on [$^{125}$I]-Insulin Binding to Term Placental Cells

Term placental cell suspensions (100 ug cell protein) were incubated at 4 C for 20 h with 5-6 x $10^4$ cpm [$^{125}$I]-insulin together with increasing concentrations (0-5 x $10^3$ ng/ml) of native insulin. PBS, KRB and buffer G inhibited insulin binding; specific binding at pH 7.5 was reduced (22.9$\pm$3.6%, N=12) compared to that observed in Hepes or Tris-$H_2O$ buffer (Table I.1). Of the buffers evaluated, PBS, KRB and buffer G were excluded from further consideration on the basis of marked inhibition in [$^{125}$I]-insulin binding to term placental cells. Binding of [$^{125}$I]-insulin to human term placental cells was very similar in both Tris-$H_2O$ and Tris-NaCl buffers (Fig. I.1). In the presence of bacitracin (125 ug/tube), there was a 35% increase in binding (Fig. I.2 B) in both buffer systems. In addition, proinsulin

produced parallel displacement curves to insulin but was approximately 100 fold less potent in the presence (Fig. I.2B) or absence of bacitracin (Fig. I.2A).

Insulin Degradation

Insulin degradation was determined at steady state binding conditions for 20 h at 4 C for both Tris-$H_2O$ and Tris-NaCl buffers by examing the ability of [$^{125}$I]-insulin remaining in the incubation medium to be precipitated by 10% TCA. The presence of bacitracin (125 ug/tube) (Fig. I.2B) or trasylol (500 KIU/ml) with human term placental cells (100 ug cell protein) increased the cell associated radioactivity and increased TCA precipitation of [$^{125}$I]-insulin in the medium to 85-90% (Fig. I.3). In the absence of bacitracin or trasylol, TCA precipitation of [$^{125}$I]-insulin in the cell conditioned medium was 55-60% with 0 to 100 ng/ml of native insulin, and increased to 75% at 1,000 ng/ml. In the presence of a large excess of insulin (50 ug/ml) tracer degradation could be saturated in both buffer systems. (Hepes assay buffer and detailed studies of [$^{125}$I]-insulin degradation are presented in Chapter II)

Morphology Studies

Under standard binding conditions, the precise sequence of cellular changes occurring in the cell remain obscure. Term placental cells before (Fig. I.4) or after 20 h (Fig. I.5) incubation at 4 C with constant shaking in Hepes assay buffer were fixed with 3% glutaraldehyde and processed for morphologic evaluation with electron microscopy: cells remained intact and showed well maintained extensive microvillar projections and an intact nuclear envelope.

## I.4 Discussion

The use of the term "physiological" to describe "in vitro" buffers is open to objection since the word often implies that the conditions are similar "in vivo". In addition, many buffers are termed "physiological" only in the sense that they are used in physiological studies. The ability of cells to survive and to adapt in various buffers (ie. hypotonic buffer) has been documented in experiments involving manipulation of ionic concentrations (127). It was of great interest to see just how tolerant term placental cells were in an hypotonic buffer such as Tris-$H_2O$ in relation to their binding properties. These cells did not behave as simple osmometers when the osmolarity of their buffer was reduced, but seemed to adapt to hypotonic conditions. After the initial swelling phase, the cell volume decreased to the original volume. The slight swelling of placental cells found in this study is in agreement with the findings of Roti Roti et al (188,189) and Dick (194). In contrast, human erythrocytes behaved as simple osmometers. The transient swelling followed by shrinking in hypotonic media suggests that not only does water enter the cell, but also that the cell membrane allows the loss of considerable solute plus osmotically obliged water over time (188). After the swelling and shrinking phases were completed (about 1.5 h) the volume was stable at 37 C for over 20 h with no loss of viability as assessed by trypan blue exclusion.

Physiological buffers such as KRB, PBS and Buffer G were included in these studies for comparison because they have been featured in many publications. Although phosphate ion is

essential for mammalian cells, [$^{125}$I]-insulin binding to term placental cells was inhibited, possibly by precipitation of the calcium salt in the assay buffer. A similar finding has been reported by Rechler et al (128) working with multiplication stimulating activity (MSA) in chicken fibroblast cells. Since extracellular $Ca^{+2}$, $Mg^{+2}$, $K^{+}$ and phosphate are the nutrients essential for key intracellular processes that control cellular metabolism, then the nutrients and proper osmolarity of the buffer are important for the study of cells in vitro. Thus, the hypotonicity of the Tris-$H_2O$ buffer seems far from ideal, despite the fact that this buffer was utilized in studies of [$^{125}$I]-insulin binding to term placentae and JEG-3 cells (190).

Hepes buffer has been found to be non-toxic to a variety of mammalian cells in culture (131). Whether any metabolic substrates need to be added to a physiological buffer depends on the energy reserves of the cells in question, the metabolic rate, the duration and conditions such as temperature of the experiment and the nature of the process to be investigated. Glucose is the substrate most often supplied as energy source. Low temperatures are helpful both in lowering metabolic rates and decreasing the degrading enzyme systems of the cells. Thus, our standard assay conditions which have been designed with Hepes assay buffer, in the presence of glucose (8 mM) at 4 C for 20 h, appeared to be suitable for studying peptide hormone binding in this cell system.

## I.5 Summary

Monolayer cultures of human term placentae have been established. Binding of [$^{125}$I]-insulin was undertaken in whole cell radioreceptor assays at 4 C for 20 h in various buffer systems. Maximal percent specific binding occurred with Hepes, Tris-$H_2O$ and Tris-NaCl buffers. On the contrary, phosphate buffered saline (PBS), Krebs-Ringer Bicarbonate buffer (KRB) and buffer G inhibited labeled insulin binding to term placental cells.

The pancreatic trypsin inhibitor, trasylol, at 500 KIU/ml and the antibiotic peptide bacitracin (125 and 500 ug/tube) were found to enhance [$^{125}$I]-insulin binding to term placental cells by 30-40%. The effect of trasylol and bacitracin on insulin binding correlated inversely with degradation of labeled insulin in our assay system.

Human term placental cells adapted to hypotonic buffer (Tris-$H_2O$) and similar binding was observed as with physiological buffers. Hepes buffer was chosen for our routine cell assay system due to the observed high specific binding and non-toxicity to our cells as judged by morphologic studies.

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Portions of Chapter I have been published.
(Deal CL, Guyda HG Endocrinology 112:1512,1983

## I.6 Figures and Table

Fig. I.1 The effect of different buffers on [$^{125}$I]-insulin binding to human term placental cells. Cells were incubated at 4 C with 100 ug cell protein in the presence of 500 ug/tube bacitracin with [$^{125}$I]-insulin for 20 hrs. A final concentration range of 0-1,000 ng/ml was tested in the assay since 0.1 ml aliquots of the insulin were added to each assay tube in which the final volume was 0.5 ml. Each point represents the mean of three determinations. Non-specific binding in all buffer systems tested averaged 1% of the total labeled insulin added.

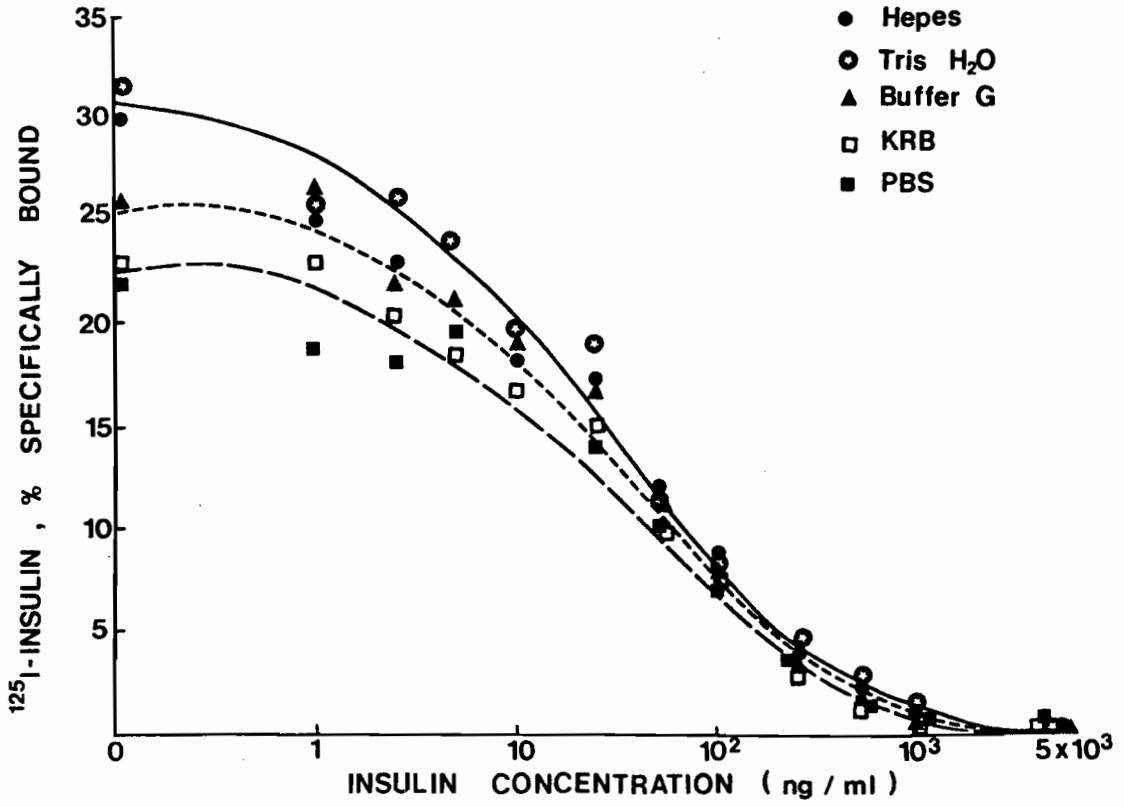
Hepes
Tris $H_2O$
Buffer G
KRB
PBS
$^{125}$I-INSULIN , % SPECIFICALLY BOUND
35
30
25
20
15
10
5
0
1
10
$10^2$
$10^3$
$5 \times 10^3$
INSULIN CONCENTRATION ( ng / ml )

Fig. I.2 Inhibition effect of porcine insulin and proinsulin on [$^{125}$I]-insulin binding to term placental cells at 4 C for 20 h in Tris-$H_2O$ or Tris-NaCl buffer. All data are corrected for non-specific binding. A final concentration range of 0-1,000 ng/ml was tested in the assay since 0.1 ml aliquots of the insulin were added to each assay tube in which the final volume was 0.5 ml.

A). Top panel, in the absence of bacitracin.

B). Lower panel, in the presence of bacitracin (125 ug/tube).

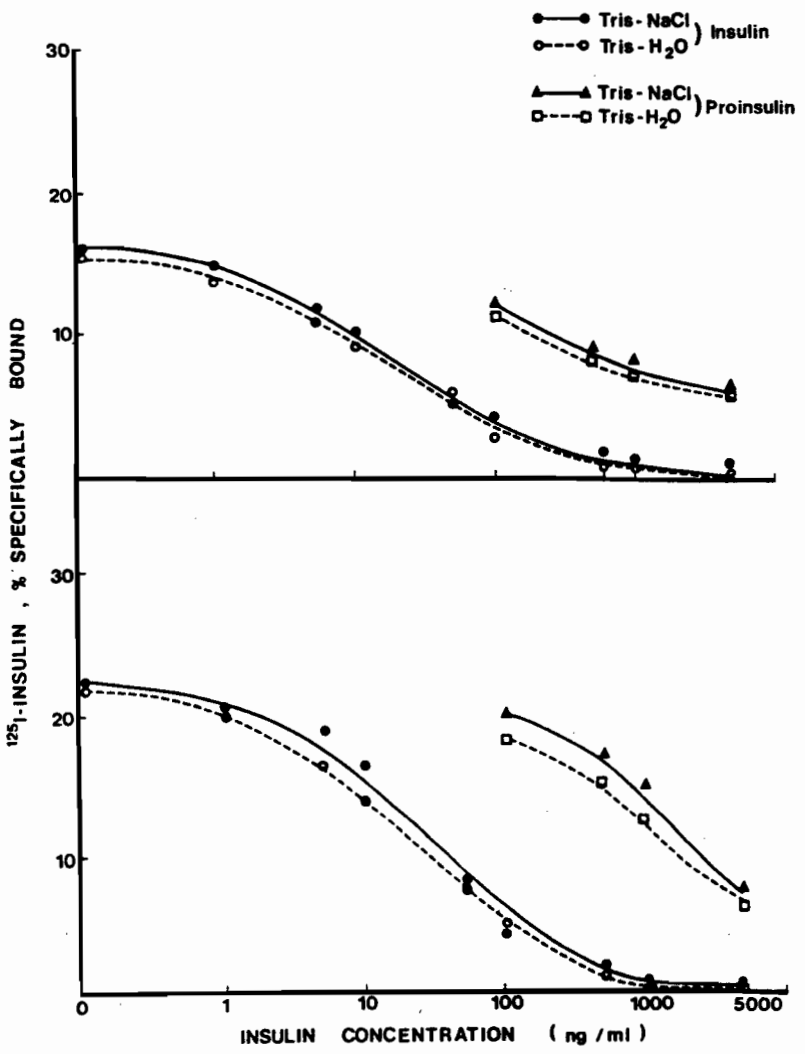
Tris - NaCl
Tris - H2O
Insulin
Tris - NaCl
Tris - H2O
Proinsulin
30
20
10
30
20
10
0
1
10
100
1000
5000
125I-INSULIN , % SPECIFICALLY BOUND
INSULIN CONCENTRATION ( ng / ml )

Fig. I.3 Ability of term placental cells to influence TCA precipitation of [$^{125}$I]-insulin as a function of insulin concentration. Degradation was assessed by the ability of [$^{125}$I]-insulin remaining in the medium to precipitate with 10% TCA.

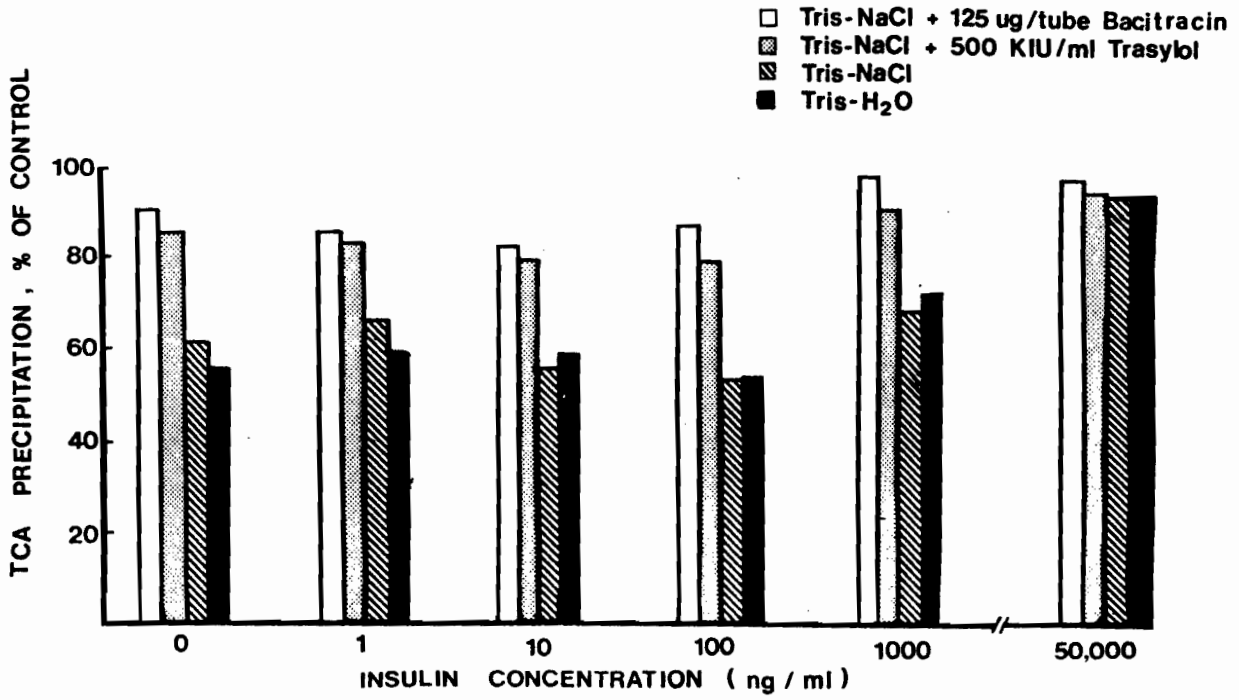
Tris-NaCl + 125 ug/tube Bacitracin
Tris-NaCl + 500 KIU/ml Trasylol
Tris-NaCl
Tris-H2O
TCA PRECIPITATION, % OF CONTROL
100
80
60
40
20
0
1
10
100
1000
50,000
INSULIN CONCENTRATION ( ng / ml )

Fig. I.4 Electron micrograph of uni- and multi-nucleated human term placental cells after 72 h in Ham's F-10 medium with 10% FBS at 37 C and before incubation under assay conditions. Cells were prepared as described in "Methods" and fixed with 3% glutaraldehyde after washing with 0.1 M Hepes assay buffer (Magnification 5000 x).

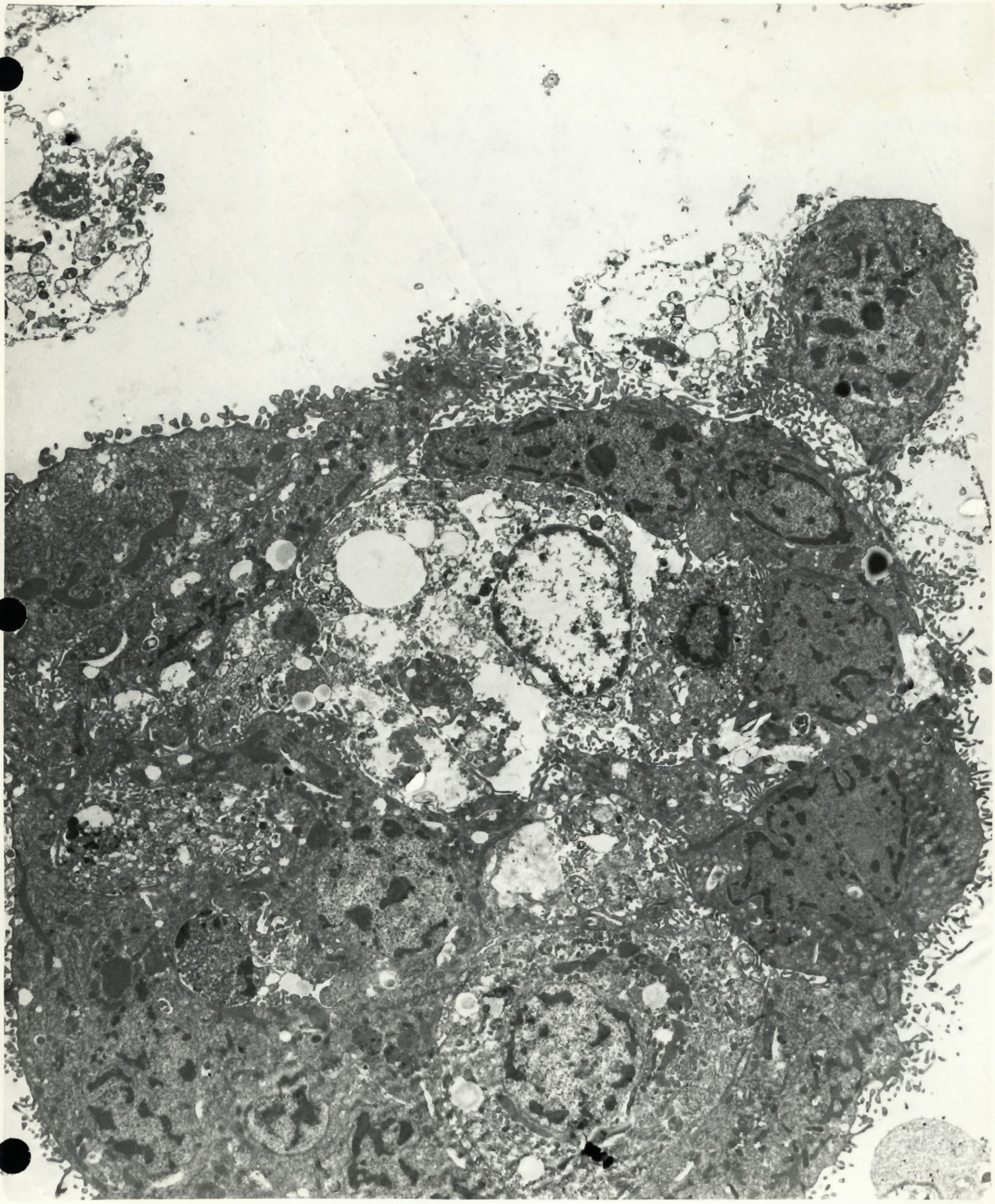

Fig. I.5 Electron micrograph of uni- and multi-nucleated human term placental cells after 72 h in culture. Cell suspensions were incubated with 0.1 M Hepes assay buffer for 20 h and fixed with 3% glutaraldehyde as described in "Methods" (Magnification 5000 x).

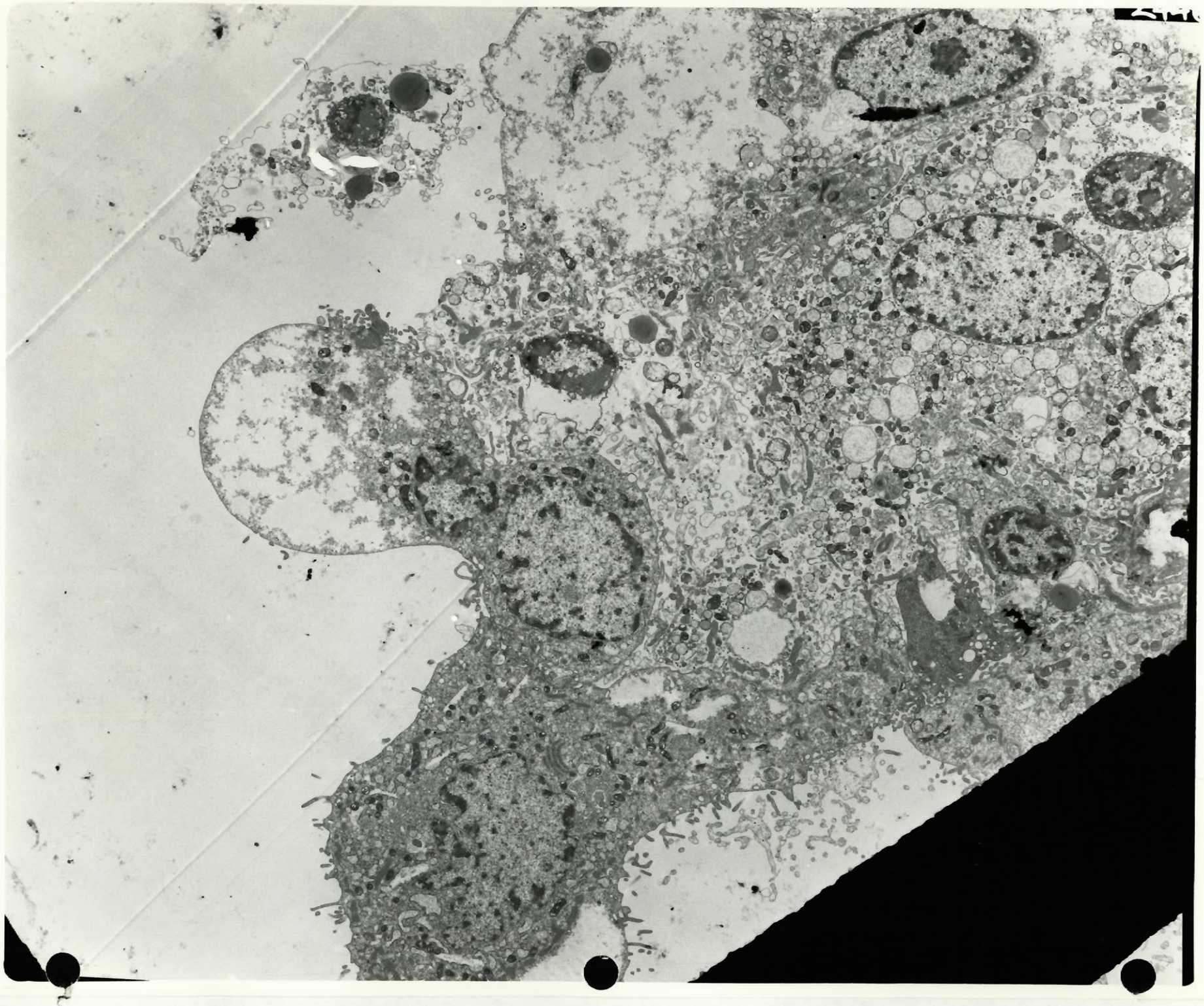

Table I.1 Effect of different buffers on [$^{125}$I]-insulin binding to term placental cells.

| Buffer | % SB | % of Control |
|---|---|---|
| Hepes | 30.1±2.1(4)* | 100.0** |
| Tris-$H_2O$ | 30.4±2.4 (4) | 101.0 |
| Buffer G | 25.1±1.6 (4) | 83.4 |
| KRB | 22.9±1.9 (4) | 76.1 |
| PBS | 21.6±2.1 (4) | 71.8 |

* Mean ± S.E.

** Maximal binding was seen with Hepes buffer which was designated as control.

## Chapter II Insulin-Induced Receptor Regulation in Early Gestation and Term Human Placental Cell Cultures

### II.1 Introduction

The presence of insulin-degrading activity in placental cell cultures has seriously interfered with attempts to study insulin receptor binding and regulation in this system (195). Interpretation of the possible relationship between insulin binding and action must be cautious unless the extent of insulin degradation has been considered. It became apparent to us, during preliminary studies on insulin receptors in human placental monolayer cultures where this degradative activity was present (190), that effective inhibitors for insulin proteolytic enzymes must be found. The potential usefulness of bacitracin, which had been shown to be an inhibitor of glucagon (196,197) and insulin degradation (115), was brought to our attention during studies on the insulin receptor in human erythrocytes (198). We have therefore investigated the effect of bacitracin, a known inhibitor of proteolytic activity, on degradation of insulin by human placental cells in culture.

Numerous reports have appeared describing that insulin can inversely regulate the number of cell-surface insulin receptors. The direct evidence for this regulatory mechanism was provided by the studies of Gavin et al.(199), who described that incubation of cultured human lymphocytes with insulin *in vitro* results in a time-dependent loss of insulin receptors. Deal et al (190) have demonstrated an insulin-induced receptor loss in human term placental and choriocarcinoma cell cultures. However due to the

presence of competing side reactions, notably insulin degradation, it could not be determined whether the observed decrease in [$^{125}$I]-insulin binding was the result of a decrease in total number of insulin receptors or an alteration in receptor affinity for insulin, or both in this study (190).

In the present study we report the effective inhibition of insulin degradation by bacitracin. We have further examined insulin binding characteristics as well as receptor regulation in both early gestation and term placental cell cultures, using bacitracin in our in vitro test system.

## II.2 Materials and Methods

### Chemicals and Hormones

Bacitracin, N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes), cycloheximide and purified bovine serum albumin (BSA) were purchased from Sigma, (St. Louis, MO), trasylol from Miles Pharmaceuticals, (Rexdale, ONT), porcine crystalline zinc insulin (24.4 units/mg) from Connaught Laboratories, (Toronto, ONT) and glucagon from Eli Lilly Co. (Indianapolis, IN). Human growth hormone (GH HS2243E), human prolactin (hPRL I-6), human ACTH (1-39) and human chorionic gonadotropin (hCG) were obtained from NIADDK, NIH (Bethesda, MD). Insulin-like growth factor II (IGF-II) was initially extracted from human plasma concentrates (Cohn Fraction IV-4) by acid-ethanol, and then further purified by Sephadex acid chromatography and high pressure liquid chromatography (HPLC) at pH 2 - 2.5 (Posner, B.I. and Guyda, H.J. manuscript in preparation). Epidermal growth factor (EGF) and nerve growth factor (NGF) were purchased from Collaborative

Research (Waltham, MA).

Cell Culture and Receptor Studies

Normal term (38-41 wk; N=25) human placentae were obtained at the time of caesarean section; early gestation (8-18 wk; N=17) placentae were obtained at the time of therapeutic abortion by vacuum aspiration. The method for preparing placental monolayers has been previously described in detail (190,191). On day 3, the cells were washed with Tris washing buffer (25 mM Tris, 10 mM $MgCl_2$,0.12 M NaCl; pH 7.5) and then gently removed from the culture dishes with a rubber policeman. Cell suspensions were pelleted at 4 C for 6 min at 70 x g and cell protein was determined by the method of Lowry et al (192).

Unless otherwise specified, the standard binding assay consisted of 0.1 M Hepes-BSA assay buffer (0.1 M Hepes,0.12 M NaCl, 5mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose with 0.1 % purified BSA; pH=7.5), 100 ug cell protein, 5-6 x $10^4$cpm [$^{125}$I]-insulin, 500 ug/tube bacitracin, and the presence or absence of unlabeled insulin (100 ug/ml) in a final volume of 0.5 ml in 12 x 75 polystyrene tubes (Simport Ltd., Montreal, PQ). Incubations were performed with constant shaking at 4 C. Binding assays were terminated after 18-20 h by the addition of 3 ml cold Hepes washing buffer (0.1% crude BSA; Fraction V, ICN Chemicals, Montreal,PQ) and centrifuged at 2000 x g for 30 min at 4 C. Specific binding was calculated from the difference between radioactivity in the presence (100 ug/ml) or absence of native insulin. Cell viability was over 85% as assessed by trypan blue exclusion as well as with electron microscopy: cells showed well-maintained extensive microvillar projections, endoplasmic

reticulum, Golgi, mitochondria and an intact nuclear membrane.

Iodination of Insulin

Iodination of insulin with Na $^{125}I$ (New England Nuclear Corp., Boston, MA) was undertaken by a modification of the chloramine-T method. The insulin was iodinated immediately before each binding assay and had a specific activity of 160-180 uCi/ug and was greater than 95% TCA precipitable. Integrity of [$^{125}I$]-insulin was also evaluated by rebinding to human placental microsomes prior to each binding assay (71).

Preincubation of Cells with Insulin

Early gestation and term placental cells were plated as confluent monolayers in 100 x 20 mm culture dishes with Ham's F-10 supplemented with 10% fetal bovine serum (FBS) and antibiotics. Two days after plating, medium was removed, and unlabeled insulin in various concentrations was added with fresh medium in the presence or absence of bacitracin (1mg/ml) in a final volume of 5 ml. After incubation at 37 C for various periods of time, the medium was aspirated and the monolayers were washed three times with phosphate buffered saline (PBS), pH=7.5, at room temperature, with 10 min incubation periods between each wash. Three more rapid washes were routinely carried out, although after the third wash step insulin was undetectable by RIA in the washing medium. Subsequently, cells were processed for the standard radioreceptor assay procedure as described above.

Statistical Methods

All experiments were performed using at least two different placental culture preparations. All points on each figure

represent the mean of triplicate determinations in one representative experiment. The data were analyzed by Student's t-test.

## II.3 Results

### Insulin Degradation by Human Placental Cells

A wide range of bacitracin concentrations were tested (Fig. II.1). As little as 25 ug per tube effectively inhibited degradation and increased specific binding. As the concentration of bacitracin was increased to $10^3$ ug/tube there was a further inhibition of [$^{125}$I]-insulin degradation and a corresponding increase in binding. At 0.5-2.5 x $10^3$ug per tube, bacitracin inhibited degradation to about 1-5%, as assessed by TCA precipitability or rebinding to placental microsomes, both methods giving equivalent results. Very high concentrations (greater than 2.5 x $10^3$ug/tube) also inhibited degradation but supressed insulin binding to the cells.

The effects of bacitracin on inhibiting insulin degradation and increasing specific insulin binding were reversible by removal of the bacitracin (data not shown). Furthermore, we assessed whether the increase in binding of [$^{125}$I]-insulin to the placental cells in the presence of bacitracin was simply the result of the presence of more intact labeled insulin or whether an effect on the insulin receptor could also be playing a role. Cells were incubated with [$^{125}$I]-insulin at 4 C and 500 ug per tube of bacitracin was added at various time periods before or after the addition of the [$^{125}$I]-insulin (Fig. II.2). These data suggest that bacitracin increased specific binding primarily by a direct inhibition of [$^{125}$I]-insulin degradation rather than by

direct effects of bacitracin on the insulin receptor, especially since preincubation of cells with bacitracin for up to 2 h did not significantly increase [$^{125}$I]-insulin specific binding to the cells.

In a further study, term placental cells which had been in the presence or absence of bacitracin (500 ug/tube) for various time periods (0-20 h) at 4 C, were removed and the cell-free supernatant assay buffer was then incubated with labeled insulin as described in the standard radioreceptor assay procedure. Insulin degradation was assessed by TCA precipitation. We found that in buffer from those cells incubated for 3 to 20 h without bacitracin, the TCA precipitation of labeled insulin was 62% as compared to control. However, if the cells were incubated with bacitracin, the TCA precipitation of labeled insulin was over 95%, even after a 20 h incubation. These data suggest that when labeled insulin is incubated with term placental cells most of the degradation takes place in the medium and that bacitracin can block this activity effectively.

Table II.1 shows [$^{125}$I]-insulin integrity as assessed by TCA precipitation and by rebinding to placental membrane receptors. The supernatant radioactivity in the presence of bacitracin was 97% TCA precipitable, and without bacitracin decreased to 57% as compared to controls. In the presence of excess unlabeled insulin (100 ug/ml) the TCA precipitability was increased, suggesting that the enzyme(s) responsible for tracer degradation could be saturated. In the rebinding studies, the integrity of the supernatant radioactivity with bacitracin was 95% of control

when compared to 42% without bacitracin. The cell-eluted tracer in the presence of bacitracin showed TCA precipitation that was 97% of control compared to 75% in the absence of bacitracin. Furthermore, as judged by its ability to rebind to placental membranes, cell eluted [$^{125}$I]-insulin remained intact in the presence of bacitracin.

The pancreatic trypsin inhibitor, trasylol, at 500 KIU/ml, was also tested and found to enhance [$^{125}$I]-insulin binding to term placental cells by 25-30%; the effects of trasylol on insulin binding correlated inversely with degradation of labeled insulin in our assay system. However, because of the high cost of trasylol, bacitracin was used for the more detailed characterization studies.

Characterization of Insulin Receptors in Human Term Placental Cells

Binding of [$^{125}$I]-insulin to human term placental cells at 4 C was a time- dependent process. With or without bacitracin (500 ug/tube), specific binding reached a steady state by 12 h which persisted for at least another 8 h (Fig. II.3). At early times (less than 2 h) bacitracin had little effect on insulin binding, but as time elapsed, potentiation of binding by bacitracin increased: maximal [$^{125}$I]-insulin binding with bacitracin was twice that observed in its absence. Nonspecific binding was about 25% without bacitracin and 2-4% with bacitracin.

In the presence of bacitracin, binding of insulin to term placental cells was reversible in a time dependent fashion: dissociation of bound insulin was observed following dilution, and this process was accelerated in the presence of excess

unlabeled insulin (100 ug/ml) (Fig. II.4).

Binding of [$^{125}$I]-insulin to term placental cells was dependent on the concentration of cell protein present (Fig. II.5). Bacitracin (500 ug/tube) caused a 40-50% increase in insulin specific binding at low cell protein concentrations (less than 100 ug per tube membrane protein). As the concentration of cell protein was raised, potentiation of binding by bacitracin remained at a similar level (ie. at 400 ug per tube, bacitracin caused a 40% increase in binding). Nonspecific binding remained relatively constant below 200 ug cell protein, but rose progressively with increasing cell protein concentration, whether bacitracin was present or not (data not shown). Binding of [$^{125}$I]-insulin was a saturable process with respect to [$^{125}$I]-insulin concentration (Fig. II.6). Maximal binding occurred at pH 7.5 in the presence or absence of bacitracin (500 ug/tube) (Fig. II.7).

The inhibition of [$^{125}$I]-insulin binding by different concentrations of porcine insulin (0-5 x $10^3$ ng/ml) is shown in Fig. II.8. At low concentrations of insulin (1 ng/ml), bacitracin maintained a 30-40% increase in insulin binding. As the insulin concentration was raised, the relative enhancement of binding caused by bacitracin decreased. With concentrations of insulin greater than 500 ng/ml, which saturated the insulin-receptor binding sites, addition of bacitracin caused virtually no increase in specific binding. Bacitracin decreased the concentration of [$^{125}$I]-insulin giving half-maximal binding from 2 x $10^{-9}$ to 1 x $10^{-9}$ M. Thus, bacitracin increased the apparent

affinity of the insulin-receptor interaction.

Binding of labeled insulin to human term placental cells was specific. Unrelated hormones such as glucagon (10 ug/ml), ACTH (10 ug/ml), prolactin (10 ug/ml), hCG (1 x $10^3$ mU/ml), NGF (2 ug/ml), EGF (2 ug/ml) and growth hormone (10 ug/ml) showed negligible competitive inhibition (Fig. II.8). With or without bacitracin, proinsulin showed parallel displacement to insulin but was 100 fold less potent.

Ontogeny of Insulin Receptors in Human Placental Cells in Culture

Since little is known about the developmental changes in insulin binding to placental cells at different gestational ages, we have also compared the binding of [$^{125}$I]-insulin to early gestation and term placental cell cultures in order to evaluate the possible contribution of insulin to feto-placental function.

Insulin specific binding to placental cells was significantly greater ($p<0.001$) at term (38-41wk; N=25) as compared to early gestation (8-18 wk; N=17). This was due primarily to an increase in number of receptors with no significant change in affinity (Fig. II.9 and Table II.2).

Regulation of Insulin Receptors in Human Placental Cells

When placental cells were preincubated with insulin at 37 C, insulin receptor loss was found to be dependent on the insulin concentration from $10^{-6}$ to $10^{-7}$ M, the range tested in early gestation and $10^{-6}$ to $10^{-9}$ M tested in term placental cells (Fig. II.10). The maximum loss of receptors occurred at an insulin concentration of $10^{-6}$ M, when binding was reduced by 70%. Preincubation of term placental cells with insulin doses as high as $10^{-5}$M did not produce a further decrease in insulin binding

(data not shown). The inclusion of 1 mg/ml of bacitracin during the preincubation period increased the extent of down-regulation produced by each concentration of insulin studied, implying that more intact insulin was present in the incubation medium (data not shown). From analyses of equilibrium binding parameters, it was apparent that the insulin-induced loss of receptors was due to a decreased number of both high and low affinity receptors per mg cell protein in both early gestation and term placental cells, with no significant change in affinity (Fig. II.9).

The effectiveness of insulin in producing receptor loss was specific for the insulin receptor. IGF-II and proinsulin, polypeptides having similar chemical but less potent biological properties when compared to those of insulin, were less effective than porcine insulin in inducing insulin receptor loss (Fig. II.10). This could be accounted for by the difference in their binding affinities for insulin receptors in human term placental cells. Cells pretreated with unrelated peptides such as growth hormone, EGF or NGF at 37 C for over 18 h showed negligible receptor loss.

Figure II.11 shows the time course of insulin-induced down-regulation of insulin receptors in the presence of bacitracin (1mg/ml). The maximum decrease in [$^{125}$I]-insulin specific binding (70%) due to insulin-induced down-regulation occurred at 8-12 h after the addition of $10^{-6}$ M insulin, and remained at this level for up to 16 h.

Cycloheximide at 10 ug/ml did not inhibit insulin binding by itself and in the presence of cycloheximide (10 ug/ml) and

insulin ($10^{-6}$M), a rapid decrease in insulin receptors still occurred. We found that down-regulation of insulin receptors was reversible in both early gestation (Fig. II.12) and term placental cells (Fig. II.11, II.12). However, when insulin pretreated cells were washed and incubated in insulin-free medium in the presence of cycloheximide, receptor recovery in both early gestation (II.12) and term placental cells was inhibited (Fig. II.11).

## II.4 Discussion

Bacitracin has been shown to block EGF receptor-mediated endocytosis by inhibiting the transglutaminase enzyme (200). Since bacitracin has also been demonstrated to enhance glucagon binding and to inhibit glucagon degradation in liver membranes (196), it is not certain that bacitracin acts at intracellular sites or only on the membrane surface. It is of interest to note that bacitracin can either inhibit non-receptor-mediated insulin degradation in isolated adult hepatocytes (201) or act as an effective inhibitor of the receptor-mediated cellular degradation process in culture fetal hepatocytes (115), or can act in both pathways in the same cell system as reported by Hammons et al in rat adipocytes (202).

The present experiments demonstrate that bacitracin produces an increase in the specific binding of [$^{125}$I]-insulin to early gestation and term placental cell cultures.Our data suggest that bacitracin increases specific binding primarily by a direct effect of increasing intact insulin in the incubation medium. Further investigation is required to clarify this point.

The coefficient of variation (CV=9.3%; N=25) for interassay

variability of [$^{125}$I]-insulin binding to term placental cells indicates that the assay system is highly reproducible. This may be due to the minimal degradation of insulin in our assay system with the use of bacitracin.

The characteristics of insulin binding to human placental cells are quite similar to those reported for other cell systems (203-206); these include saturable and reversible binding that is highly specific for insulin, higher maximal binding at low (28.2 $\pm$ 0.6%, N=25 at 4 C) than at high temperature (5.4$\pm$ 0.3%, N=3 at 37 C), and a sharp pH optimum at pH 7.5. Insulin binding is also dependent on time and cell protein as well as ligand concentrations. Furthermore, kinetic analysis of this hormone-receptor interaction suggests the presence of both high affinity, low capacity and low affinity, high capacity sites. Although some of the assumptions inherent in the Scatchard analyses are currently unproven, the data reported herein are consistent with the results obtained with placental membrane preparations by us (71,163) and others (207), and with other cell systems (208,209).

The increase in binding of insulin to human placental cells from early gestation to term was approximately 3- fold. This observation is in agreement with our findings in early gestation and term placental membrane preparations (163). The difference in binding between early gestation and term is primarily due to an increase in the number of binding sites per cell with no significant change in apparent affinity.

In the present chapter we have extended previous studies in this laboratory on insulin-induced receptor loss in term human

placental cell cultures. Both early gestation and term placental cells, exposed to various concentrations of insulin at 37 C for times ranging from 2 to 24 h, exhibit loss of insulin receptors with no apparent change in their affinity. The maximal decrease in receptor number was 70%. This phenomenon is similar to what has been observed with other hormone receptor systems (210,211). The decrease in receptors was greater in the presence of bacitracin at each insulin concentration studied. A similar finding has been reported by Sorge and Hilf (208) who were working with adenocarcinoma cells. They have also suggested that the greater decrease in insulin receptors with bacitracin is due to an increase in the concentration of intact insulin in the preincubation culture medium.

The ability of insulin to induce receptor loss was not inhibited by cycloheximide (10 ug/ml); this observation suggests that insulin-induced receptor down-regulation does not depend on protein synthesis (212). Furthermore, our findings are consistent with those of Prince (214) and Kosmakos et al (211) who have reported that this receptor loss is reversed by removing insulin from the incubation medium. However, recovery of insulin receptors in both early gestation and term placental cells did not occur in the presence of cycloheximide, supporting the thesis that protein synthesis is required for receptor recovery (210,212,213).

In addition to these data, we also demonstrated that the effectiveness of insulin in inducing receptor loss was a specific effect of insulin. Preincubation of term placental cells with either insulin ($10^{-6}$M) or EGF (200 ng/ml) results in a decrease

in insulin or EGF receptors respectively (see Chapter IV, Table 5). Neither insulin nor EGF changed the total number or affinity of receptors for the other hormone.

The role of insulin in the metabolic activity of the human placenta is not clear, but a number of insulin effects have been demonstrated. Avruch et al (214) and Kasuga et al (117,118) reported that insulin stimulates tyrosine phosphorylation of the insulin receptors. Wheeler et al (215) and Bhaumick et al (216) have demonstrated correlations between the binding of labeled insulin and the ability of insulin to stimulate aminoisobutyric acid uptake by embryonic heart cells and human placental organ cultures, respectively. These studies suggest that the placental cell insulin receptor is coupled to biologically significant events.

The physiological significance of insulin receptors in human early gestation and term placental cells is uncertain. Although the human placenta is not regarded as a major site for insulin action, alterations in fetal growth have been associated with gestational hyperinsulism or congenital absence of insulin (153,217). Fetal growth and development require a continuous supply of nutrients from the maternal circulation. The microvillous surface membrane of the human placental syncytiotrophoblast is bathed in maternal blood and is in a position to mediate the transfer of metabolites between mother and fetus. It has also been found to be markedly enriched in insulin receptors by Whitsett et al (218). Autoradiographic studies of placental monolayers confirm Whitsett's findings (218)

showing that the major cell type binding insulin is syncytiotrophoblast in origin (W. Lai, H.J. Guyda and J. Bergeron, manuscript in preparation).

In conclusion, both human early gestation and term placental cell cultures possess insulin receptors that display many of the characteristics common to other well-studied culture systems. The finding of a correlation between insulin binding and receptor regulation at different gestational ages suggest that the placental culture system could serve as an important model for physiologic studies of insulin action in the feto-placental unit.

## II.5 Summary

Human placentae of different gestational ages have been used to investigate the binding and degradation of insulin as well as the regulation of insulin membrane receptors. Bacitracin was found to be an effective inhibitor of insulin degradation in human early gestation and term placental cell cultures. The increase in insulin binding to placental cells observed with bacitracin can be explained by an increase in intact insulin present in the incubation medium. In the presence of bacitracin, [$^{125}$I]-insulin bound rapidly and reversibly : maximal binding occurred at 4 C, with a sharp pH optimum at 7.5, and exhibited a high degree of specificity. The extent of binding was proportional to cell protein and [$^{125}$I]-insulin concentrations.

Term (38-41wk; N=25) placental cell cultures possessed receptors for insulin that were 3- fold increased compared to early gestation (8-18 wk; N=17).This was due to an increase in receptor number with no significant alteration in affinity.

A decrease in insulin binding in both early gestation and term placental cells was related to the insulin concentration present during 12-20 h of preincubation at 37 C. Cell cultures degraded significant amounts of insulin at 37 C, but the addition of bacitracin to the culture medium decreased the amount of degradation and increased the extent of receptor loss at each insulin concentration. The receptor loss was due to a decrease in the number of receptors per mg cell protein with no apparent change in their affinity. The maximal decrease in receptor number after insulin incubation was 70%. The insulin-mediated decrease in receptor number in both early gestation and term placental

cells was reversible. Following removal of insulin from the culture medium, 60% of the receptors were regenerated within 16 h. Cycloheximide did not inhibit down-regulation but prevented recovery of the insulin receptor, suggesting the need for protein synthesis for this latter phenomenon only.

We conclude that in our in vitro test system the addition of bacitracin is capable of decreasing the degradation of insulin. This will permit a more precise study of the metabolic effects of insulin in both early gestation and term placentae.

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This chapter will be submitted for publication (1984).

Fig. II.1 Binding (●) and degradation (○) of [$^{125}$I]-insulin by human term placental cells as a function of bacitracin concentration. [$^{125}$I]-insulin was incubated with term placental cells (100 ug cell protein) in 0.1 M Hepes assay buffer at 4 C for 20 h with varying concentrations of bacitracin. Degradation of [$^{125}$I]-insulin was assessed by TCA precipitability.

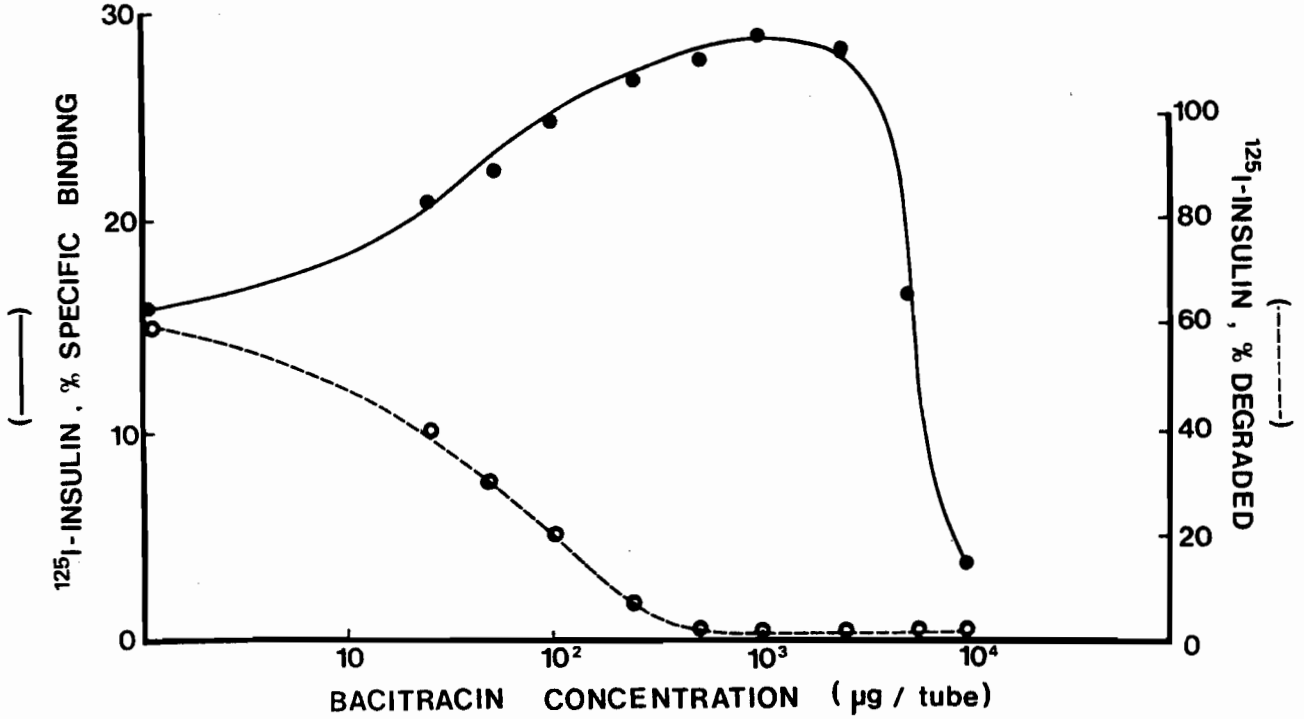

30
20
10
0
100
80
60
40
20
0
10
$10^2$
$10^3$
$10^4$
$^{125}$I-INSULIN , % SPECIFIC BINDING (——)
$^{125}$I-INSULIN , % DEGRADED (- - - - -)
BACITRACIN CONCENTRATION ( µg / tube)

Fig. II.2 Human term placental cells (100 ug cell protein) were incubated with [$^{125}$I]insulin at 4 C : 500 ug/tube of bacitracin were added at various times before or after the addition of the [$^{125}$I]-insulin.

A. % specific binding of insulin to the cells.

B. TCA precipitability of labeled insulin in the medium at the end of the incubation.

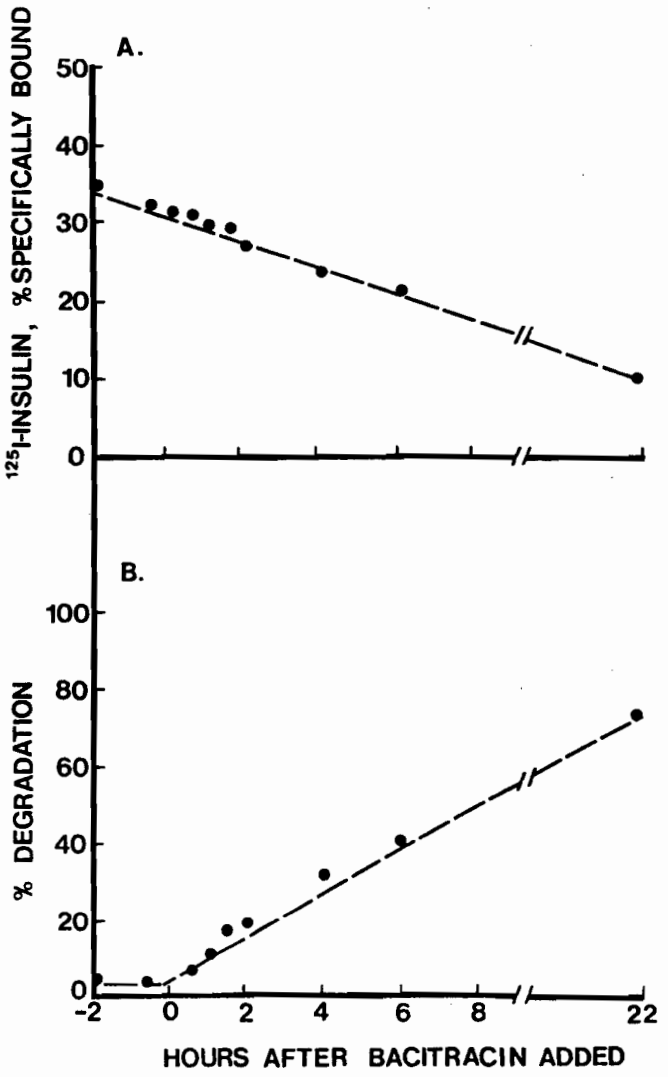
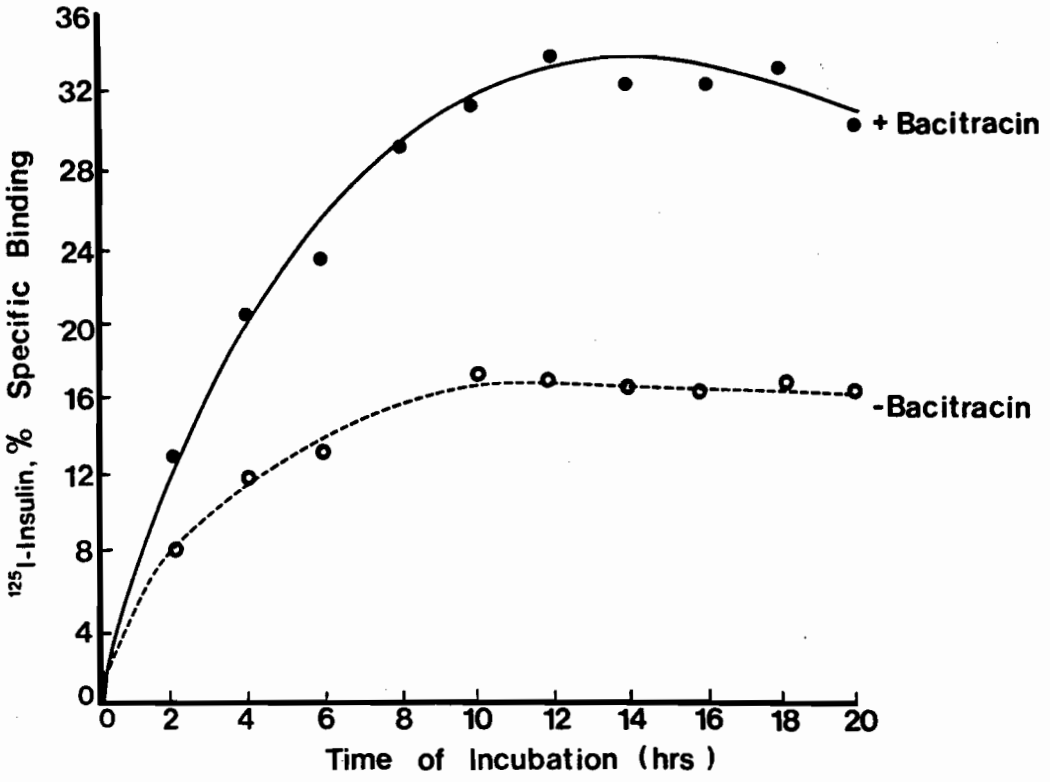

A.
$^{125}$I-INSULIN, % SPECIFICALLY BOUND
50
40
30
20
10
0
B.
% DEGRADATION
100
80
60
40
20
0
-2
0
2
4
6
8
22
HOURS AFTER BACITRACIN ADDED

Fig. II.3 Effect of bacitracin on the specific binding of [$^{125}$I]-insulin to human term placental cells (100 ug cell protein). Cells were incubated at 4 C with [$^{125}$I]-insulin in the presence or absence of bacitracin (500 ug/tube). Specific binding was determined at the indicated time points.

Fig. II.4 Dissociation of [$^{125}$I]-insulin at 4 C in the presence and absence of unlabeled insulin (100 ug/ml). Bound [$^{125}$I]-insulin at a given time is expressed as a percentage of zero time value.


36
32
28
24
20
16
12
8
4
0
$^{125}$I-Insulin, % Specific Binding
0 2 4 6 8 10 12 14 16 18 20
Time of Incubation (hrs)
+ Bacitracin
-Bacitracin

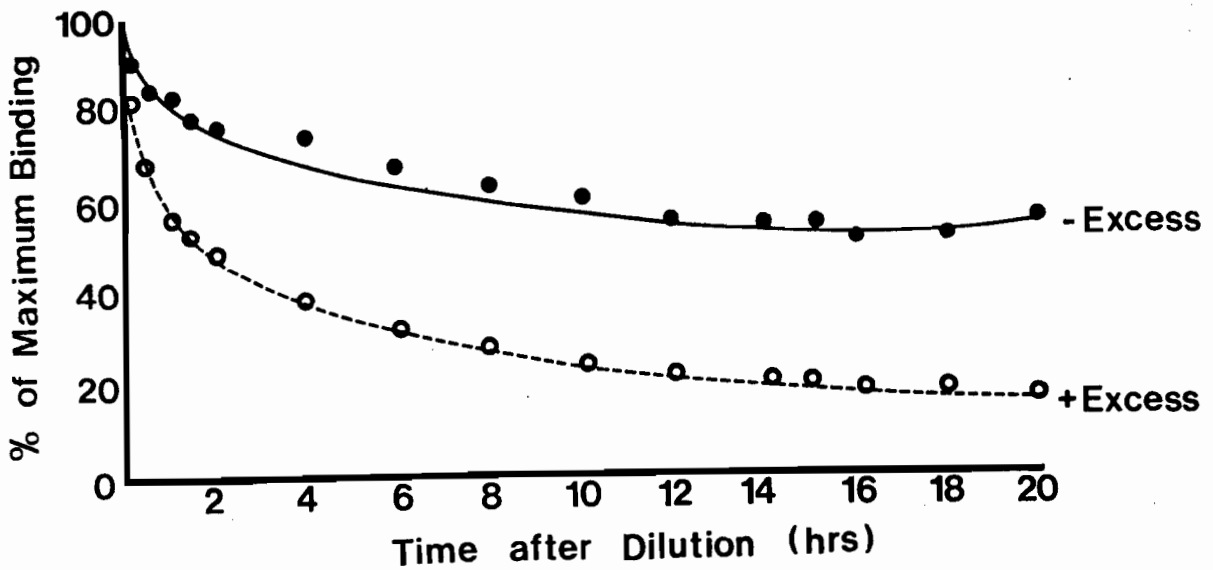

100
80
60
40
20
0
% of Maximum Binding
2 4 6 8 10 12 14 16 18 20
Time after Dilution (hrs)
- Excess
+ Excess

Fig. II.5 Effect of cell protein concentration on [$^{125}$I]insulin binding to human term placental cells. Incubations were performed for 20 h at 4 C in 0.1 M Hepes assay buffer, in the presence and absence of bacitracin (500 ug/tube) with various concentrations of cell protein ranging from 50 to 400 ug per assay tube.

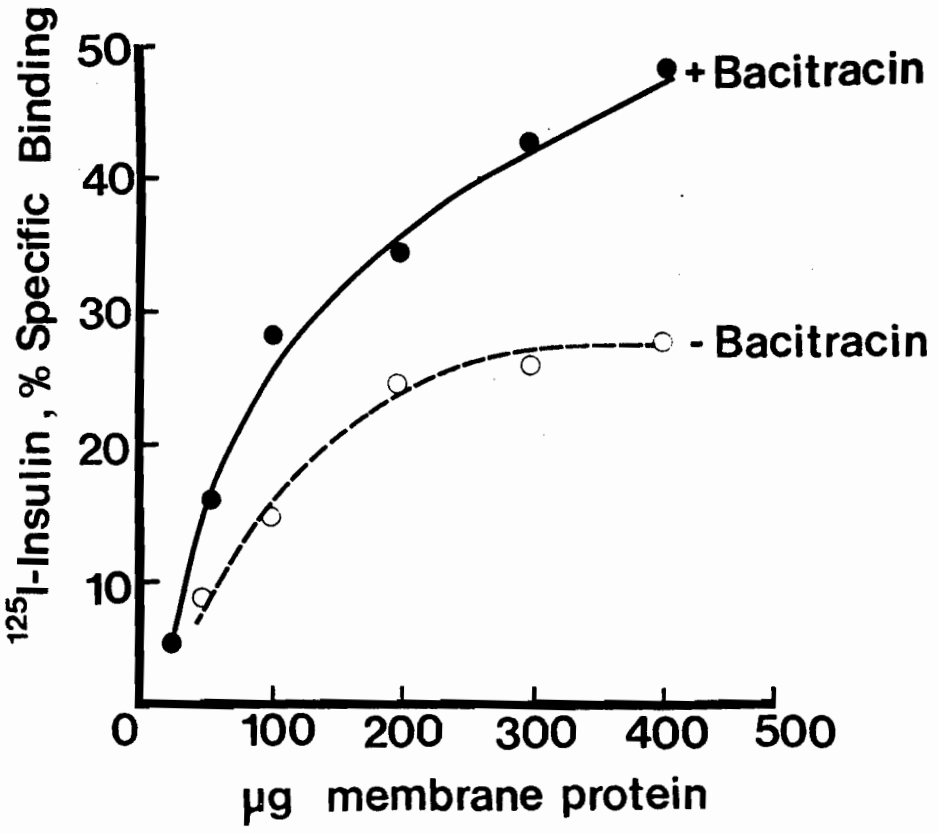

$^{125}$I-Insulin, % Specific Binding
50
40
30
20
10
0
100
200
300
400
500
µg membrane protein
+ Bacitracin
- Bacitracin

Fig. II.6 Effect of ligand concentration on [$^{125}$I]-insulin binding to human term placental cells. Cells (100 ug cell protein) were incubated in Hepes assay buffer (pH 7.5) at 4 C for 20 h.

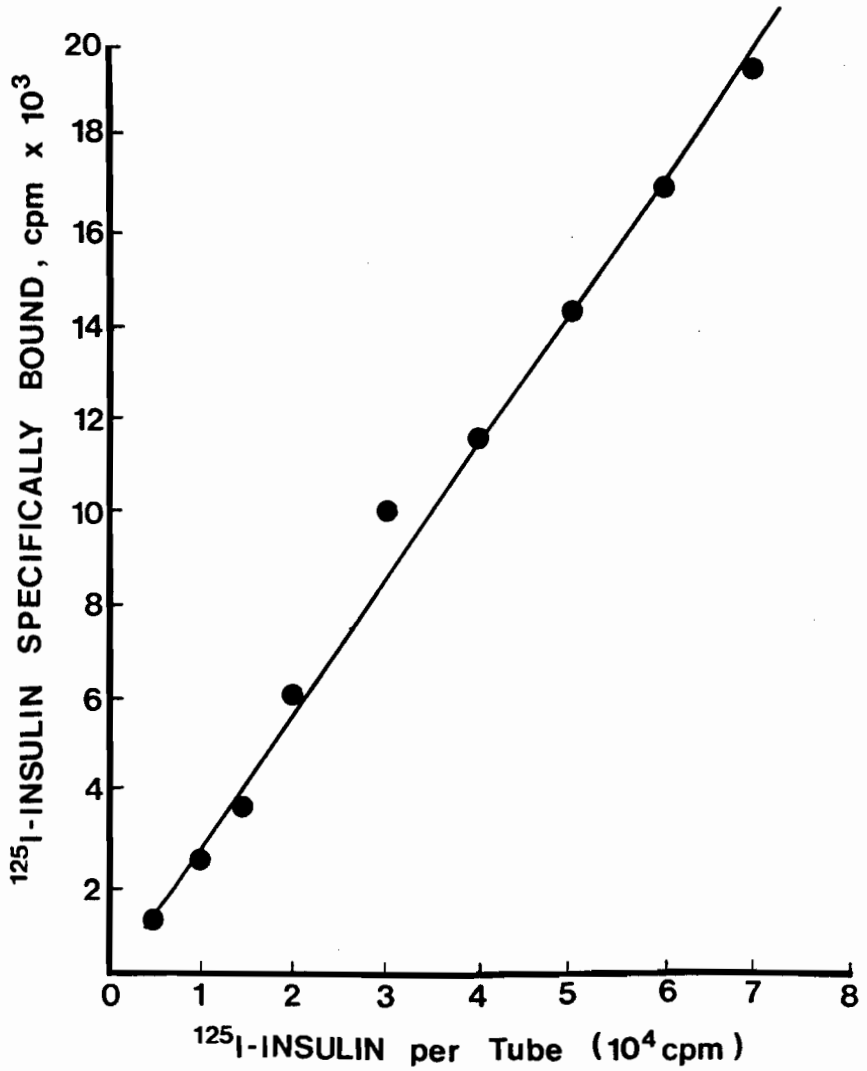

$^{125}$I-INSULIN SPECIFICALLY BOUND, cpm x $10^3$
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
$^{125}$I-INSULIN per Tube ($10^4$ cpm)

Fig. II.7 Effectof pH on [$^{125}$I]-insulin binding to human term placental cells (100 ug cell protein) at 4 C for 20 h in the presence and absence of bacitracin (500 ug/tube). The pH of the buffer was adjusted to values from 6-8.5 as indicated in the figure. At each pH the percent [$^{125}$I]insulin specifically bound was determined.

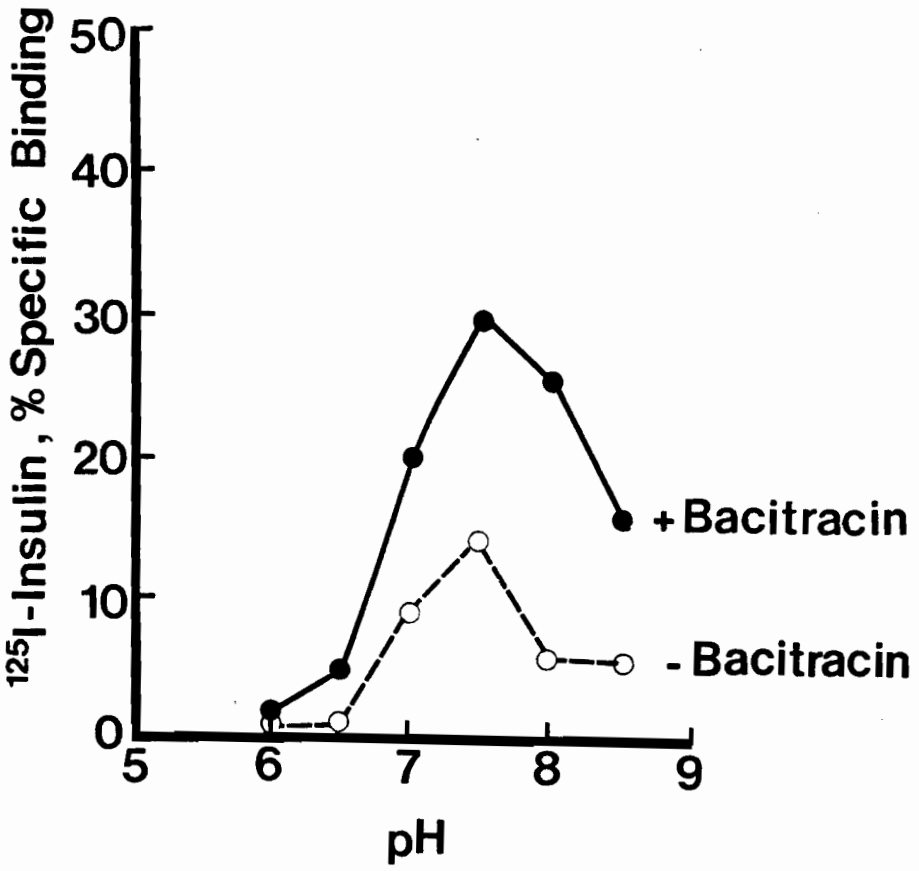

$^{125}$I-Insulin, % Specific Binding
50
40
30
20
10
0
5
6
7
8
9
pH
+Bacitracin
-Bacitracin

Fig. II.8 Effect of bacitracin and various peptides on [$^{125}$I]-insulin binding to term placental cells (100 ug cell protein). Labeled insulin was incubated with cells for 20 h at 4 C in the presence and absence of bacitracin, or the various peptides indicated. A final concentration range of 0-1,000 ng/ml in the assay since 0.1 ml aliquots of the insulin were added to each assay tube in which the final volume was 0.5 ml. At the end of the incubation the cell bound [$^{125}$I]-insulin was determined. The data were plotted as the percentage of labeled insulin bound.

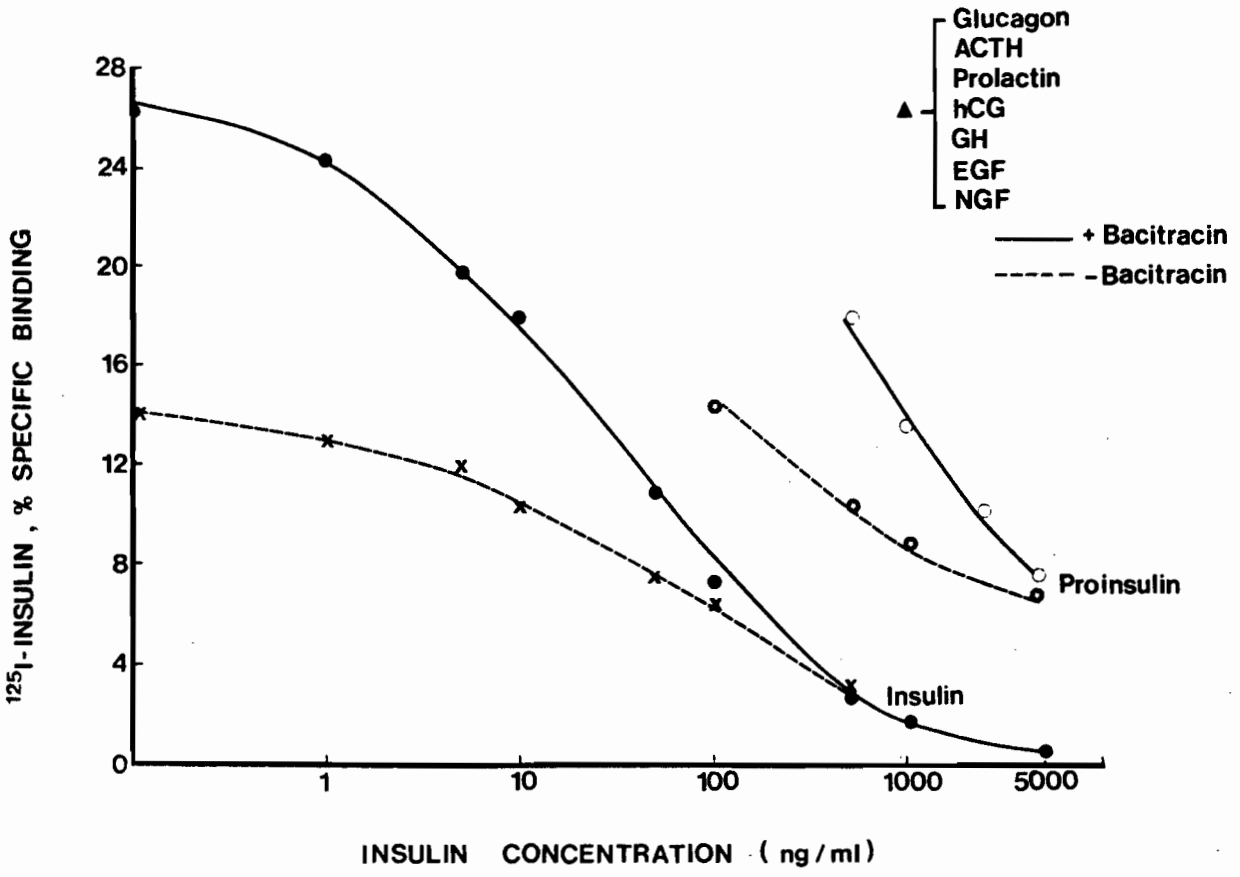

Glucagon
ACTH
Prolactin
hCG
GH
EGF
NGF
+ Bacitracin
- Bacitracin
Proinsulin
Insulin
$^{125}$I-INSULIN , % SPECIFIC BINDING
INSULIN CONCENTRATION ( ng / ml )
0
4
8
12
16
20
24
28
1
10
100
1000
5000

Fig. II.9 Scatchard plot of insulin binding after down-regulation in 18 wk and term placental cells. Confluent cultured placental cells in 100 x 20 mm cultured dishes were incubated for 18 h at 37 C in culture medium alone (○ 18 wk, ✪ term), or medium plus $10^{-6}$ M unlabeled insulin (★ 18 wk, ● term). Cells were washed and insulin binding was assessed over the insulin concentration of 0-5 x $10^3$ ng/ml. This gave a final concentration range of 0-1 x $10^3$ ng/ml in the assay since 0.1 ml aliquots of the insulin were added to each assay tube in which the final volume was 0.5 ml. No difference in nonspecific binding was observed in down-regulated cells.

The calculated binding parameters were: (a) for control term placental cells: $K_1$= 0.90 x $10^9 M^{-1}$, $K_2$= 30.4 x $10^6 M^{-1}$, total receptor number of 15638 fM/mg of cell protein; (b) for term placental cells exposed to $10^{-6}$M insulin: $K_1$=0.76 x $10^9 M^{-1}$, $K_2$= 28.1 x $10^6 M^{-1}$, 4621 fM/mg of receptors, (c) for control 18 wk early gestation placental cells : $K_1$=0.51 x $10^9 M^{-1}$, $K_2$=22.0 x $10^6 M^{-1}$, 7040 fM/mg of receptors; and (d) for 18 wk early gestation placental cells exposed to $10^{-6}$M insulin: $K_1$= 0.62 x $10^9 M^{-1}$, $K_2$ = 26.1 x $10^6 M^{-1}$, 1821 fM/mg of receptors.

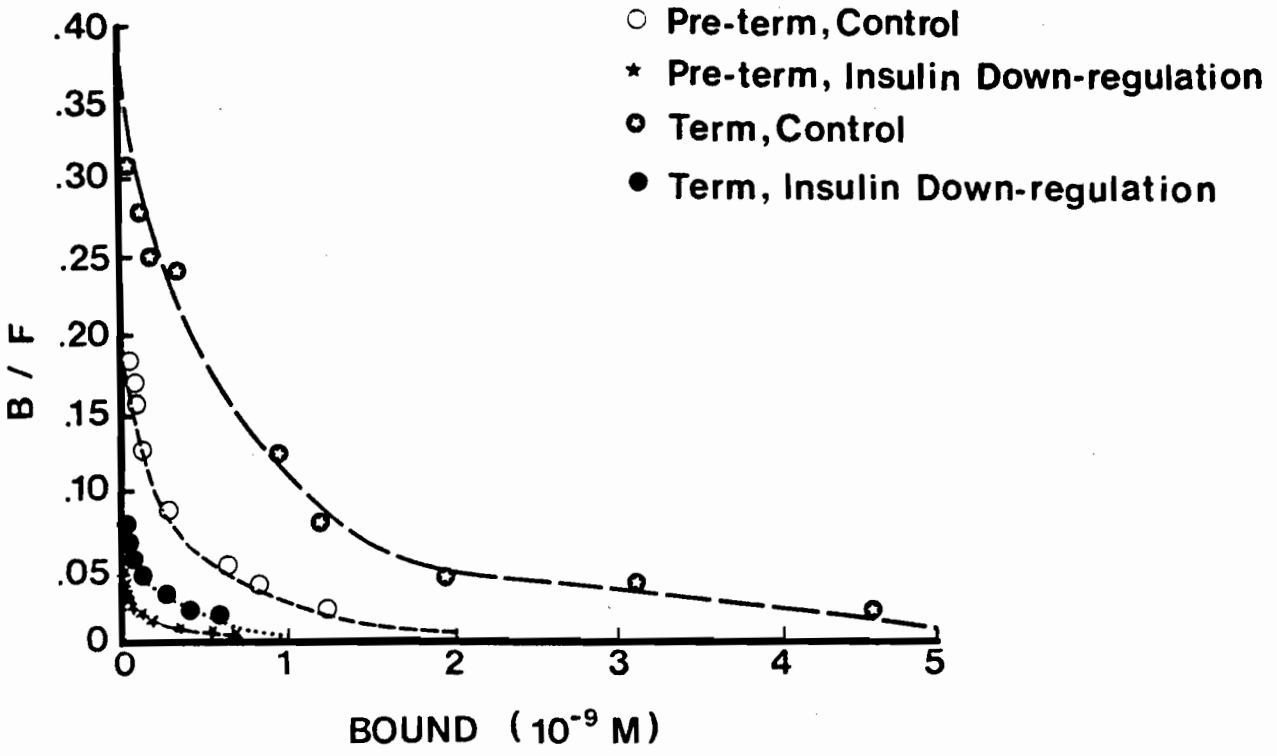

○ Pre-term, Control
★ Pre-term, Insulin Down-regulation
⊙ Term, Control
● Term, Insulin Down-regulation
B / F
.40
.35
.30
.25
.20
.15
.10
.05
0
0
1
2
3
4
5
BOUND (10⁻⁹ M)

Fig. II.10 Insulin-induced receptor down-regulation was tested in early gestation (18 wk) and term placental cells in the presence of bacitracin (1 mg/ml) at 37 C for 18 h with various concentrations of native insulin as indicated.

Specificity of insulin-induced receptor down-regulation was examined with cultured term placental cells only at 37 C for 18 h in the presence of 1 mg/ml bacitracin with various concentrations of unlabeled proinsulin, IGF-II ($10^{-8}$M), and various growth factors and peptides at the concentration of $10^{-8}$M as indicated.

After preincubation the cells were washed free of exogeneous peptides and assayed for [$^{125}$I]-insulin specific binding. The data are expressed as percent of control (ie. cells not exposed to native insulin). Each point is the mean and the vertical line represents the S.E. from three different experiments.

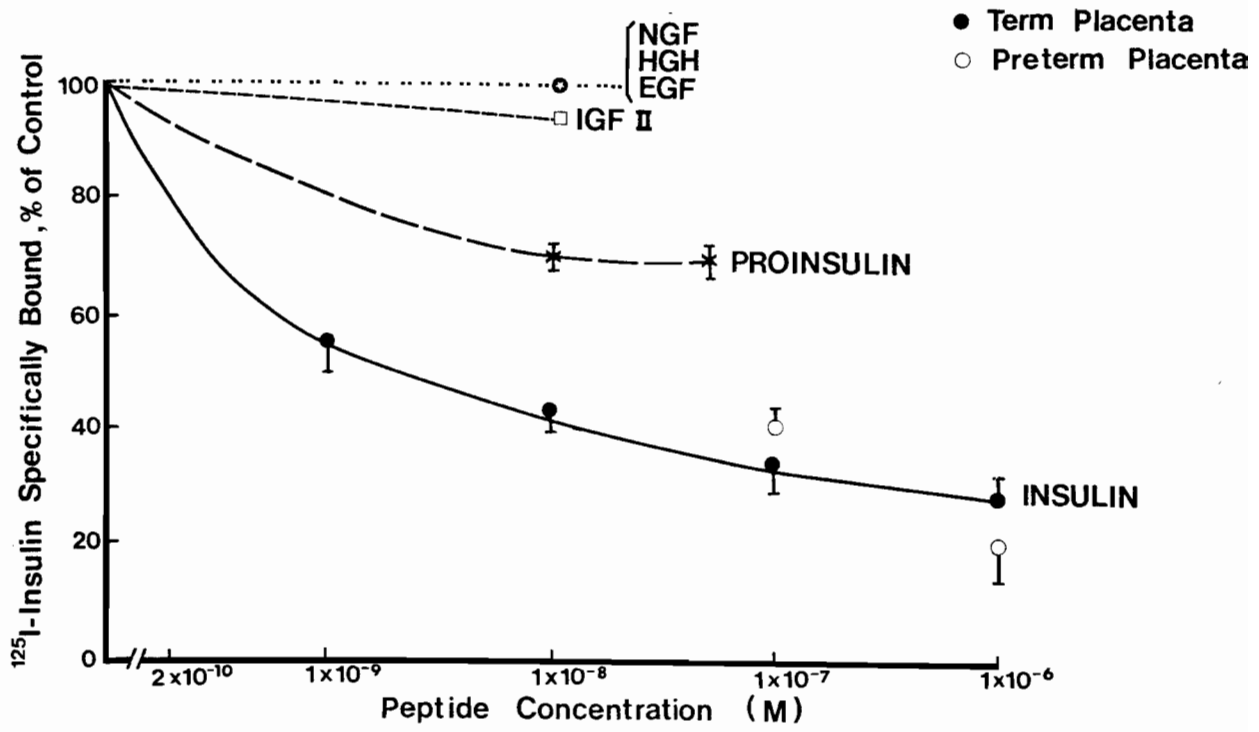

● Term Placenta
○ Preterm Placenta
NGF
HGH
EGF
IGF II
PROINSULIN
INSULIN
$^{125}$I-Insulin Specifically Bound, % of Control
100
80
60
40
20
0
2x10$^{-10}$
1x10$^{-9}$
1x10$^{-8}$
1x10$^{-7}$
1x10$^{-6}$
Peptide Concentration (M)

Fig. II.11 Cultured term placental cells were preincubated with $10^{-6}$ M insulin or $10^{-6}$ M insulin plus 10 ug/ml cycloheximide at 37 C for 16 h in the presence of bacitracin (1mg/ml). The percent of [$^{125}$I]-insulin specifically bound was determined at several time points. In order to study receptor recovery following preincubation with insulin,the human term placental cells which had been preincubated with only $10^{-6}$M insulin and 1 mg/ml bacitracin alone were washed thoroughly and then resuspended in fresh insulin-free Ham's F-10 medium with or without 10 ug/ml cycloheximide. Percent specific binding of [$^{125}$I]-insulin was determined when the cells were resuspended in fresh media (indicated by arrow) and at the various time intervals shown, up to 32 h. The data are expressed as the percent of specific binding compared to cells not preincubated with insulin (ie. % of control).

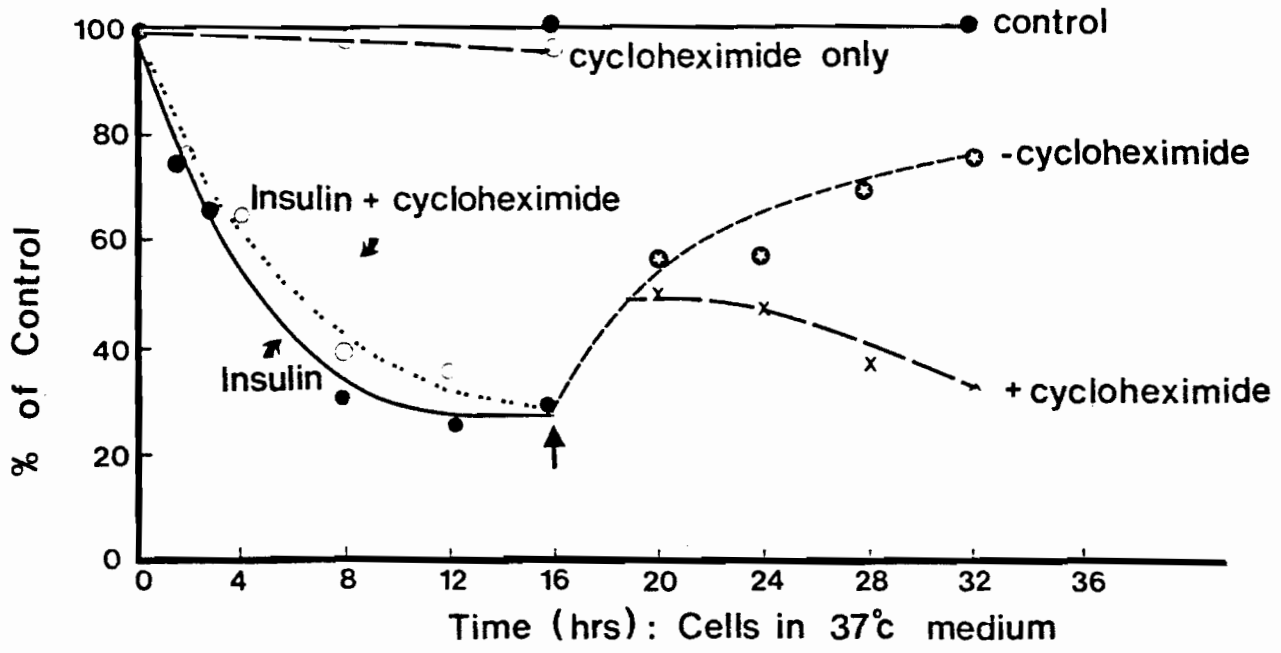

100
80
60
40
20
0
% of Control
0
4
8
12
16
20
24
28
32
36
Time (hrs): Cells in 37°c medium
control
cycloheximide only
-cycloheximide
Insulin + cycloheximide
Insulin
+ cycloheximide

Fig. II.12 Receptor recovery following preincubation with insulin. 18 wk and term placental cells which had been preincubated with insulin ($10^{-6}$M) for 16 h at 37 C were washed and then resuspended in fresh insulin-free Ham's F-10 medium with or without 10 ug/ml cycloheximide. The level of binding is expressed as the percent of binding compared to cells not exposed to insulin. The specific [$^{125}$I]-insulin binding was measured when the cells were initially resuspended in fresh media and 16 h later.

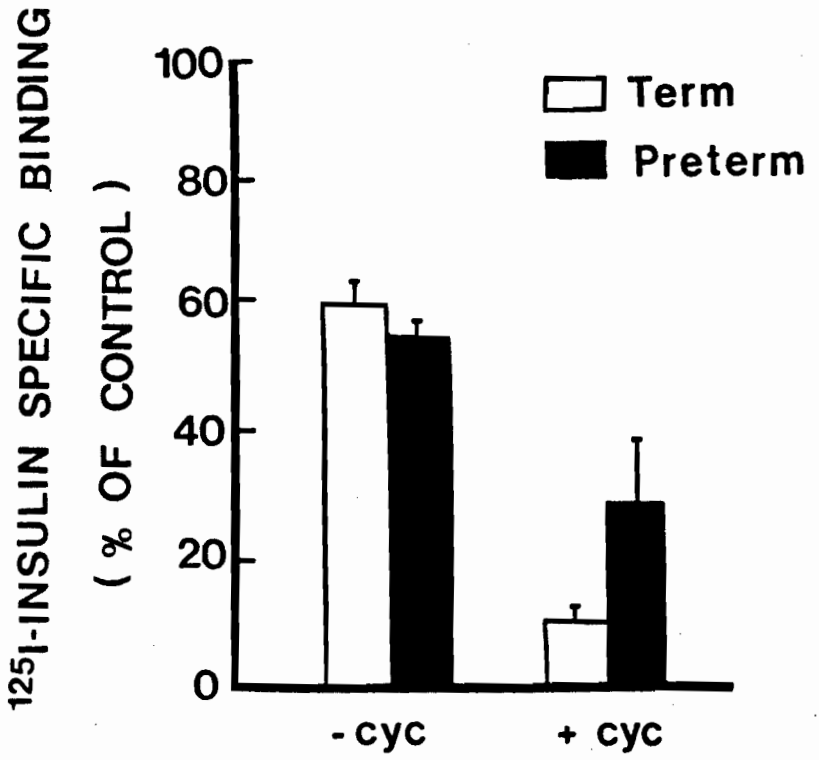

$^{125}$I-INSULIN SPECIFIC BINDING
( % OF CONTROL )
100
80
60
40
20
0
Term
Preterm
- cyc
+ cyc

Table II.1 Degradation of [125 I]-Insulin: Term Placental Cells

| | + Bacitracin | | - Bacitracin | |
|---|---|---|---|---|
| | % total | % control | % total | % control |
| **Supernatant Radioactivity** | | | | |
| **TCA Precipitation** | | | | |
| | * | | | |
| (1) tracer | 95 ± 0.4 | 100 ± 0.3 | 91 ± 0.5 | 100 ± 0.4 |
| (2) + cells | 92 ± 0.3 | 97 ± 0.3 | 52 ± 0.7 | 57 ± 0.8 |
| (3) + cells + unlabeled insulin | 95 ± 0.1 | 100 ± 0.1 | 81 ± 0.3 | 89 ± 0.4 |
| **Rebinding to membranes** | | | | |
| (1) tracer | | 100 ± 0.2 | | 100 ± 0.3 |
| (2) + cells | | 95 ± 0.4 | | 42 ± 0.7 |
| **Radioactivity Eluted from from Pellet** | | | | |
| **TCA Precipitation** | | | | |
| (1) tracer | 96 ± 0.2 | 100 ± 0.2 | 92 ± 0.3 | 100 ± 0.2 |
| (2) + cells | 93 ± 0.4 | 97 ± 0.3 | 69 ± 0.5 | 75 ± 0.7 |
| (3) + cells + unlabeled insulin | 92 ± 0.1 | 96 ± 0.0 | 73 ± 0.6 | 79 ± 0.6 |
| **Rebinding to membranes** | | | | |
| (1) tracer | | 100 ± 0.1 | | 100 ± 0.2 |
| (2) + cells | | 100 ± 0.0 | | 78 ± 0.8 |

The percentage of intact tracer was determined for supernatant and cell-eluted [$^{125}$I]-insulin by 10% TCA precipitation and rebinding to 100 ug human placental membrane. Control refers to tracer not pre-incubated with cells. Values are mean ± S.E. for at least triplicate determinations of two term placental cultured cell preparations.

* Mean ± S.E.

Table II.2 Percent Specific Binding and Binding Parameters of $[^{125}I]$-Insulin to Human Placental Cells.

| Gestational age | % Specific binding | Receptor number (fM/mg) | $K_1$ $1 \times 10^{-9}$ M | $K_2$ $1 \times 10^{-6}$ M |
|---|---|---|---|---|
| Early gestation | 10.4 ± 1.4 (17)* | 9,220 ± 2,639 (7) | 0.48 ± 0.2 (7) | 21.97 ± 4.9 (7) |
| Term gestation | 28.2 ± 0.6 (25) | 18,262 ± 1,059 (7) | 0.91 ± 0.4 (7) | 30.43 ± 11.8 (7) |
| t test | $P < 0.0001$ | $P < 0.0001$ | NS | NS |

* Mean ± S.E. (N).

NS = not significant.

# Chapter III Characterization of Receptors for Insulin-Like Growth Factors (IGFs) I and II in Human Placental Cells in Culture

## III.1 Introduction

Insulin-like growth factors (IGFs) are a family of polypeptides isolated from human plasma or conditioned media. They are acid-stable and have a molecular weight of about 7-8,000 daltons (154). IGFs have been shown to have many insulin-like biologic activities, including the stimulation of glucose oxidation, lipogenesis (219,220) and cell growth (221-223). Studies indicate that the anabolic effect of the IGFs are mediated through the growth factor receptor and the insulin-like effects of these peptides are secondary phenomena, resulting from lower affinity binding to the insulin receptor (104,224). This is true for some, but not all cell types. Recently, Foley et al (225) suggested that insulin stimulated proteoglycan synthesis in chondrosarcoma chondrocytes by acting via the insulin receptor, whereas the IGFs appeared to act through their own receptors.

The human placenta has been recognized as playing a selective and active role in transfer of substances essential for feto-placental growth. Receptors for IGFs have been identified in human placentae (154,190). Cultured placental monolayers offer an excellent opportunity for evaluation of IGF binding and action since these cells are easily obtained, can be grown in culture and lend themselves to experimental manipulation. The following studies were initiated to characterize the IGF receptors in human placental cultures of different gestational ages in order to elucidate the possible contribution of IGFs to feto-placental unit function.

## III.2 Materials and Methods

### Cell Culture and Receptor Studies

Normal term (38-41 wk;N=10) human placentae were obtained at the time of caesarean section; early gestation (8-18 wk; N=8) human placentae were obtained at the time of therapeutic abortion by vacuum aspiration. The method for preparing placental monolayers and cell suspensions for receptor studies has been described in detail (190,191, Chapters I and II).

Unless otherwise specified, the standard binding assay consisted of 0.1 M Hepes-BSA assay buffer (pH 7.5), 100 ug cell protein, 2-3 x $10^4$ cpm of [$^{125}$I]-IGF-I or [$^{125}$I]-IGF-II, 500 ug/tube bacitracin, and the presence or absence of unlabeled semi-purified IGF standard (100 ng eq/ml, vide infra) in a final volume of 0.5 ml in 12 x 75 polystyrene tubes (Simport Ltd, Montreal, PQ). Incubations were performed with constant shaking at 4 C. Binding assays were terminated after 18-20 h by the addition of 3 ml cold Hepes washing buffer and centrifuged at 2000 x g for 30 min at 4 C.

### Hormones

IGF-I and IGF-II were extracted from human plasma Cohn fraction IV-4 (Connaught Research Laboratories; Willowdale, ONT) by acid-ethanol and further purified on Sephadex G-75 eluted with 1.0 M acetic acid followed by high pressure liquid chromatography (HPLC) at pH 2.0-2.2 (Posner BI and Guyda HJ, manuscript in preparation). A semi-purified IGF preparation [Sephadex insulin-like activity (ILAs)] (143,154) was used in all displacement curves and as unlabeled excess (100 ng eq IGF/ml), with potency determined in an insulin radioreceptor assay using human placental membranes as

receptor and porcine insulin as standard (143), where 1 ng eq IGF equals 25 uU porcine insulin. The preparations had a specific activity of 9.3-12.7 mU/mg protein and contained both IGF-I and IGF-II in a ratio of approximately 1:4.

Iodination

IGF-I

Iodination of IGF-I with $Na^{125}I$ (New England Nuclear Corp., Boston, MA) was undertaken by a modification of the chloramine T method (122) with further purification on a Sephadex G-50 column at pH 7.5 to a specific activity of 150-180 uCi/ug. The integrity of the labeled IGF-I was tested by trichloroacetic acid (TCA) precipitation and binding to human placental membranes prior to use in the receptor assays.

IGF-II

IGF-II was radioiodinated by either chloramine T (122) or the lactoperoxidase (226) method. Labeled IGF-II was then purified on a Sephadex G-50 column at pH 7.5 or by absorption to and elution from human placental membranes as previously described (143).

a). Chloramine T Method

IGF-II (85 ng eq/25 ul) was pipeted into a 12 x 75 mm polystyrene tube and mixed with 10 ul 0.5 M phosphate buffer (pH 7.5) and 1 mCi (10 ul) of carrier-free $Na^{125}I$. The following reagents (all in 0.05 M phosphate buffer, pH 7.5) were then added with hand agitation: 25 ul of chloramine T (0.5 mg/ml) well mixed for 20 seconds, 100 ul of sodium metabisulfite (0.2 mg/ml), 100 ul potassium iodide (10 mg/ml) and 0.5 ml of purified BSA (2.5%) (Sigma; St Louis, MO).

b). Lactoperoxidase Method

IGF-II (85 ng eq/25 ul) was pipeted as above. Lactoperoxidase (0.2 ug/ul) (Fisher, Fair Lawn, NJ) was dissolved in 0.5 M phosphate buffer (pH 7.5) and added with 1 mCi (10 ul) of a carrier-free $Na^{125}I$, followed by 20 ul $H_2O_2$ (0.2 mg/ml) (Fisher; Fair Lawn, NJ) and mixed for 20 seconds. Finally 925 ul of 0.1 % purified BSA in 0.05 M phosphate buffer was added.

Preparation of Cell Membranes and Erythrocytes

Normal human term placental membranes were processed according to Posner (71). Rat liver membranes were from young fasted female Sprague-Dawly rats (150 g) processed as previously described (88) (courtesy of B.Patel). Fractionated young erythrocytes were prepared from normal non-obese healthy male volunteers as previously described (198) (courtesy of C. Polychronakos).

III.3 Results

Binding of [$^{125}$I]-IGF-II with Various Receptor Preparations

We have compared the ability of placental cells, liver membranes, placental membranes and fractionated young red blood cells (RBC) to bind [$^{125}$I]-IGF-II prepared with different iodination as well as purification methods. [$^{125}$I]-IGF-II binding was determined at steady state binding conditions at 4 C for 20 h in all membranes and isolated cells in the presence of bacitracin (500 ug/tube). It was found that tracer iodinated by the chloramine T method and further purified with absorption to and elution from placental membranes gave highest steady state binding with all four receptor preparations (Table III.1). The percent specific binding of labeled [$^{125}$I]-IGF-II purified with Sephadex G-50 chromatography was generally lower than that observed with tracer purified by human

tracer purified by human placental membranes. The chloramine T iodination method produced better results with both placental membrane and Sephadex G-50 purification methods. On the basis of these studies, all subsequent experiments utilized [$^{125}$I]-IGF-II iodinated by the chloramine T method and further purified by absorption to and elution from human placental membranes.

Characterization of IGF Receptors in Human Term Placental Cells

The effect of temperature and time on [$^{125}$I]-IGF-II binding to human term placental cells in the presence of bacitracin (500 ug/tube) is depicted in Fig.III.1. Both the amount of binding at steady state and the rate of binding were temperature dependent. At 4 C, total binding of labeled IGF-II to placental cells reached a plateau at 12 h (percent specifiic binding around 5-6%) and persisted for at least another 8 h. Under these conditions, the nonspecifically bound [$^{125}$I]-IGF-II remained minimal during the 12 h incubation period, and thus, specific binding increased as a function of increased total binding. At 22 and 37 C, binding was more rapid, however, nonspecific binding increased linearly throughout the incubation period (Fig.III.1). It seemed likely that the apparent decrease in specific binding at higher temperature might be due to ligand degradation (vide infra).

Maximal specific binding in 0.1 M Hepes buffer occurred at pH 6.5-8.5 in the presence of bacitracin (500 ug/tube) (Fig.III.2) and the pH optimum was broad in contrast to the very sharp pH optimum for insulin (Chapter II) under similar conditions.

Increments of cell protein up to 100 ug resulted in linear increases in total binding of labeled IGF-II (Fig.III.3). Nonspecific binding rose progressively with high cell protein

concentration, whereas total binding decreased; thus specific binding decreased.

Binding of [$^{125}$I]-IGF-II to human term placental cells was specific. Unrelated hormones such as glucagon (GLU) (10 ug/ml), ACTH (10 ug/ml), prolactin (PRL) (10 ug/ml), growth hormone (GH) (10 ug/ml) , human chorionic gonadotropin (hCG) (1 x $10^3$mU/ml), nerve growth factor (NGF) (2 ug/ml), epidermal growth factor (EGF) (2 ug/ml) showed negligible competitive inhibition (Fig. III.4). Insulin showed about 30% inhibition at 0.5 ug/ml for the IGF-II receptor (Fig.III.4).

Data on binding of [$^{125}$I]-IGF-I were very similar to IGF-II except that the magnitude of binding was lower. At 4 C, in the presence of bacitracin (500 ug/tube), total binding of [$^{125}$I]-IGF-I to term placental cells plateaued as early as 2 h at 2-3 % and persisted for another 14 h (Fig.III.5). At 37 C, in the presence of bacitracin (500 ug/tube), total binding increased but nonspecific binding increased more markedly, resulting in a decrease in specific binding at 2 h (Fig.III.5).

The pH optimum of [$^{125}$I]-IGF-I binding to term placental cells was examined. A broad plateau of maximal binding was seen over the pH range from 6.5-8.5 in the presence of bacitracin (500 ug/tube) (Fig.III.6).

Degradation

A wide range of bacitracin concentrations was tested (Fig. III.7). As the concentration of bacitracin increased to 0.5 mg/tube, there was an inhibition of labeled IGF-II degradation and a corresponding increase in specific binding. At 0.5-2.0 mg/tube,

bacitracin inhibited degradation to less than 5% as assessed by TCA precipitability. Very high concentrations of bacitracin (greater than 1.5 mg per tube) inhibited degradation but also suppressed total IGF-II binding to the cells.

The pancreatic trypsin inhibitor trasylol at 500 KIU/ml and insulin (0.2 ng/ml) were also tested and found to enhance labeled IGF-II binding to term placental cells by about 25%; degradation in the medium assessed by TCA precipitability was less than 5%. In the presence of bacitracin (500 ug/tube), degradation of labeled IGF-I was also decreased to less than 5% in the medium as assessed by TCA precipitation. Thus, bacitracin has been utilized routinely in all subsequent studies of both IGF-I and IGF-II receptors in human placentae.

IGF-I and IGF-II Receptors in Mid-Gestation and Term Placental Cells

Hormone binding was assessed in mid-gestation (12-18 wk; N=5) and term placentae (38-41 wk; N=6). Binding of labeled IGF-I and IGF-II to each cell preparation was assessed at 100 ug cell protein per tube. Specific IGF-I and IGF-II binding was found at all gestational ages we examined. Percent specific binding of both IGF-I and IGF-II appeared to be similar at the two gestational ages studied (Table III.2).

III.4 Discussion

IGF-I and IGF-II have been shown to stimulate proliferation of a variety of cultured cells (227). All the IGFs have insulin-like activity as well as anabolic effects on cells in culture. Zapf et al (227) proposed that there is a complementary effect between insulin and IGFs with respect to their interaction with cell

surface receptors. The structural homology between IGFs and insulin, as well as their overlapping biological actions, would suggest that they either act via the same receptor, or via two separate but closely related receptors in regard to spatial orientation and chemical structure. The structure of the [$^{125}$I]-IGF-I binding site is similar to that of the insulin receptor; the specificity of complex formation, however, clearly establishes the [$^{125}$I]-IGF-I binding site as a subtype of IGF receptor rather than an insulin receptor (228).

It is well accepted that polypeptide hormone action is initiated when hormone binds to its specific receptor on the cell membrane. Studies of IGF receptors require a satisfactory radioactive ligand probe. A modification of the original chloramine T method first applied to growth hormone (122) was described in "Materials and Methods". In addition, in parallel, we also prepared labeled IGF-II with the lactoperoxidase method. Almost without exception the percent specific binding of IGF-II prepared with chloramine T exceeded binding of tracer prepared with lactoperoxidase, though the specific activities in both preparations were similar (140-160 uCi/ug). In our early binding assays with cells or membranes, [$^{125}$I]-IGF-II tracer showed variable binding with each iodination. Therefore, in order to achieve better and more consistent results, two different purification methods were tested: Sephadex G-50 chromatography and human placental membrane absorption and elution. The specific binding of labeled IGF-II to the four different membrane receptor preparations was highest with chloramine T iodination, followed by

membrane absorption and elution. Due to the limited supply of purified IGF-II, we were unable to demonstrate that labeled IGF-II has the same biological properties as native IGF-II, even though we appreciate that altered biological activity may result from iodination of different tyrosines.

The demonstration of specific binding of labeled IGF-II to placental receptors that was relatively free of competition by insulin (seen only at 10 ug/ml) and other hormones indicates that IGF-II has its own specific receptor in addition to that shared with insulin. Both [$^{125}$I]-IGF-I and IGF-II bound rapidly to their receptors in placental cells; maximal [$^{125}$I]-IGFs binding occurred at 4 C. The pH optimum for IGF binding was broad (6.5-8.5), in contrast to the very sharp pH optimum we observed for insulin. We were unable to carry out meaningful studies of the differential competitive effect of unlabeled IGFs on IGF-I and IGF-II receptors due to lack of purified materials. We, therefore, have chosen not to estimate the binding parameters with the current data. Other essential binding characteristics, such as thorough specificity studies with all members of the family of IGF polypeptides, must await additional supplies of purified peptides.

The placental cell is extremely rich in degrading activity for insulin and IGFs (229). In our in vitro test system bacitracin was essential for the study of IGF receptors.

IGFs, a group of growth hormone dependent plasma peptides which are thought to promote post-natal growth, may also play a stimulatory role in fetal growth. We have studied the ontogeny of IGF-I and II receptors in human placentae of various gestational ages. We observed significant but similar percent specific binding

of both IGFs at both gestational ages. Thus, similar specific binding of IGFs at different gestational ages suggests that maximum binding is already reached by early gestation, and therefore IGFs may be important very early in placental development.

III.5 Summary

An initial experiment was designed for the purpose of preparing radio-labeled [$^{125}$I]-IGF-II of high tracer quality. Following iodination, each tracer was monitored with binding to four membrane receptor preparations. It appeared that IGF-II prepared by the chloramine T procedure and purified by absorption to and elution from human placental membranes was better suited for use in the human placental cell system.

We have characterized the binding properties of the IGF-I and IGF-II receptor in human placental cells in culture. Maximal binding occurred at 4 C, with broad pH optima (pH 6.5-8.5) for both [$^{125}$I]-IGF-I and [$^{125}$I]-IGF-II and exhibited a high degree of specificity.

In the presence of bacitracin (500 ug/tube), [$^{125}$I]-IGF-I and [$^{125}$I]-IGI-II appeared to be physically intact following exposure to placental cells for 20 h at 4 C, as assessed by trichloroacetic acid precipitability.

The ontogeny of IGF receptors in human placental cells was investigated. Percent specific binding of [$^{125}$I]-IGF-I and [$^{125}$I]-IGF-II appeared to be similar at the two gestational ages studied. The presence of specific IGF-I and IGF-II receptors at both gestational age groups, with little ontogenic change, suggests that the IGFs may be involved in growth and development of the feto-placental unit.

## III.6 Figures and Tables

Fig. III.1 Effect of time and temperature on [$^{125}$I]-IGF-II binding to human term placental cells (100 ug cell protein). Association was conducted in the presence of bacitracin (500 ug/tube) at 4, 22 and 37 C. The data are plotted as the percent of [$^{125}$I]-IGF-II bound for total (TB) and nonspecific binding (NSB).

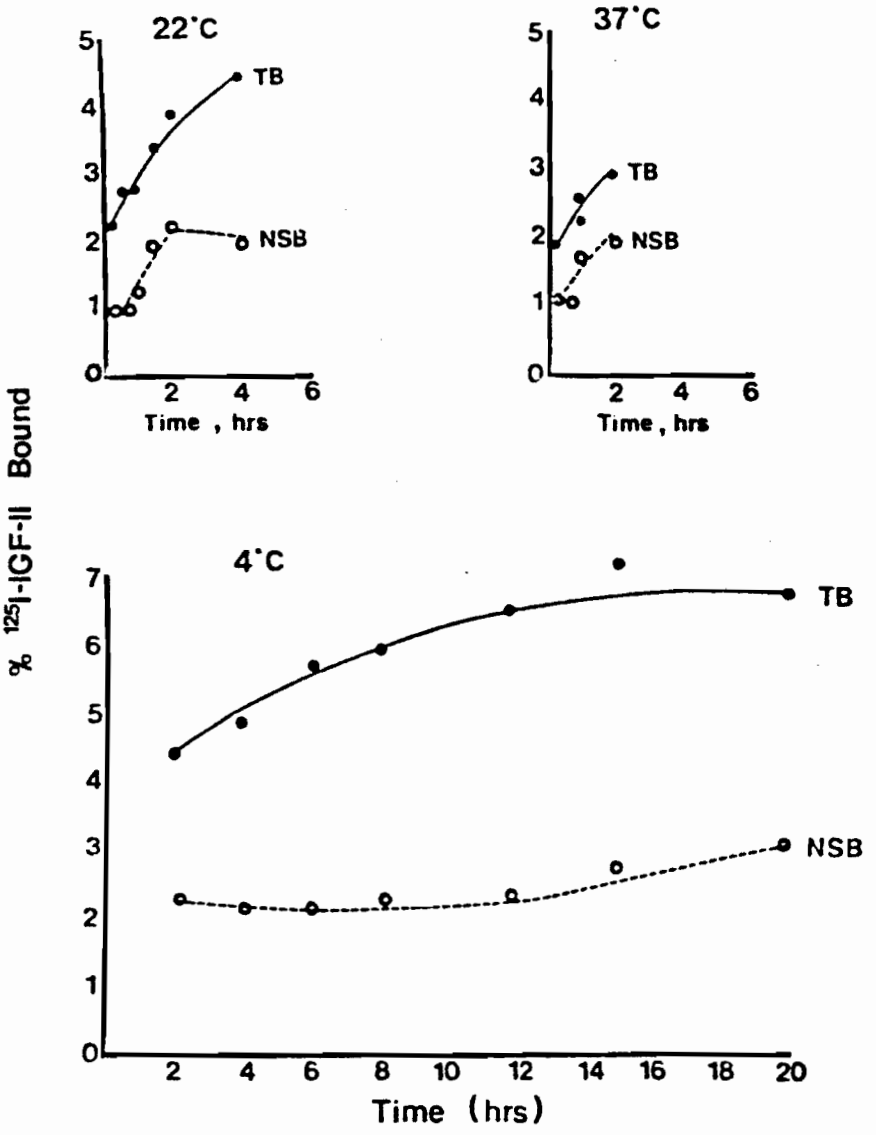
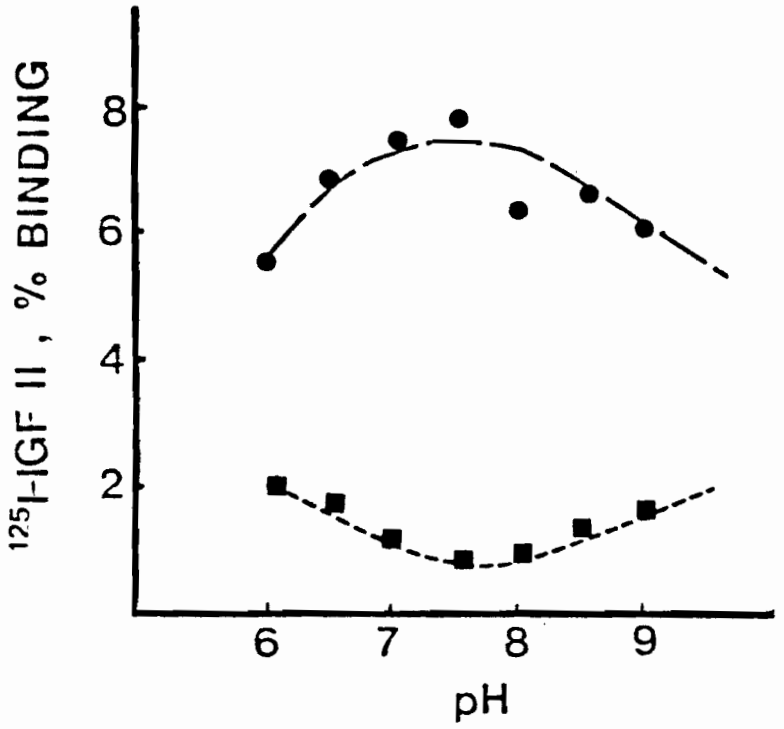

22°C
37°C
4°C
TB
NSB
% 125I-IGF-II Bound
Time , hrs
Time (hrs)

Fig. III.2 Effect of pH on [$^{125}$I]-IGF-II binding to term placental cells (100 ug cell protein). The pH of the buffer was adjusted to values from 6.0 to 9.0 as indicated in the figure. The data were plotted as the percent of [$^{125}$I]-IGF-II bound for total ( ● ) and nonspecific binding ( ■ ).

Fig. III.3 Effect of cell protein concentration on [$^{125}$I]-IGF-II binding. Labeled IGF-II was incubated for 20 h at 4 C with various concentrations of cell protein in the presence and absence of unlabeled semi-purified IGF preparations (Sepahdex ILAs, 100 ng eq/ml) in the presence of bacitracin (500 ug/tube). The data are plotted as the percent of [$^{125}$I]-IGF-II bound for total ( ● ) and nonspecific binding ( ■ ).


$^{125}$I-IGF II , % BINDING
8
6
4
2
6
7
8
9
pH

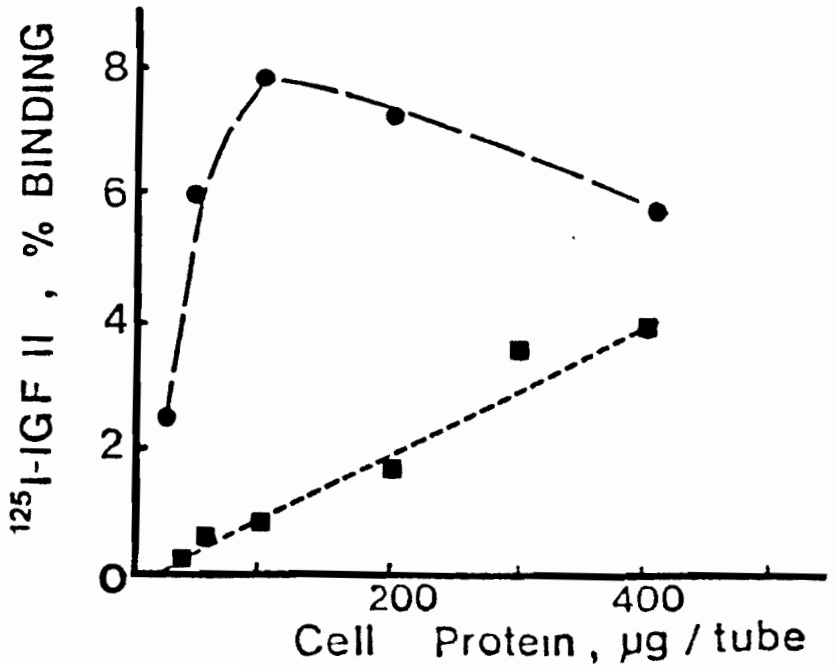

$^{125}$I-IGF II , % BINDING
8
6
4
2
0
200
400
Cell Protein , µg / tube

Fig. III.4 Specificity of [$^{125}$I]-IGF-II binding to term placental cells (100 ug cell protein) for 20 h at 4 C was tested with various polypeptides in the presence of bacitracin (500 ug per tube). The IGF-II preparation was purified by HPLC. A final concentration range of 0-100 ng/ml was tested in the assay since 0.1 ml aliquots of the IGF-II and insulin were added to each assay tube in which the final volume was 0.5 ml.

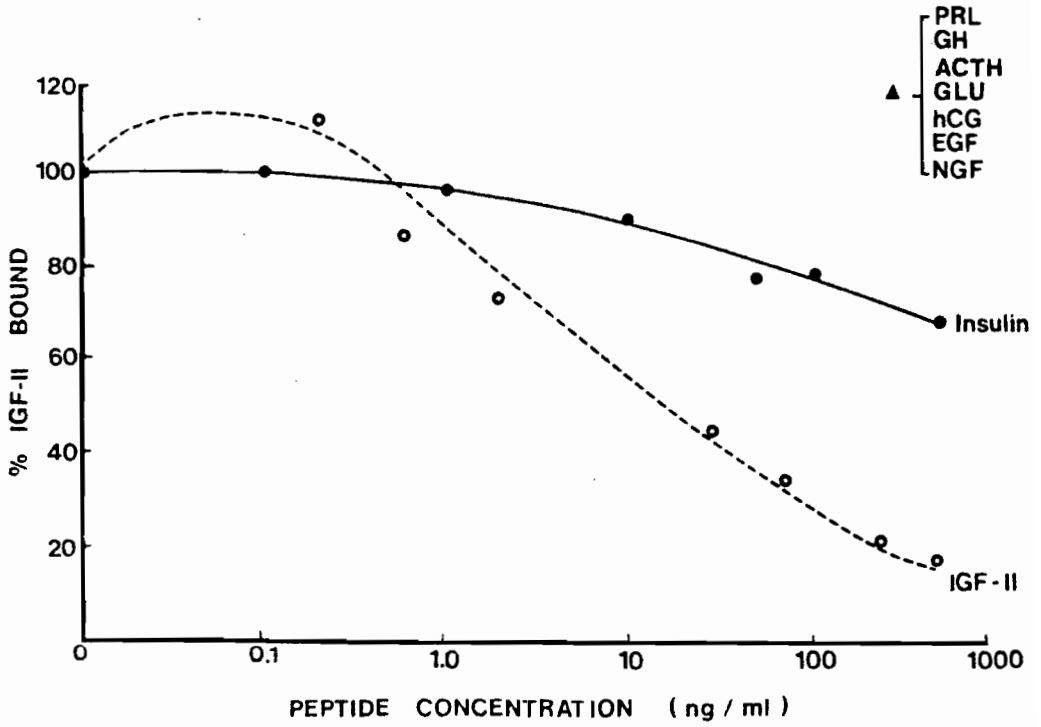

PRL
GH
ACTH
GLU
hCG
EGF
NGF
Insulin
IGF-II
% IGF-II BOUND
120
100
80
60
40
20
0
0.1
1.0
10
100
1000
PEPTIDE CONCENTRATION (ng / ml)

Fig. III.5 Association of [$^{125}$I]-IGF-I with human term placental cells (100 ug cell protein), plotted as a function of duration of incubation. Experiments were performed at 4 and 37 C as indicated.

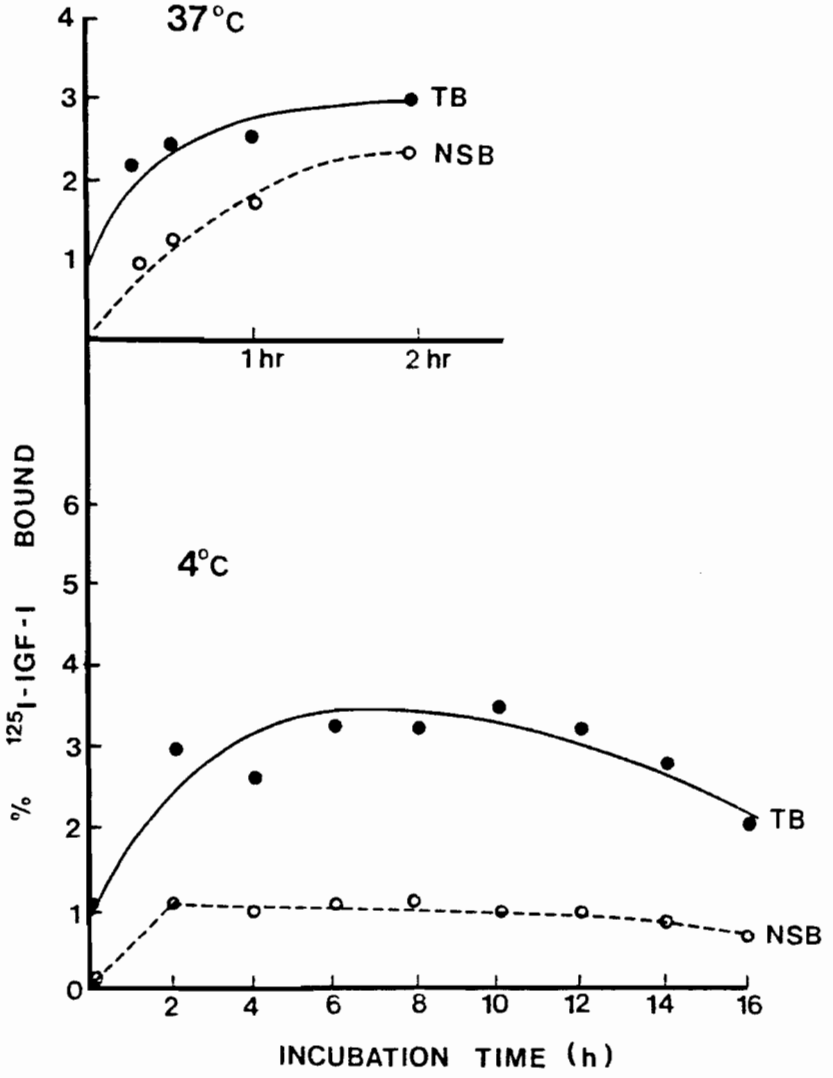

37°C
TB
NSB
1 hr
2 hr
4°C
TB
NSB
% $^{125}$I-IGF-I BOUND
INCUBATION TIME (h)

Fig. III.6 Effect of pH on total binding of [$^{125}$I]-IGF-I to term placental cells. Cultured cells (100 ug cell protein) were incubated with labeled IGF-I in 0.1 M Hepes assay buffer. The pH of the buffer was adjusted to the values from 6.0 to 9.0 as indicated. The data are plotted as the percent of [$^{125}$I]-IGF-I bound for total (TB) and nonspecific binding (NSB).

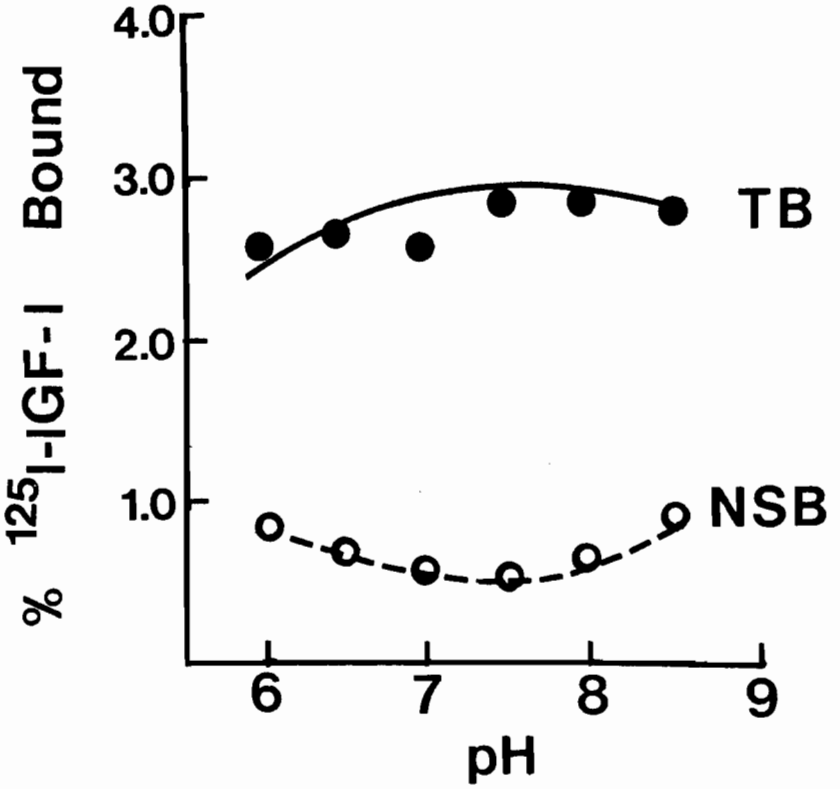

% $^{125}$I-IGF-I Bound
4.0
3.0
2.0
1.0
TB
NSB
6
7
8
9
pH

Fig. III.7 Binding of [$^{125}$I]-IGF-II by human term placental cells as a function of bacitracin concentration. [$^{125}$I]-IGF-II was incubated with term placental cells (100 ug cell protein) in 0.1 M Hepes assay buffer (pH 7.5) at 4 C for 20 h with varying concentrations of bacitracin. The data are plotted as percent of [$^{125}$I]-IGF-II bound for total (TB) and nonspecific binding (NSB).

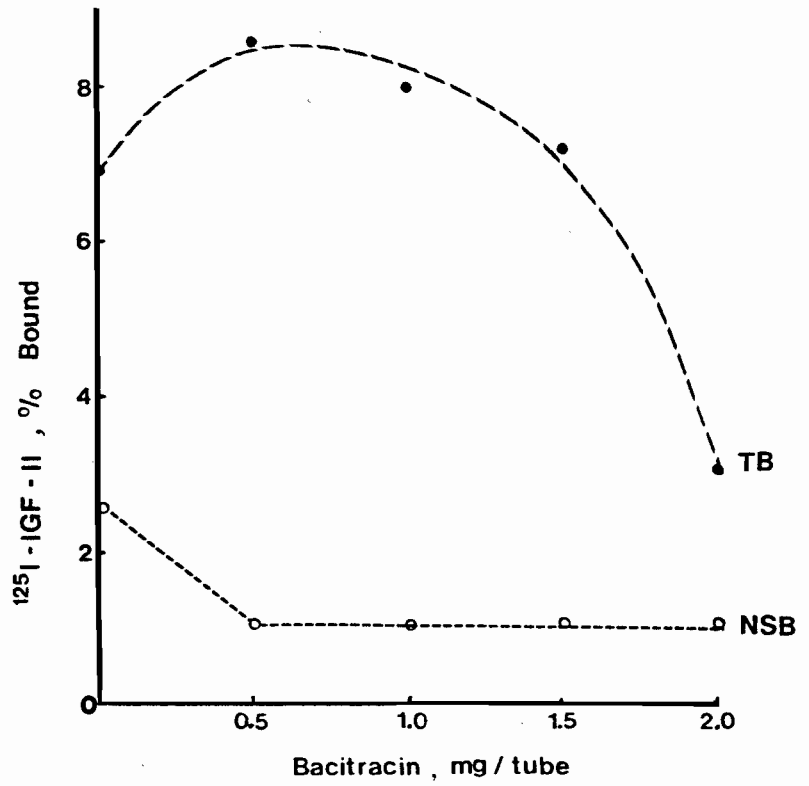

$^{125}$I - IGF - II , % Bound
8
6
4
2
0
0.5
1.0
1.5
2.0
TB
NSB
Bacitracin , mg / tube

Table III.1 Percent Specific Binding of [$^{125}$I]-IGF-II in Various Preparations

| | | Cells or Membranes (cell protein or cell number per tube) | | | |
|---|---|---|---|---|---|
| | | Placental cells | Liver membranes | Placental membranes | Fractionated RBC |
| Iodination | Purification | 100 ug | 150 ug | 150 ug | $1 \times 10^6$ cells |
| Lacto-peroxidase | Placental membrane | 5.9 ± 0.7 (N=3) * | 14.7 | 20.3 | 0.4 |
| | G-50 Sephadex | 1.9 ± 0.6 (N=3) | 4.3 ± 1.4 (N=3) | 7.4 ± 2.9 (N=3) | 4.7± 0.6 (N=3) |
| Chloramine T | Placental membrane | 6.5 ± 1.2 (N=2) | 21.8 | 39.6 ± 3.4 (N=3) | 8.4 |
| | G-50 Sephadex | 1.6 ± 0.2 (N=3) | 7.8 ± 1.1 (N=3) | 16.5 ± 3.4 (N=2) | 3.4 ± 0.4 (N=3) |

* Mean ± S.E. (N)

Table III.2 Percent Specific Binding of [$^{125}$I]-IGF-I and [$^{125}$I]-IGF-II to Human Placental Cells

| Peptides | IGF-I | IGF-II |
|---|---|---|
| Placenta | | |
| Early gestation | 2.1 ± 0.5 (2)* | 4.7 ± 0.4 (4) |
| Term gestation | 2.3 ± 0.3 (3) | 5.5 ± 0.8 (4) |

* Mean ± S.E. (N).

# Chapter IV Characterization and Regulation of Epidermal Growth Factor Receptors in Human Placental Cell Cultures

## IV.1 Introduction

Epidermal growth factor (EGF) is a polypeptide of 6040 daltons isolated from mouse submaxillary glands (146) and human urine (140). EGF has a number of biological responses in target cells. These include the ability to act as a potent mitogen (230), to phosphorylate membrane proteins (120,231), and to increase RNA and DNA synthesis and cell multiplication in a wide variety of cell types (168,232).

EGF has been reported to stimulate human chorionic gonadotropin (hCG) secretion in a human choriocarcinoma cell line (233). Huot et al (234) tested EGF effects on hCG secretion from midterm placental organ cultures and did not find a significant effect. In this laboratory we have established short term monolayer cultures of human placentae as a model system that offers the advantage of obtaining a well differentiated population of cells. Since the effects of EGF on hCG and human placental lactogen (hPL) secretion in early and term human placental cell cultures have not been examined, we have studied these EGF effects in detail.

The human placenta is a relatively rich source of receptors for several polypeptide hormones which include insulin (71), EGF (235), and the family of insulin-like growth factors (IGFs): somatomedin A and C (Sm A and C) (207,226,236), insulin-like growth factors I and II (IGF I and II) (154,229), and multiplication

stimulating activity (MSA) (223). Placental receptors for insulin (218) and EGF (Lai WH, Guyda HJ, Bergeron J, manuscript in preparation) have been found to be located in the microvillous membrane of the syncytiotrophoblast where they may interact with hormones in the maternal circulation. In this study we have evaluated the presence and modulation by exogenous EGF of EGF receptors in intact whole placental cells.

## IV.2 Materials and Methods

### Cell Culture

Normal human placentae were obtained following caesarean section at term (N=18) or therapeutic abortion at midterm (N=10) between 7 1/2 to 18 weeks of gestation. The cell culture procedures were previously described (190,191). Briefly, 20 g wet weight of tissue were dispersed with 0.25% trypsin (hog pancreas, ICN Chemicals, Montreal, PQ) and harvested cell crops were plated into 60 x 15 mm or 100 x 20 mm tissue culture dishes. Cells were cultured at 37 C with 95% air and 5% $CO_2$ in Ham's F-10 (Microbiological Associates via Johns Scientific, Toronto, ONT), with heat inactivated 10% fetal bovine serum (FBS) (Grand Island Biologicals Co., Grand Island, N.Y.) and antibiotics: Penicillin G sodium (200 IU/ml, Glaxo Lab., Montreal, PQ); Gentamycin sulfate (40 ug/ml, Schering Inc., Montreal, PQ); Amphotericin B (5 ug/ml, Squibb and Sons, Montreal, PQ). Media were changed daily prior to use in receptor binding assays.

### Hormones

EGF and nerve growth factor were purchased from Collaborative Research, Inc. (Waltham, MA), with EGF stated to be 99% pure by SDS gel electrophoresis and mitogenic activity in a cell proliferation

assay using human skin fibroblasts. Porcine crystalline zinc insulin (24.4 units/mg) was from Connaught Laboratories, Ltd. (Toronto, ONT) and glucagon was from Eli Lilly Co. (Indianapolis, IN). Human growth hormone (GH HS2243E), prolactin (CU5), FSH (HS-1), and LH (1-1) were obtained from NIADDK, NIH. IGF-I and IGF-II were initially extracted from human plasma concentrates (Cohn Fraction IV-4) by acid-ethanol, and were then further purified by Sephadex acid chromatography and high pressure liquid chromatography (HPLC) at pH 2-2.5 (Posner BI, Guyda HJ, manuscript in preparation).

Iodination of EGF

Iodination of EGF with $Na^{125}I$ (New England Nuclear Corp., Boston, MA) was carried out by a modification of the chloramine-T method (122). This method yielded specific activities of 140 to 160 uCi/ug. The integrity of [$^{125}$I]-EGF was evaluated by trichloroacetic acid (TCA) precipitability and rebinding to human placental microsomes (163).

Standard Receptor Binding Assay

Unless otherwise specified, assays for [$^{125}$I]-EGF binding to placental cells were undertaken at 4 C in 12 x 75 mm polystyrene tubes (Simport, Ltd., Montreal, PQ). On day 3, cells were washed with 25 mM Tris, 10 mM $MgCl_2$, 0.12 M NaCl (pH 7.5) and were gently removed from culture dishes with a rubber policeman. Cell suspensions were pelleted at 4 C for 6 min at 70 x g and viability (over 85%) was assessed by trypan blue exclusion. Cell protein was measured by Lowry et al (192). The Hepes-BSA binding assay mixture consisted of 0.1 M Hepes buffer (0.1 M Hepes, 0.12 M NaCl, 5 mM

KCl, 1.2 mM $MgSO_4$, 8 mM glucose, pH 7.5) with 0.1% purified bovine serum albumin (BSA) (Sigma Chemicals Co., St. Louis, MO), 4 to 6 x $10^4$ cpm [$^{125}$I]-EGF, 100 ug cell protein, in the presence or absence of unlabeled EGF (2 ug/tube) in a final volume of 0.5 ml. Incubation was performed with shaking at 4 C. Assays were terminated after 18-20 h by the addition of 3.0 ml of cold Hepes washing buffer - 0.1% crude BSA (fraction V, ICN Chemicals, Montreal, PQ) and centrifugation at 2000 x g for 30 min at 4 C. Specific binding is expressed as the difference between radioactivity bound in the absence (total binding) or presence (nonspecific binding) of excess unlabeled EGF. Nonspecific binding accounted for 2-7% of total binding.

Dissociation of Binding

[$^{125}$I]-EGF (5 x $10^6$ cpm) was incubated for 18 h at 4 C with 10 mg cell protein in a final volume of 40 ml of Hepes-BSA assay buffer. The cells were recovered by centrifugation for 6 min at 70 x g and washed with cold Hepes-BSA assay buffer. The final pellet was resuspended in 40 ml Hepes-BSA assay buffer and divided into two 20 ml volumes. To one portion EGF was added to give a final concentration of 1 ug/ml. Aliquots of each suspension (0.25 ml) were dispensed into assay tubes containing 3.75 ml of Hepes-BSA assay buffer and kept shaking at 4 C. Triplicate tubes were removed at each time interval from 0 to 20 h, immediately centrifuged for 30 min at 2000 x g and rapidly separated to obtain radioactivity bound to cells.

Degradation Studies

Cells were treated the same as for the standard radioreceptor assay procedure, except at the end of incubation (20 h), bound and

free tracer were separated by centrifugation for 30 min at 2000 x g without dilution. After decanting and saving the supernatant, the cell pellets were resuspended in 0.1 N HCl - 0.1% purified BSA, incubated at 4 C with constant shaking for 90 min and pelleted for 30 min at 2000 x g. The resulting eluate was neutralized with 1 N NaOH and with the decanted supernatant was assessed for TCA precipitability and rebinding to placental membranes (100 ug cell protein) for 2 h at 4 C. Control unexposed tracer was processed in the same manner.

Pre-incubation of Cells with EGF

a).Measurement of hormones: Confluent placental cell cultures were maintained in 60 x 15 mm culture dishes containing Ham's F-10 medium with 10% FBS at 37 C. 24 hours after plating fresh medium with or without various concentration of EGF was added to each monolayer in a final volume of 3 ml. The medium was collected daily for 6 days. The concentrations of immunoreactive hCG and hPL were determined by double antibody RIAs with reagents provided by NIADDK. All cultures were maintained in triplicate or quadruplicate.

b).Receptor regulation: Confluent term placental monolayers were plated in 100 x 20 mm cultured dishes with Ham's F-10 supplemented with 10% FBS. Two days after daily feeding, medium was removed, and unlabeled EGF in various concentrations was added with fresh medium to each monolayer in a final volume of 5 ml. After incubation at 37 C for various periods of time, the medium was aspirated and the monolayers were washed three times with phosphate buffer-saline (PBS) (pH = 7.5) at room temperature, with 10 min incubation periods between each wash, followed by three more

rapid washes. Subsequently, cells were processed for the standard radioreceptor assay (RRA) procedure, as described above.

Analysis of Data

All experiments were performed using at least two different placental culture preparations. All data presented are the mean of triplicate determinations in one representative experiment unless otherwise stated. Statistical analyses were performed with Student's t-test. Scatchard analyses were based on the EGF displacement data using a Hewlett-Packard computer system for a two-site model, as previously described (135,198).

## IV.3 Results

### Effects of EGF on hCG and hPL Release from Midterm and Term Placental Monolayers

Placental cells were exposed to various concentrations of EGF for 24 h in Ham's F-10 medium with 10% FBS. The presence of EGF (200 ng/ml) for 24 h increased hCG release in midterm (Fig.IV.1A) and term placentae (Fig.IV.2A). In both, maximal stimulation was reached after 24 h of EGF treatment. The effect of EGF (200 ng/ml) on hPL release was less than that observed for hCG, but a significant increase relative to the levels found in control cultures occurred in both midterm and term placentae (Fig.IV.1B and 2B): maximal stimulation was observed after 24 h of EGF treatment in midterm and 72 h in term placentae. A lower EGF concentration (100 ng/ml) had a significant effect on hCG release on day 2 only and showed no effect on hPL release in term placental cultures (Fig. IV.2A and 2B). Concentrations of EGF higher than 200 ng/ml did not further increase the hCG or hPL release in term placentae (data not shown).

Kinetic and Specificity Studies

At 4 C, specific binding of [$^{125}$I]-EGF to term placental cells plateaued at 50% by 8 h (Fig. IV.3A) and persisted for another 10 h. Specific binding was similar in the presence or absence of bacitracin (500 ug/tube). Binding of labeled EGF was reversible; the presence of excess unlabeled EGF accelerated the rate of dissociation at 4 C (Fig. IV.3B)

Figure IV.4 depicts the time course of specific binding of [$^{125}$I]-EGF to term placental cells at 22 C and 37 C. The rate of binding was more rapid at 22 C and 37 C when compared to 4 C (Fig. IV.3A). At 22 C and 37 C, binding plateaued by 45 min and persisted at that level for at least another hour. At 37 C, EGF binding at steady state was lower than that at 22 C. Half maximal displacement was observed with a final EGF concentration of 10 ng/ml at 37 C in term, and at 8 ng/ml at 4 C in midterm and term placental cells.

There was a proportional relationship between the extent of binding and the cell protein concentration in the incubation medium (Fig. IV.5). Specific binding of [$^{125}$I]-EGF increased over a 3-fold range from 25 to 300 ug cell protein per tube, and plateaued at 300 ug. Nonspecific binding remained low (less than 3%) at high and low cell protein concentrations. In addition, binding of EGF was proportional to labeled ligand concentrations (Fig. IV.6). Optimal pH of [$^{125}$I]-EGF binding to its receptor occurred at pH 7.5; below pH 7.5 binding decreased sharply (Fig. IV.7).

Binding of [$^{125}$I]-EGF to term placental cells was specific. Unrelated hormones such as glucagon, prolactin, human growth

hormone, nerve growth factor, insulin, and IGF-I and IGF-II showed negligible competitive inhibition (Table IV.1).

Degradation

Placental cell cultures are extremely rich in degrading activity for insulin, IGF-I and IGF-II, but not for EGF (229). In the presence or absence of bacitracin at 4 C, specific binding was similar (Fig. IV.3A). Labeled EGF exposed to placental cells at 4 C was physically intact as assessed by TCA precipitation or rebinding to placental membranes (Table IV.2). In the supernatant medium, without bacitracin, [$^{125}$I]-EGF was degraded approximately 5 to 12% more than cell eluted [$^{125}$I]-EGF. It thus appears that proteolytic inhibitors such as bacitracin are not essential to control degradation of added ligand in this assay system and thus have not been utilized routinely.

Ontogeny

Early gestation placentae may serve as a useful model for studying early events involved in embryonic differentiation and fetal development. To study the possible ontogenic role of the EGF cell surface receptor in mediating effects, we studied EGF receptor interaction in different gestational placentae. Representative displacement curves for labeled EGF from 15 wk and term placental cell receptors, as well as mean percent specific binding of [$^{125}$I]-EGF in early and term placentae, are seen in Fig. IV.8 and Table IV.3. Specific binding of [$^{125}$I]-EGF increased two-fold as the gestational age increased. Incubations were undertaken at 4 C for 20 h, when degradation was minimal and binding was at a steady state. Scatchard plots of [$^{125}$I]-EGF binding performed on early gestation and term placental cell displacement curves were

curvilinear. This has been interpreted as receptor heterogeneity with respect to affinity for hormones. Based on a two-site model, displacement curves were resolved into two populations of receptors. EGF receptors were significantly increased in term compared to early gestation, and this is due primarily to an increase in receptor number with no significant change in affinity (Table IV.3).

Receptor Regulation

Recently it was demonstrated in mouse calvaria (237) and 3T3 cells (230) that the concentration of EGF receptors per cell was inversely related to the ambient EGF concentration to which the cells were exposed. This process of hormone receptor loss has been termed "down-regulation". In studies of ligand-induced loss of receptors, one of the major concerns is to distinguish residual occupancy by the ligand, that is, decreased binding due to exogenous EGF, from true receptor loss. Thus, prior to actual down-regulation experiments, the efficacy of a series of washing procedures used to remove unlabeled EGF was tested as described under Methods. After the third wash step, EGF was undetectable by RRA (Table IV.4). Thus, residual occupancy by EGF from the initial incubation was eliminated.

The loss of EGF receptors in human term placental cells was dependent on the EGF concentration over the range of 20 to 2000 ng/ml ($2.3 \times 10^{-9}$ to $10^{-7}$M). The maximum loss of receptors occurred at an EGF concentration of 200 to 2000 ng/ml, where binding was reduced by 80% (Fig. IV.9). There was a plateau beyond which receptor number did not decrease. The effectiveness of EGF

in producing receptor loss was a specific effect on the EGF receptor since pre-incubation of placental cells with insulin (1 x $10^{-6}$M) did not alter the specific binding of EGF (Fig. IV.9 inset). In addition, pre-incubation of placental cells with EGF (200 ng/ml) did not change the concentration of insulin receptors (Table IV.5). From equilibrium binding parameters, EGF-induced loss of receptors was due to a decreased number of both high and low affinity receptors per mg cell protein, with no significant change in apparent affinity (Fig. IV.10).

Figure IV.11 shows the time course of EGF-induced down-regulation and receptor recovery. The maximum decrease of EGF-induced down-regulation (80%) occurred at 16 h after the addition of 200 ng/ml EGF. In the presence of only cycloheximide (10 ug/ml), there was no effect on EGF binding during 16 h pre-incubation with cells at 37 C. However, when cells were incubated simultaneously with EGF (200 ng/ml) and cycloheximide (10 ug/ml), cycloheximide did not block the effect of EGF-induced down-regulation at 4 h but did so at 16 h. At 16 h, monolayer cells were washed and then incubated an additional 16 h in fresh EGF-free Ham's F-10 medium with 10% FBS in the presence or absence of cycloheximide (10 ug/ml). Down-regulation of EGF receptors was reversible; recovery of EGF receptors continued up to 32 h (16 h incubation in EGF-free medium) reaching 50% of control value. Later time periods were not assessed to determine when 100% recovery was achieved. In the presence of cycloheximide EGF binding was markedly decreased to 10% of control at 32 h. These results indicate that ligand-induced receptor loss and receptor recovery in this cell system are dependent on new protein

synthesis.

## IV.4 Discussion

Monolayer cultures of human placental cells have lost the ability to divide; this may be a consequence of terminal differentiation or of inadequate culture environment. However, within the study time period (5-7 days), most cells were viable and secreted biochemical markers such as hCG, hPL and steroid hormones (238).

hCG and hPL are synthesized in the human placenta from the earliest stages, with peak levels at the end of the first trimester for hCG and at term for hPL (59). In our studies human midterm and term placental cells responded to EGF with significant increase in hCG and in hPL release. These responses occurred at an EGF concentration (30 ng/ml per 100 ug cell protein) that was similar to that for half-maximal inhibition of binding, suggesting a link between receptor binding and biological activity. These findings are different from those of Huot et al (234), who studied human midterm placental organ cultures. The difference in culture conditions employed by these authors may have contributed to the poor response of hCG to EGF that they reported.

Fibroblast contamination of our cultures was less than 5%. While the limitations in this experimental system due to the use of trypsinization and the possibility of multiple origin of cell types have to be considered, steps were taken to minimize these difficulties: 1) to avoid possible effects of trypsinization during culture preparation on the structure of EGF receptors, a minimum equilibration period of 36 h before harvesting the cells for

binding assays was employed, and 2) the extensive washing procedure and tissue mincing made it unlikely that maternal decidual cells contributed significantly to the cultured cell population. The increase in hCG levels was not due to cell replication or change of total protein content (appendix 1). This is in agreement with the studies of Boyd et al (14) who observed that the syncytial nuclei at the end of their functional life did not show any mitotic activity even though syncytial villi were identified as an important organ of protein synthesis.

We have used protein concentration as the binding assay unit rather than cell number due to the heterogeneous cell sizes obtained from syncytiotrophoblast dispersion. From different placental preparations, EGF specific binding was very similar and reproducible (interassay coefficient of variation = 7.7%, N=10). In addition, cells remained intact after the 20 h binding assay as judged by trypan blue exclusion (over 85%), as well as by morphologic evaluation with electron microscopy: cell cultures showed well maintained extensive microvillar projections, intact nuclear envelopment, rER, Golgi apparatus and mitochondria (W. Lai, unpublished observations). Thus, these cells were well suited for the evaluation of receptor binding, receptor regulation and metabolic action of peptide hormones.

[$^{125}$I]-EGF bound rapidly and reversibly to term placental cells; maximal binding occurred at 4 C and exhibited a high degree of specificity, with the same pH optimum as for insulin and IGF (229). The non-linearity of the Scatchard analysis is in agreement with the findings of Osborne et al (239) and suggests that EGF binding to cultured placental cells does not involve a single class

of receptors. Analysis of term cell data with a two-site model resolved EGF receptors into high and low affinity binding sites with binding characteristics similar to those of EGF receptors we described in placental membrane preparations (163).

The present study shows a two-fold increase in specific binding of labeled EGF in term gestation compared to early gestation, primarily due to a 30-fold increase in receptor number with little change in apparent affinity. From a developmental point of view there is a progressive diminution of the cytotrophoblast cells throughout gestation and at term the syncytial layer is the dominant trophoblastic component. In midterm placental cells there is the possibility of contamination with cytotrophoblast or nonparenchymal derived cell types. However no data are available as to the binding ability of these cell types. Our radioautographic studies demonstrate that [$^{125}$I]-EGF binding sites are mainly localized in the syncytial layer (W. Lai, H. Guyda. J. Bergeron, manuscript in preparation). Despite the possible greater heterogeneity in midterm cells compared to term placental cells, Scatchard analyses suggest that the EGF receptors were of a very similar nature with respect to their affinity characteristics in early gestation versus term placentae. The greater EGF effect observed on hCG and hPL secretion in term placentae was not due to a higher affinity for EGF, but was due to a greater number of receptors at term. However a direct concordance between receptor number and biologic response was not observed for the two gestational time periods studied. The presence of EGF receptors and the ontogenic changes observed suggest that this

growth factor may be involved in the regulation of growth of the feto-placental unit. Evidence to support these speculations will require studies correlating EGF membrane interactions with intracellular biological processes in the early and term placentae. D'Ercole (240) has recently reviewed evidence in support of EGF having a stimulatory function in fetal growth and development.

Exposure of confluent cultured placental cells to various concentrations of EGF at 37 C led to 80% reduction in the binding capacity for [$^{125}$I]-EGF. This finding is similar to the results obtained with 3T3 cells (230). Our studies demonstrate that hormone-induced receptor loss can readily be detected in monolayer cell cultures and this phenomenon is of high specificity; insulin induced down-regulation of the insulin receptor in the same cells without affecting EGF receptors. When the magnitude of receptor loss was quantified as a function of time and hormone concentration, it was found that after exposure to EGF, maximal receptor loss reached a plateau after 16 h at a new cell surface receptor concentration. After initial cell surface binding, EGF is internalized, and it has been suggested that this internalization pathway may be related to the process of receptor regulation (210,213).

In the presence of cycloheximide, receptor loss and receptor recovery were inhibited. Cycloheximide has previously been shown to lead to significant inhibition of protein synthesis (212). With cycloheximide, we observed a significant inhibition of EGF-induced receptor loss in human placental cells during 8 - 16 h, while there was no effect at 4 or 8 h. It appears that cycloheximide promotes recovery of EGF binding from 8 to 16 h whereas it blocks recovery

upon removal of EGF. Our results are similar to Prince et al (213) and Kosmakos et al (210) obtained in studies on insulin down-regulation in human fibroblasts and IM-9 lymphocytes respectively, but different to the results of Aharonov et al (230) on EGF down-regulation studies in 3T3 cells, where cycloheximide had no effect. The reasons for the differences in these results are unclear but may relate to methodologic and cell type differences. EGF receptor loss proceeded at a relatively rapid rate (t1/2 = 2 h at an EGF concentration of 200 ng/ml). On the contrary, receptor recovery proceeded in a linear fashion over a slower period. The difference in the rate of these two processes suggests that receptor loss and receptor recovery involve different cellular processes; receptor recovery does not simply imply the absence of ongoing receptor loss (213).

The human placenta has been recognized as playing a selective and active role in providing nutrients essential for fetal growth and development (241). Although the physiological role of EGF has not been clarified in this study, the presence of specific, high affinity EGF receptors in human placental cells that increase during gestation, and the EGF effects on hCG and hPL secretion observed, suggest a significant role for EGF in fetal development.

## IV.5 Summary

We have confirmed that cultured human placental cells rapidly release human chorionic gonadotropin (hCG). Pre-incubation with epidermal growth factor (EGF) for 24 h significantly increased the amount of hCG release and also increased human placental lactogen (hPL) release by these cells. To better understand the mechanisms of action of EGF on the feto-placental unit, we studied EGF receptor binding and regulation by examining the characteristics and specificity of EGF receptors in human placental syncytiotrophoblast cultures. Maximal [$^{125}$I]-EGF binding occurred at pH 7.5 and 4 C and exhibited a high degree of specificity. In the presence or absence of bacitracin at 4 C, specific binding was similar and labeled EGF was physically intact as assessed by TCA precipitation or rebinding to human placental membranes. The percent specific binding was proportional to cell and ligand concentrations and was significantly increased in term (52.9$\pm$1.2%; N=11) compared to early gestation placental cells (22.7$\pm$3.4%; N=7) ($P < 0.001$). Both term and midterm EGF displacement curves generated curvilinear Scatchard plots suggesting receptor heterogeneity.

Pre-treatment of cells with EGF resulted in a dose-, time-dependent decrease in specific binding which was maximal (80%) at 200 ng/ml EGF. This loss of binding was due to decrease in the number of both high and low affinity receptor sites with no significant change in the apparent affinity. The induction of EGF receptor loss by EGF was a specific effect on the EGF receptor. Pre-incubation of these same cells with insulin caused a decrease in the number of insulin receptors, while EGF receptors remained

unaltered. Conversely, pre-incubation with EGF, in a dose which down-regulated EGF receptors, did not alter insulin receptor number or affinity. Down-regulation of EGF receptors was reversible, with 50% recovery by 16 h. However, cycloheximide (10 ug/ml) blocked EGF-induced down-regulation and receptor recovery.

The presence of EGF receptors in human placental cells and the ontogenic changes found suggest that EGF may be involved in the regulation of fetal growth and development. These studies indicate the feasibility of using human placental cells in culture as a model system to probe hormonal-cell interaction in the feto-placental unit.

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Lai WH, Guyda HJ, 1984 J Clin Endocrinol Metab 58:344-352.

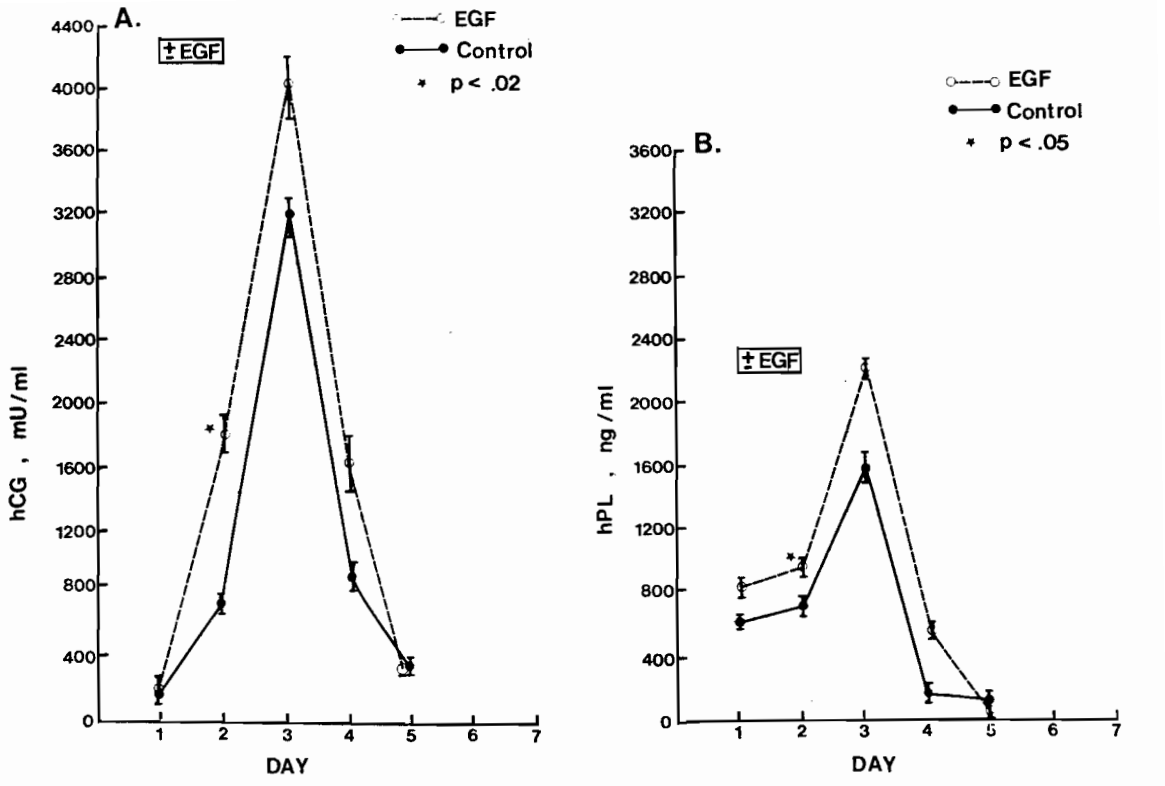
## IV.6 Figures and Tables

Fig. IV.1A Stimulation of hCG secretion by EGF (200 ng/ml) in 17 1/2 wk midterm placental cells. Results indicate the mean ± S.E. per mg cell protein per 24 h of quadruplicate determinations. (1A, 1B).

1B Stimulation of hPL secretion by EGF (200 ng/ml) in 17 1/2 wk midterm placental cells.

Fig. IV.2A Stimulation of hCG secretion by EGF in term placental cell cultures. Data shown are the mean ± S.E. per mg cell protein per 24 h of triplicate determinations (2A, 2B)

2B Stimulation of hPL secretion by EGF in term placental cell cultures.


A.
± EGF
EGF
Control
* p < .02
hCG , mU/ml
DAY
B.
EGF
Control
* p < .05
hPL , ng/ml
DAY

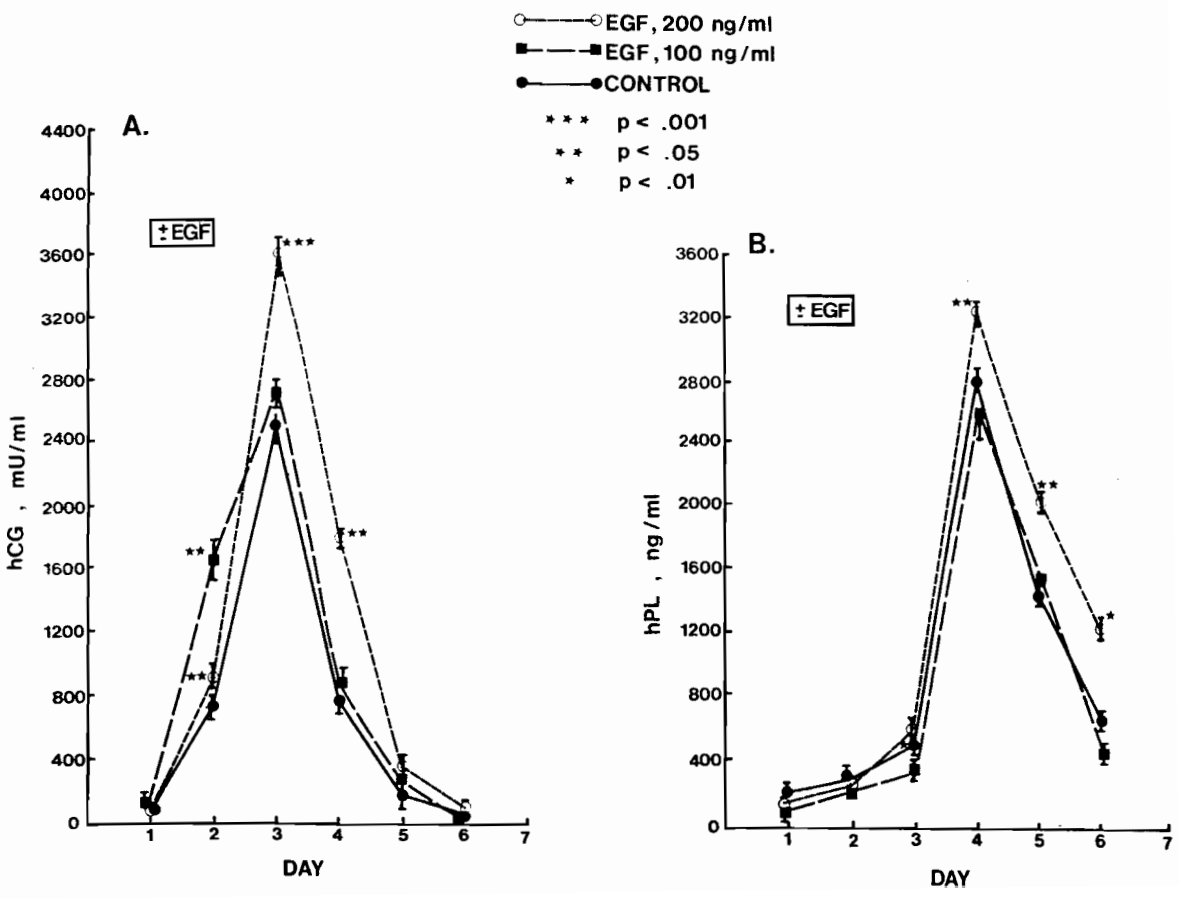

EGF, 200 ng/ml
EGF, 100 ng/ml
CONTROL
*** p < .001
** p < .05
* p < .01
A.
± EGF
hCG , mU/ml
DAY
B.
± EGF
hPL , ng/ml
DAY

Fig. IV.3A Time course of specific binding of [$^{125}$I]-EGF to human term placental cells at 4 C in the presence and absence of bacitracin (500 ug/tube).

Fig. IV.3B Dissociation of [$^{125}$I]-EGF at 4 C in the presence (1 ug/tube) and absence of unlabeled EGF. The percent specific binding at zero time was 53.8%. Bound [$^{125}$I]-EGF at a given time is expressed as a percentage of zero time value.

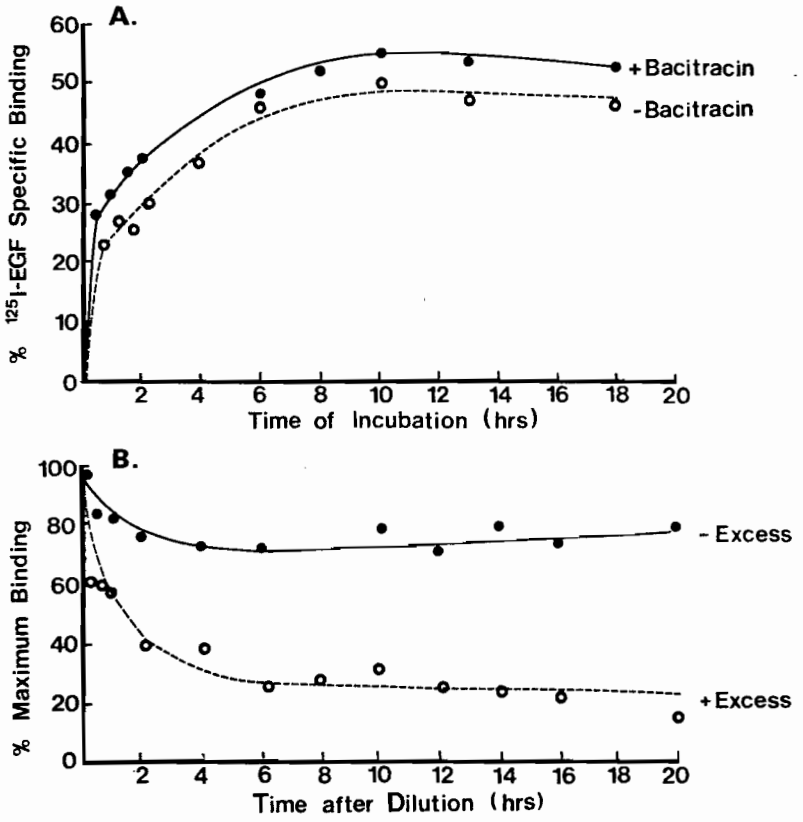

A.
% $^{125}$I-EGF Specific Binding
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
Time of Incubation (hrs)
+Bacitracin
-Bacitracin
B.
% Maximum Binding
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
Time after Dilution (hrs)
- Excess
+Excess

Fig. IV.4 Influence of incubation temperature on the time course of specific binding of [$^{125}$I]-EGF to human term placental cells. Samples were incubated at 22 C and 37 C under standard binding conditions with 100 ug/tube of cell protein.

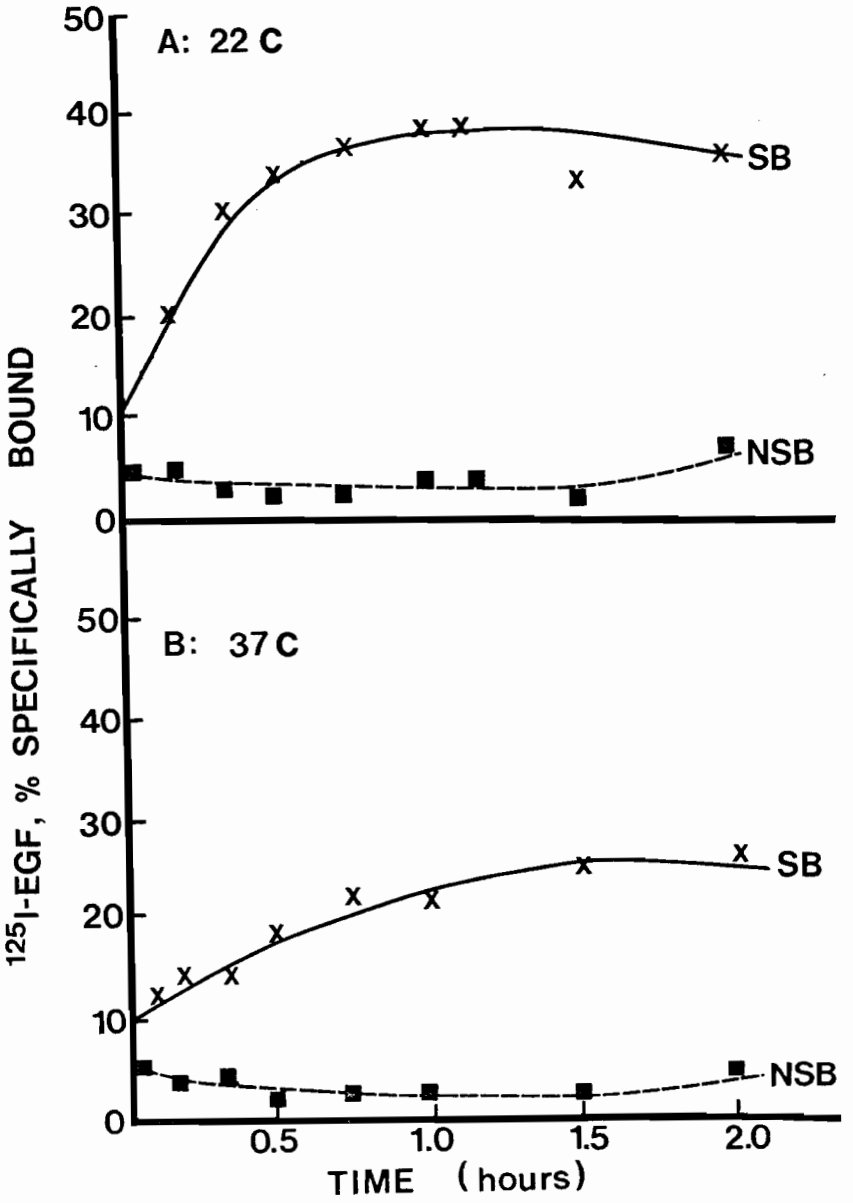
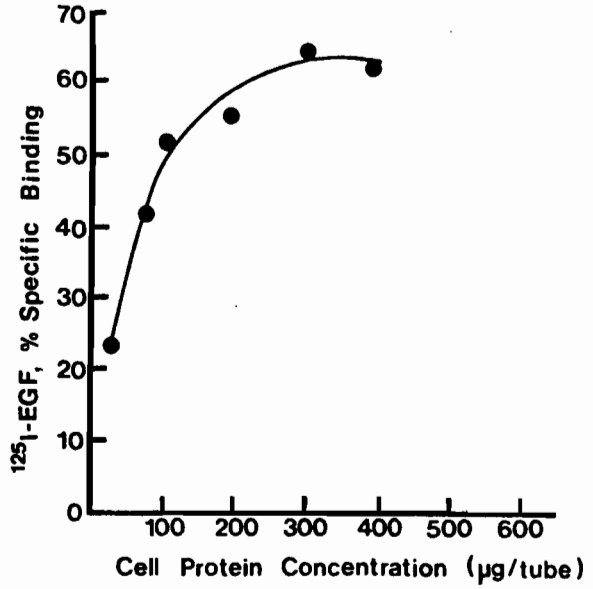

A: 22 C
B: 37 C
SB
NSB
SB
NSB
50
40
30
20
10
0
50
40
30
20
10
0
0.5
1.0
1.5
2.0
TIME (hours)
125I-EGF, % SPECIFICALLY BOUND

Fig. IV.5 Effect of term placental cell protein concentration. [$^{125}$I]-EGF specifically bound at each receptor concentration was calculated by subtracting the radioactivity not displaceable by 2 ug of unlabeled EGF.

Fig. IV.6 Effect of ligand concentration on [$^{125}$I]-EGF binding to term placental cells. Cells (100 ug cell protein) were incubated in Hepes assay buffer pH 7.5 at 4 C for 20 h.


70
60
50
40
30
20
10
0
$^{125}$I-EGF, % Specific Binding
100
200
300
400
500
600
Cell Protein Concentration (µg/tube)

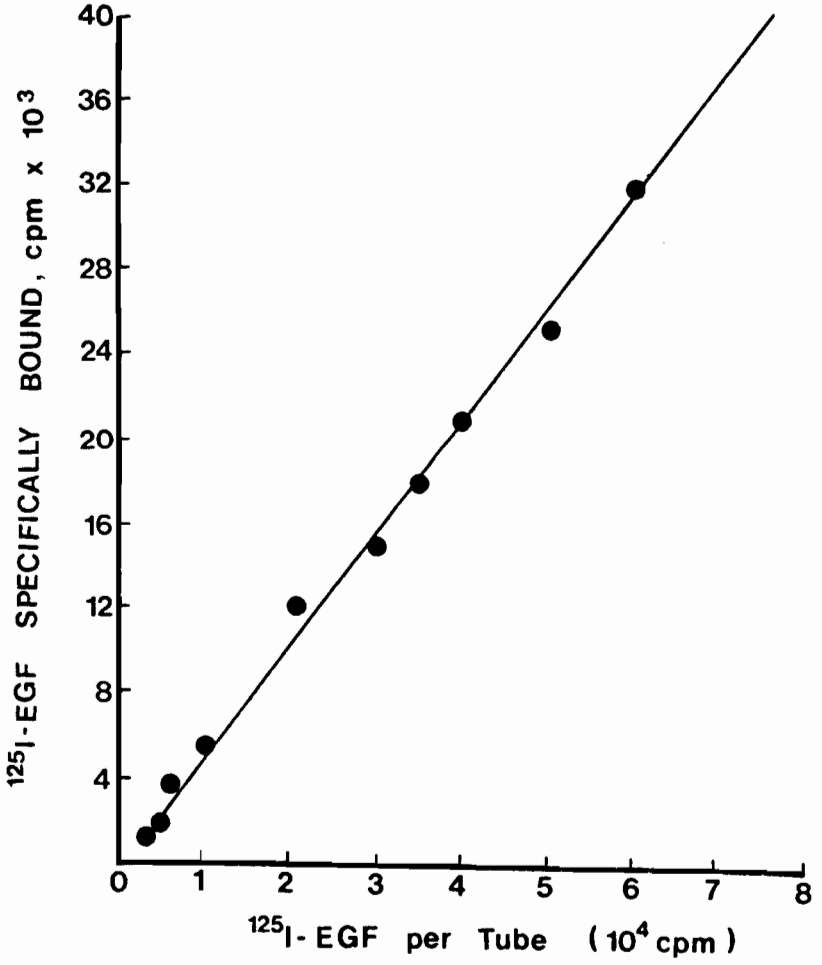

40
36
32
28
24
20
16
12
8
4
0
$^{125}$I-EGF SPECIFICALLY BOUND, cpm x $10^3$
1
2
3
4
5
6
7
8
$^{125}$I-EGF per Tube ($10^4$ cpm)

Fig. IV.7 pH dependence of [$^{125}$I]-EGF binding to term monolayer cells. The pH of the buffer was adjusted to values from 6.0 to 9.0 as indicated in the figure. At each pH, the percent [$^{125}$I]-EGF specifically bound was determined.

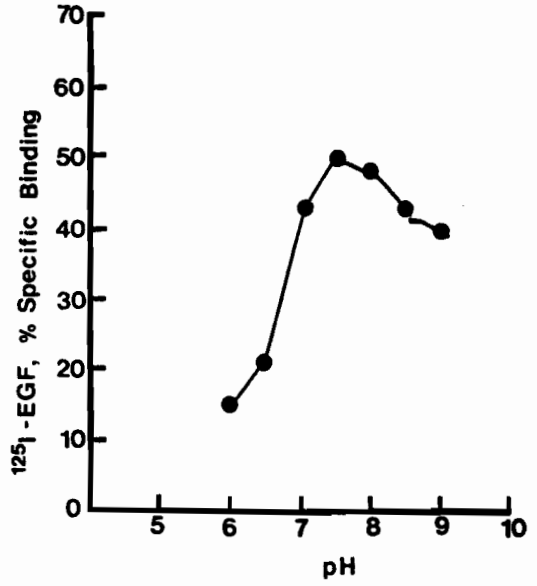
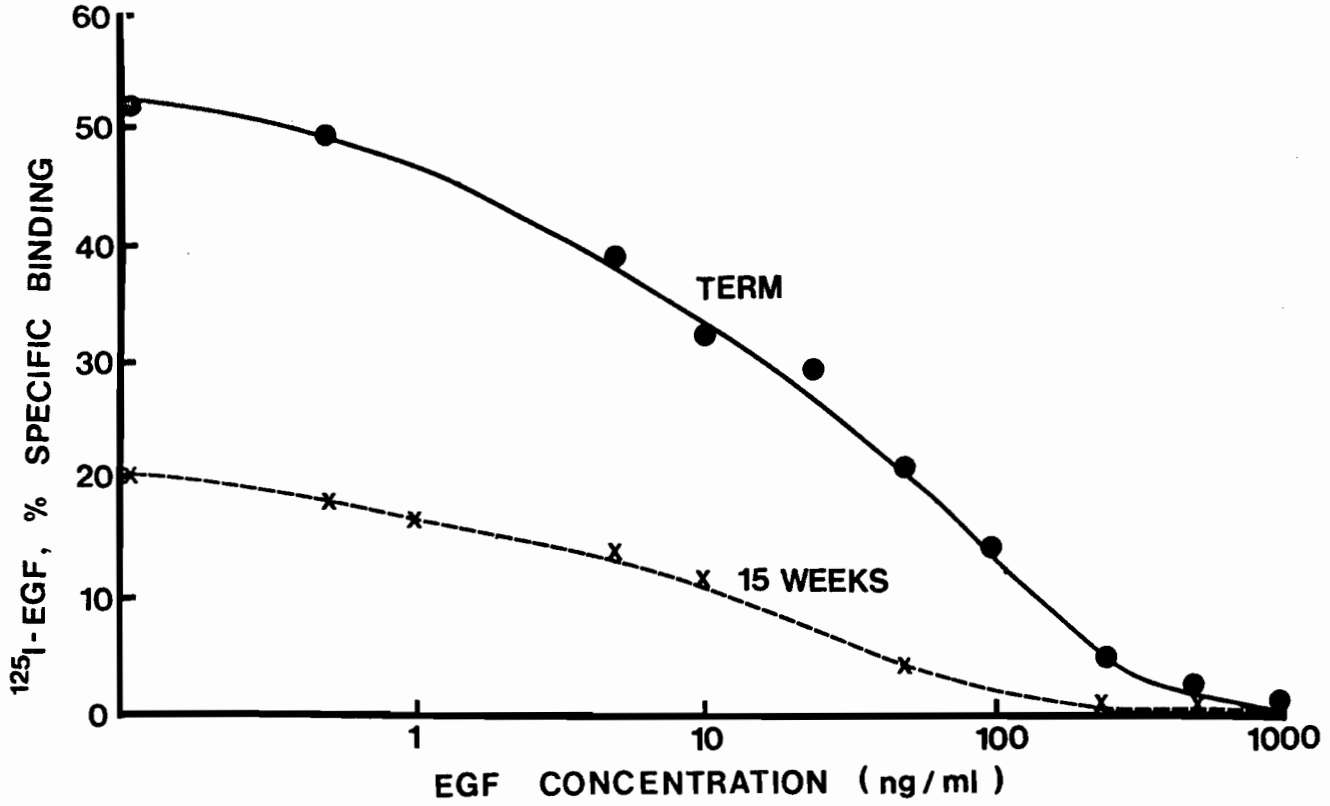

70
60
50
40
30
20
10
0
$^{125}$I-EGF, % Specific Binding
5
6
7
8
9
10
pH

Fig. IV.8 Representative displacement curves of [$^{125}$I]-EGF binding at 4 C for 20 h with 15 wk and 39 wk term placental cells. A final concentration range of 0-200 ng/ml was tested in the assay since 0.1 ml aliquots of the EGF were added to each assay tube in which the final volume was 0.5 ml.

Fig. IV.9 Exposure of confluent term placental culture cells at 37 C for 16 h with 20-2000 ng/ml EGF in 100 x 20 mm culture dish in 5 ml Ham's F-10 with 10% FBS. The maximum loss of receptors occurred at an EGF concentration of 200 to 2000 ng/ml, where binding was reduced by 80%.

Inset Pre-incubation of term monolayer cells with 200 ng/ml EGF leads to a 75-80% reduction in the binding capacity for [$^{125}$I]-EGF. Under the same conditions, pre-incubation of cells with insulin does not alter the specific binding of EGF.


60
50
40
30
20
10
0
$^{125}$I-EGF, % SPECIFIC BINDING
TERM
15 WEEKS
1
10
100
1000
EGF CONCENTRATION (ng/ml)

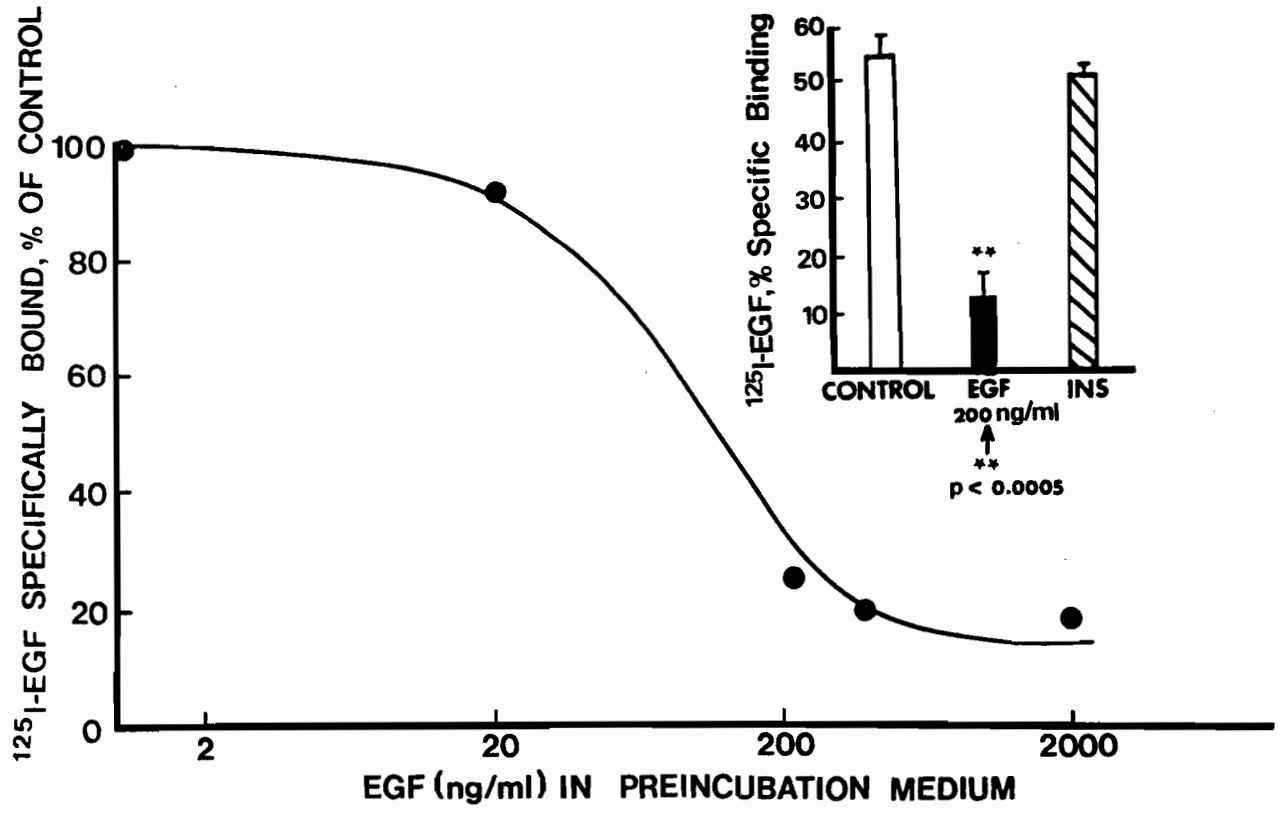
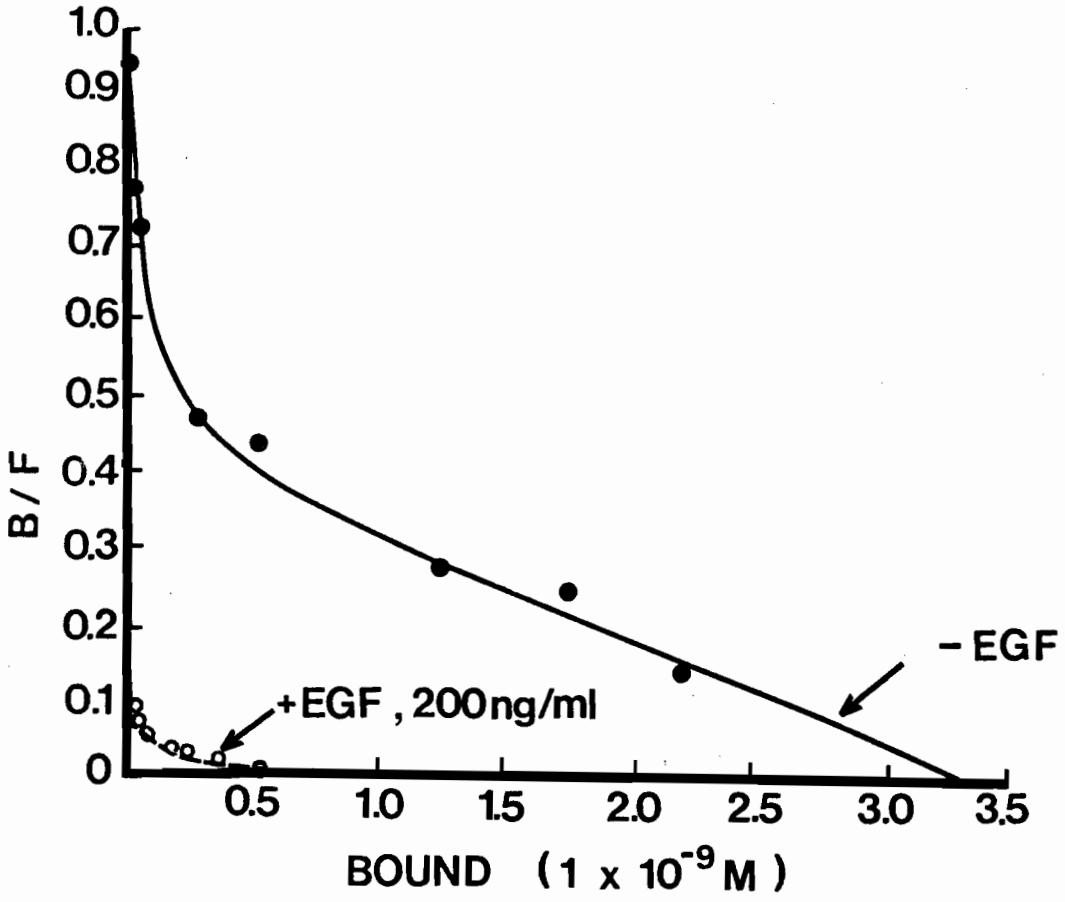

100
80
60
40
20
0
$^{125}$I-EGF SPECIFICALLY BOUND, % OF CONTROL
2
20
200
2000
EGF (ng/ml) IN PREINCUBATION MEDIUM
60
50
40
30
20
10
$^{125}$I-EGF, % Specific Binding
CONTROL
EGF
200 ng/ml
INS
**
**
p < 0.0005

Fig. IV.10 Scatchard plots of [$^{125}$I]-EGF binding at 4 C following pre-incubation of human placental cells in the presence or absence of 200 ng/ml EGF at 37 C for 18 h. Binding capacity is defined by the X-intercept. The calculated affinity for pooled data on cells incubated in the absence of EGF (K1 = 2.8 $\pm$ 1.7 X $10^9$ $M^{-1}$; K2 = 13.3 $\pm$ 1.5 X $10^7$ $M^{-1}$, N = 5) or in the presence of EGF (K1 = 1.5 $\pm$ 0.3 X $10^9$ $M^{-1}$; K2 = 14.4 $\pm$ 4.0 X $10^7$ $M^{-1}$. N = 5) were not different.

Fig. IV.11 Receptor down-regulation and recovery following pre-incubation with EGF in the presence and absence of cycloheximide is illustrated. In the recovery phase of the experiment, human placental cells which had been pre-incubated with only EGF (200 ng/ml) for 16 h at 37 C were washed with Ham's F-10 medium without FBS six times and then incubated for an additional 16 h in fresh Ham's F-10 medium with 10% FBS in the presence or absence of cycloheximide (10 ug/ml) (arrow). At indicated intervals, cells were washed with PBS (pH = 7.5) and the level of [$^{125}$I]-EGF binding was determined. Cell viability and cell protein were measured at each time point.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
B / F
- EGF
+EGF , 200ng/ml
0.5
1.0
1.5
2.0
2.5
3.0
3.5
BOUND (1 x 10⁻⁹ M)

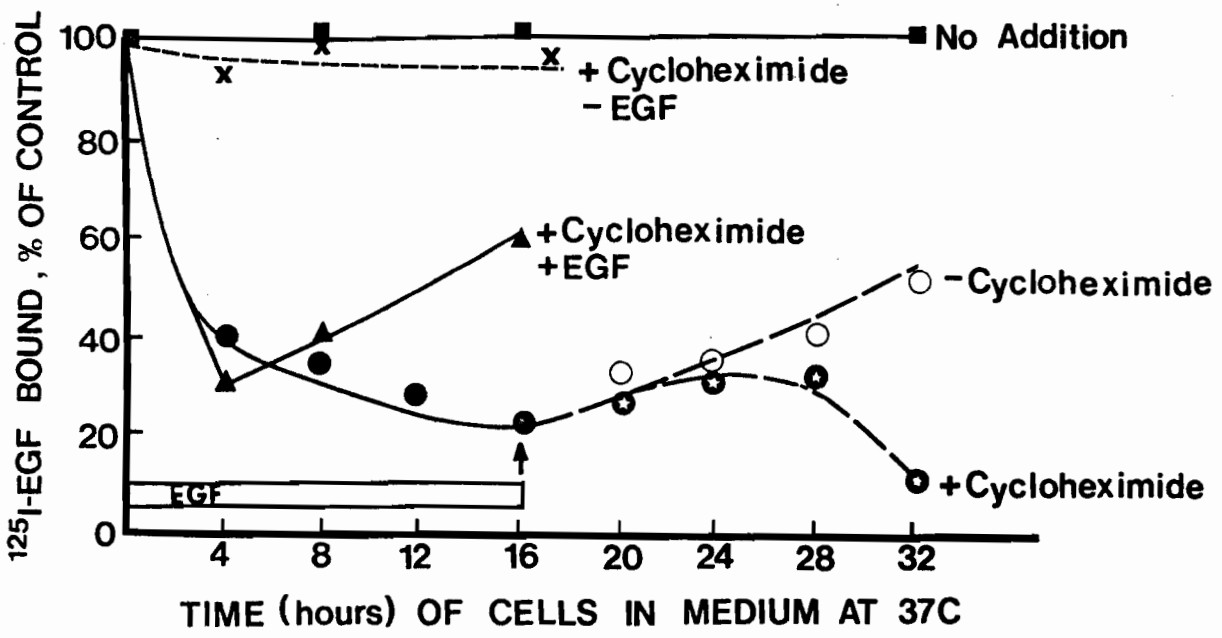

100
80
60
40
20
0
125I-EGF BOUND, % OF CONTROL
No Addition
+ Cycloheximide
- EGF
+Cycloheximide
+EGF
-Cycloheximide
+Cycloheximide
EGF
4
8
12
16
20
24
28
32
TIME (hours) OF CELLS IN MEDIUM AT 37C

Table IV.1 Effect of Peptide Hormones on the Binding of [$^{125}$I]-EGF of Human Term Placental Cells

| Addition | Concentration | %[$^{125}$I]-EGF Bound |
|---|---|---|
| no addition | | 100.0 |
| glucagon | 10 ug/ml | 99.0 ± 0.9* |
| prolactin | 10 ug/ml | 100.0 ± 1.1 |
| hGH | 10 ug/ml | 98.0 ± 1.6 |
| NGF | 2 ug/ml | 99.2 ± 0.6 |
| hCG | 1000 mU/ml | 97.6 ± 1.8 |
| insulin | 50 ug/ml | 100.1 ± 1.0 |
| IGF-I | 500 ng/ml | 98.2 ± 1.3 |
| IGF-II | 500 ng/ml | 99.3 ± 0.7 |

* Mean ± S.E. triplicate determinations of two separate placental cultures (N = 2)

Table IV.2 Degradation of [$^{125}$I]-EGF in Human Term Placental Cells at 4C

| | % Total TCA precipitation | % SB to membrane | % Control |
|---|---|---|---|
| Supernatant Radioactivity | | | |
| TCA Precipitability | | | |
| (1) tracer | 94.0 ± 1.0 | | 100.0 |
| (2) + cells | 89.0 ± 0.4 | | 94.7 |
| (3) + cells + unlabeled EGF | 92.3 ± 0.3 | | 98.2 |
| Rebinding to membranes | | | |
| (1) tracer | | 26.4 ± 1.5 | 100.0 |
| (2) + cells | | 23.1 ± 0.4 | 87.5 |
| Radioactivity Eluted from Pellet | | | |
| TCA Precipitability | | | |
| (1) tracer | 94.8 ± 0.3 | | 100.0 |
| (2) + cells | 94.8 ± 0.5 | | 100.0 |
| (3) + cells + unlabeled EGF | 94.8 ± 0.6 | | 100.0 |
| Rebinding to membranes | | | |
| (1) tracer | | 26.4 ± 0.2 | 100.0 |
| (2) + cells | | 26.0 ± 0.8 | 98.5 |

The percentage of intact tracer was determined for supernatant and cell-eluted [$^{125}$I]-EGF by 10% TCA precipitation and percent specific binding (% SB) was determined with 100 ug human term placental membrane protein as described under "Methods". Control refers to tracer not pre-incubated with cells. Values for TCA precipitable labeled EGF and % SB are mean ± for at least triplicate determinations of two placental cultured cell preparations.

Table IV.3 % Specific Binding (SB) and Binding Parameters of [$^{125}$I]-EGF with Human Placental Cells

| Placenta | SB (%) | $K_1$ ($9^{-1}$) ($1 \times 10^{M}$) | $K_2$ ($7^{-1}$) ($1 \times 10^{M}$) | N (fM/mg) |
|---|---|---|---|---|
| Midterm | 22.7 ± 3.4 (7)* | 1.5 - 2.3 | 13.1 - 16.8 | 300 - 460 |
| Term | 52.9 ± 1.2 (11)** | 1.20 - 9.50 | 14.8 - 16.0 | 9,000 - 12,000 |

* M ± SE (N); ** $P < 0.001$

Table IV.4 Efficacy of the Wash Procedure in Removing Exogenously Added EGF from Term Placental Monolayers

| Wash Procedure Step | EGF Added (ng/ml) | | | |
|---|---|---|---|---|
| | 0 | 200 | 400 | 2,000 |
| 1 | 1.2 ± 0.3 | 175.0 ± 1.3 | 380.2 ± 4.2 | 1,989.1 ± 3.2 |
| 2 | ND ** | 8.5 ± 1.2 | 20.0 ± 0.9 | 35.6 ± 0.1 |
| 3 | ND | ND | ND | ND |
| 4 | ND | ND | ND | ND |
| 5 | ND | ND | ND | ND |
| 6 | ND | ND | ND | ND |

* Mean ± S.E. of EGF concentrations (ng/ml)

** ND: not detectable

Table IV.5 Specificity of Down-Regulation of Insulin and EGF Binding to Human Term Placental cells

| Addition (concentrations) | Insulin Receptor number (fM/mg) | Insulin % of control | EGF Receptor number (fM/mg) | EGF % of control |
|---|---|---|---|---|
| Insulin ($10^{-6}$ M) | 4,621±187(6) * | 24.0 | 10,923±378(2) | 96.7 |
| EGF (200 ng/ml) | 18,573±879(2) | 96.5 | 2,621±213(2) | 23.2 |
| None | 19,253±927(7) | 100.0 | 11,293±624(7) | 100.0 |

Effect of preincubation with insulin and/or EGF on insulin or EGF binding to human term placental cells. Loss of insulin receptor is a specific effect following exposure of insulin to placental cells. Thus, preincubation of term placental cells with insulin causes a decrease in total number of insulin receptors, but preincubation with EGF does not alter the concentration of insulin receptors. Similarly, preincubation of cells with insulin does not alter the concentration of EGF receptors.

* Mean ± S.E. (N)

## Chapter V An In Situ Method for Rapid Measurement in Human Placental Monolayers of Receptor Binding and Uptake of Glucose and Amino Acid

### V.1 Introduction

Polypeptide hormone binding studies with various cell types are usually performed at unphysiological temperatures of 4 or 15 C, temperatures at which labeled ligands frequently show maximal binding. Freidenberg et al (242) performed binding of [$^{125}$I]-insulin to erythrocytes at 30 C and found a rapid time- dependent increase in specific binding. On the contrary, investigators using monocytes (243), granulocytes (244), hepatocytes (204), human term placental cells (190) and human breast cancer cells (239) have found a time- dependent decrease in insulin or epidermal growth factor (EGF) binding at physiological temperatures (37 C).

We were not able to reliably measure insulin binding to human placental cell suspensions at 37 C due to of significant degradation of insulin (Chapter II). In order to evaluate the relationship between binding and possible biological responses of target cells, it became necessary to establish a method that would permit the placental binding of various peptides at 37 C with minimal degradation. Thus, we investigated the influence of several drugs known to influence degradation: bacitracin (115), chloroquine (116) and colchicine (116), on binding of [$^{125}$I]-insulin and -EGF in this cell system.

Efficient uptake and transport of glucose and amino acids are essential to meet the nutrient requirements for rapid cell growth. Since information on the mechanism of nutrient uptake in human

cells is relatively limited, the in situ placental monolayers provided a well-controlled experimental model for the study of nutrient uptake. The functional relationship between the degradation and biological effects of insulin and EGF in the presence of various drugs has been examined previously (116,202; General Introduction: Table 1). Thus, we utilized our culture system to examine the effects of several drugs on the binding of insulin and EGF and correlated these findings with effects of EGF on nutrient uptake. Similar studies with insulin are in progress.

## V.2 Materials and Methods

### Chemicals and Hormones

Chloroquine, colchicine and bacitracin were purchased from Sigma (St Louis, MO), 3-O-methyl-D-[U$^{14}$-C]-glucose (295 mCi/mmole) ([$^{14}$C]-3-OMG) from Amersham (Oakville, ONT), and alpha-[1-$^{14}$C]-amino-isobutyric acid (53.2 mCi/mmole) (AIB) from New England Nuclear (Boston, MA). The sources of other materials and hormones were described previously (Chapters II and IV).

### Cell Culture

Normal term placentae were obtained following caesarean section at term (38-41 wk; N=12). The cell culture procedures have been previously described (190,191 and Chapter 1).

### In Situ Binding Assay

Human term placental cells were plated in 60 x 15 mm culture dishes. After day 3 of culture, medium was aspirated, and monolayers were washed 3 x with 0.1 M Hepes-BSA (purified BSA, Sigma, St Louis, MO) assay buffer. Monolayers were then incubated with [$^{125}$I]-insulin or -EGF (240,000 -300,000 cpm) in a final concentration of 1 mg cell protein/ml. Incubations were carried

out at 37 C for 1 h unless otherwise specified. At the completion of the incubation, the assay buffer was aspirated, and monolayers were washed 3 x in ice cold 0.1 M Hepes-BSA (Fraction V, ICN; Montreal, PQ) washing buffer. Monolayers were then solubilized in 0.6 N NaOH at room temperature in a final concentration of 1 mg cell protein/ml. Triplicate 100 ul aliquots (100 ug cell protein) were counted in a gamma-counter for determination of cell-associated radioactivity. Non-specific binding was defined as the percentage of [$^{125}$I]-insulin or -EGF bound in the presence of unlabeled insulin (100 ug/ml) or EGF (2 ug/ml), and was substracted from the total amount of added labeled insulin or EGF to determine specific binding.

Degradation Studies

Following incubation with [$^{125}$I]-insulin or -EGF for 1 h, the incubation media were collected and stored at 4 C. Monolayers were then washed 4 x with 0.1 M Hepes assay buffer at 4 C, and treated with 3 ml of 0.1 N HCl containing 0.1% purified BSA for 1.5 h at 4 C with constant hand agitation to remove surface bound ligands. The eluate was then removed, and was neutralized with 1 N NaOH to pH 7.4-7.5. The labeled materials from medium or cell extract were then tested for precipitation with 10% trichloroacetic acid (TCA).

Uptake Assay System

Human term placental monolayers were washed 3 x with Krebs-Ringer bicarbonate buffer (KRB) (pH 7.3-7.4) and resuspended with KRB buffer in a final concentration of 1 mg cell protein/ml containing 0.1% purified BSA. Cells were incubated for 30 min or for the designated time period at 37 C with 0.125 mM [$^{14}$C]-3-OMG or

0.1 mM [$^{14}$C]-AIB in the presence and absence of additional EGF with and without inhibitors. Uptake was terminated by washing the cells 4 x with KRB-BSA assay buffer at 4 C. Monolayers were then solubilized in 0.6 N NaOH at room temperature in a final concentration of 1 mg cell protein/ml. Triplicate 100 ul (100 ug cell protein) aliquots were mixed with 2 ml Aquasol-II (New England Nuclear; Boston, MA) in plastic pico vials (Packard Instrument, Downer's Grove, Ill) and $^{14}$C activity was determined in a LKB beta-counter.

## V.3 Results

### Binding Studies

Human placental monolayers were incubated in situ with [$^{125}$I]-EGF at 37 C. Addition of chloroquine (100 uM), colchicine (100 uM) or bacitracin (1 mg/ml) to the monolayers augmented EGF specific binding from 45% in control cultures to 60%, 60% and 65% respectively (Fig. V.1). The effect of bacitracin was detected as early as 30 min and at 60 min for chloroquine and colchicine.

Optimal concentrations for chloroquine and colchicine for increased binding of [$^{125}$I]-EGF were 100 uM and 1 mg/ml for bacitracin (Fig. V.2). Medium and cell associated [$^{125}$I]-EGF degradation was minor as evaluated by TCA precipitability (Table V.1) or rebinding to placental membranes (data not shown).

Specific binding of [$^{125}$I]-insulin rose progressively reaching a level of 12% by 60 min, followed by a 35% decrease in binding to 8% at 120 min (Fig. V.3). In the presence of bacitracin (1 mg/ml), chloroquine (100 uM), and colchicine (100 uM) [$^{125}$I]-insulin binding increased to 130% of control at 1 h and 200% at 2 h (Fig. V.3). Medium degradation evaluated at 1 h by TCA

precipitability was about 15-25% in the presence or absence of inhibitors, being lowest with bacitracin, but cell associated degradation was 6% or less under all conditions (Table V.2). At 2 h degradation was 35% in the medium in the absence of inhibitors, whereas in the presence of inhibitors, degradation remained the same as observed at 1 h (data not shown). Optimal concentrations of chloroquine and colchicine for maximal [$^{125}$I]-insulin binding to term placental monolayers were 100 uM (Fig.V.4) and 1-2.5 mg/ml for bacitracin (Fig.V.5). Preincubation of cells with chloroquine (50 and 100 uM) for up to 2 h did not increase [$^{125}$I]-insulin specific binding to the cells.

Effects of Inhibitors on Basal and EGF-Stimulated Glucose Uptake

Chloroquine, colchicine and bacitracin were examined for their effects on glucose uptake. In these experiments [$^{14}$C]-3-OMG uptake by term placental cells was measured over a 30 min incubation period in the presence or absence of EGF. An effect of EGF on [$^{14}$C]-3-OMG uptake was found (Fig. V.6) since a significant ($p<0.05$) stimulation by EGF was noted on the uptake of [$^{14}$C]-3-OMG by control cultures. This stimulatory effect of EGF was also noted when incubations were carried out in the presence of EGF and any one of the processing inhibitors ($p<0.05$). However, only chloroquine significantly augmented glucose uptake to values higher than that of EGF alone ($p<0.05$).

In addition, concentrations of EGF higher than 200 ng/ml did not further increase the [$^{14}$C]-3-OMG uptake in term placental cells in the presence or absence of these inhibitors.

Effects of Inhibitors on Basal and EGF-Stimulated Amino-Isobutyric Acid Uptake

The same agents, which have been reported to inhibit degradation of peptide hormones by intact cells, were tested for their ability to affect the uptake of [$^{14}$C]-AIB in human term placental cells. Monolayers were incubated with [$^{14}$C]-AIB for 30 min at 37 C. In the absence of EGF, basal [$^{14}$C]-AIB uptake was not affected by chloroquine or colchicine (100 uM) and bacitracin at 1 mg/ml (Fig. V.6). In the presence of EGF, none of the drugs had any significant effect on [$^{14}$C]-AIB uptake by human term placental cells, partly due to large experimental variations.

## V.4 Discussion

Three drugs with different mechanisms of action were studied: chloroquine, which is a lysosomotropic amine, is generally believed to increase lysosomal pH (245); colchicine, which blocks microtubular function and has a disorganizing effect on the Golgi apparatus leading to inhibition of endocytosis (246-248); and bacitracin, which enhances glucagon (196) and insulin (202) binding by inhibiting degradation in the medium.

The data presented demonstrated that chloroquine, bacitracin and colchicine increase human term placental cell-associated [$^{125}$I]-insulin and [$^{125}$I]-EGF in a time- and dose- related manner. The chloroquine-induced increase in cell-associated [$^{125}$I]-insulin was presumably due to the inhibition of intralysosomal degradation, and subsequent accumulation of intact [$^{125}$I]-insulin in these

vesicles (245). This phenomenon can vary among cell types. For instance, chloroquine exhibited a significant effect in increasing cell-associated [$^{125}$I]-insulin in adipocytes but had little or no effect when isolated human erythrocytes or cultured IM-9 lymphocytes were studied under similar conditions (245). Thus, although all of these cell types possess insulin receptors, these differences indicate that a lysosomal pathway for insulin processing and degradation is not uniformally seen. The lack of effect of chloroquine on erythrocytes is not surprising since these cells do not possess lysosomes, and this further indicates the specific nature of the chloroquine effect. On the other hand, cultured IM-9 lymphocytes have lysosomes. However, this transformed cell line does not have a demonstrated biologic action for insulin and has very limited activity to degrade insulin. Thus, the absence of a lysosomal pathway for insulin processing and degradation may be related to a unique functional difference of this cell type.

In cultured placental monolayers, the antibiotic peptides bacitracin as well as colchicine were also found to increase insulin and EGF binding and to decrease [$^{125}$I]-insulin degradation at 2 h in the same way as chloroquine. Bacitracin is known to inhibit insulin and glucagon degradation in intact liver cell preparations (196). However, it is not certain that this drug acts at intracellular sites. Furthermore, bacitracin is not known to accumulate in lysosomes and/or to inhibit lysosomal enzymes.

Colchicine has been suggested to act by retarding the translocation of [$^{125}$I]-insulin to intracellular degrading sites in hepatocytes (248). However, the mechanism of colchicine's

inhibitory effect on intracellular translocation is not clear.

In order to interpret studies of hormone-receptor interaction, it may not be sufficient to only satisfy the criteria necessary to determine optimum binding characteristics; it should also be possible to correlate binding and the biological response of the cells studied. In the present study, the binding of EGF to its specific membrane receptors can be related to a biological response of these cells to EGF, namely stimulation of [$^{14}$C]-3-OMG uptake. There was a good correlation between the binding and biological response, despite the fact that binding and biological activity were measured under different conditions of buffer and time (Chapter IV, Discussion). This assay measures the total uptake of radiolabeled 3-OMG and is based on the principle that it cannot be further metabolized. Chloroquine enhanced EGF-stimulated [$^{14}$C]-3-OMG uptake in human term placental monolayers ($p<0.05$). It is interesting to note that their effect on EGF binding and an EGF induced hormonal action occurred within the same concentration range of the drugs and that chloroquine, colchicine and bacitracin did not alter basal uptake.

The uptake of AIB, a non-metabolizable amino acid, into human placental slices has been reported (249). In contrast with glucose uptake, EGF was a poor stimulant of AIB uptake in human placental monolayer cells in the presence or absence of inhibitors.

No relationship could be established between the inhibition of EGF degradation by inhibitors and the biological effects of EGF, such as stimulation of [$^{14}$C]-3-OMG, as reported by other investigators (General Introduction: Table I). Whether these results reflect differences in fundamental mechanisms by which EGF

elicits its various effects in different tissues or methodological differences is not yet known.

The present study provides a methodological basis for the examination of insulin, EGF and IGF receptor-mediated functions in human placental monolayers. These data also serve as a prelude to a detailed study of the binding, degradation, and possible metabolic effects of these hormones in this cell system. Further studies on receptor binding, internalization and degradation of the cell membrane bound hormone should help to elucidate the mechanisms which couple the biological response to hormone binding.

## V.5 Summary

The present study demonstrated that at physiological temperature (37 C) [$^{125}$I]-labeled insulin and EGF bind to human term placental monolayers "in situ".

Addition of bacitracin, chloroquine or colchicine increased the initial rate of association of [$^{125}$I]-labeled insulin and EGF with term placental monolayers in a time- and dose-dependent fashion. During the initial 60 min, chloroquine and colchicine did not affect the degradation of labeled insulin or EGF in the incubation media or eluted from the cells. However insulin degradation was increased after 90 min, and these inhibitors decreased degradation of [$^{125}$I]-insulin but not of [$^{125}$I]-EGF in both media and cell elutes.

EGF stimulated [$^{14}$C]-3-OMG, but not AIB uptake in term placental monolayers. These data indicate that degradation and biological effects are not directly associated for EGF in this system, and thus do not support hypothesis that intracellular degradation of EGF is necessary for its metabolic effects.

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Portions of Chapter V have been submitted in Abstract form (Appendix 2) Lai WL, Guyda HG, Bergeron JJM 7th International Congress of Endocrinology, Quebec City,1984

## V.6 Figures and Tables

Fig. V.1 Time course of [$^{125}$I]-EGF binding to human term placental cells (100 ug cell protein) in the presence or absence of inhibitors. The monolayers were incubated at 37 C for the indicated time intervals, washed and the cell associated radioactivity was determined as described in "Materials and Methods". Each point represents the mean of triplicate determination from one representative experiment.

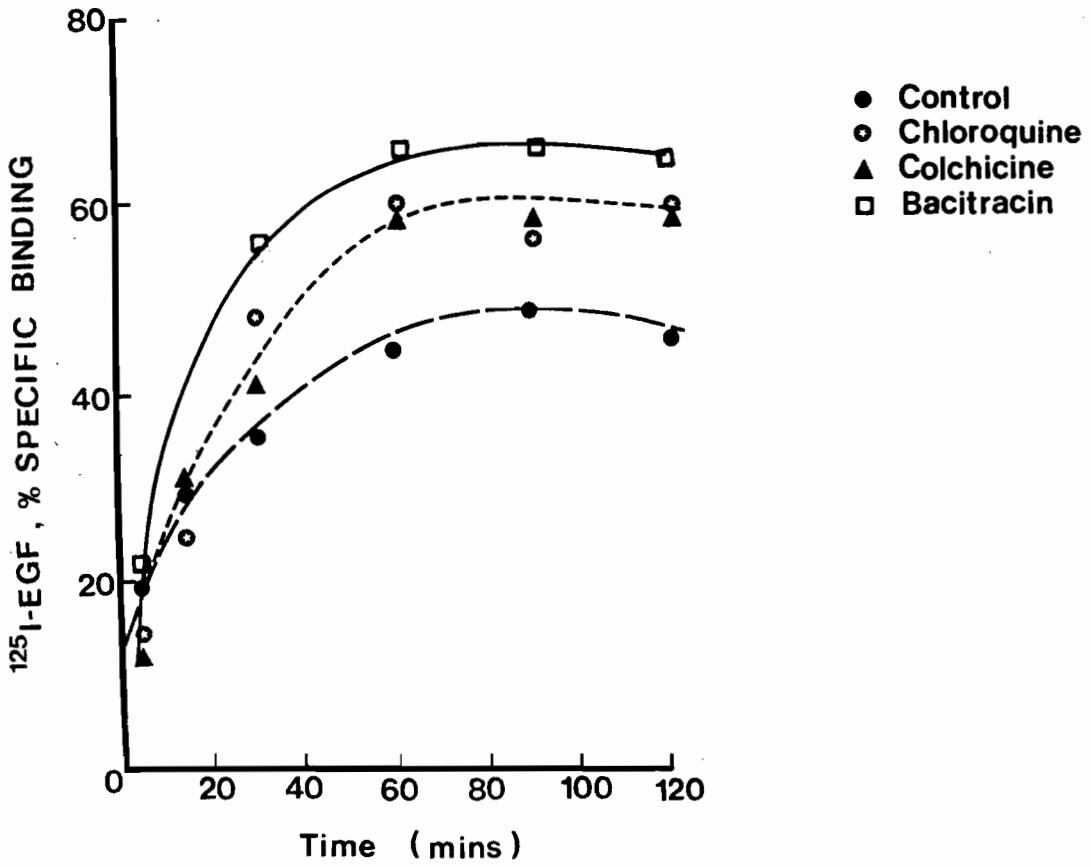

80
60
40
20
0
20
40
60
80
100
120
$^{125}$I-EGF , % SPECIFIC BINDING
Time ( mins )
Control
Chloroquine
Colchicine
Bacitracin

Fig. V.2 Dose response curves for the effects of a). chloroquine, colchicine and b). bacitracin on the specific binding of [$^{125}$I]-EGF to human placental cells as indicated in the figure. Human term placental cells (1mg/ml) were incubated at 37 C for 1 h. At the end of incubation, cell associated radioactivity was determined.

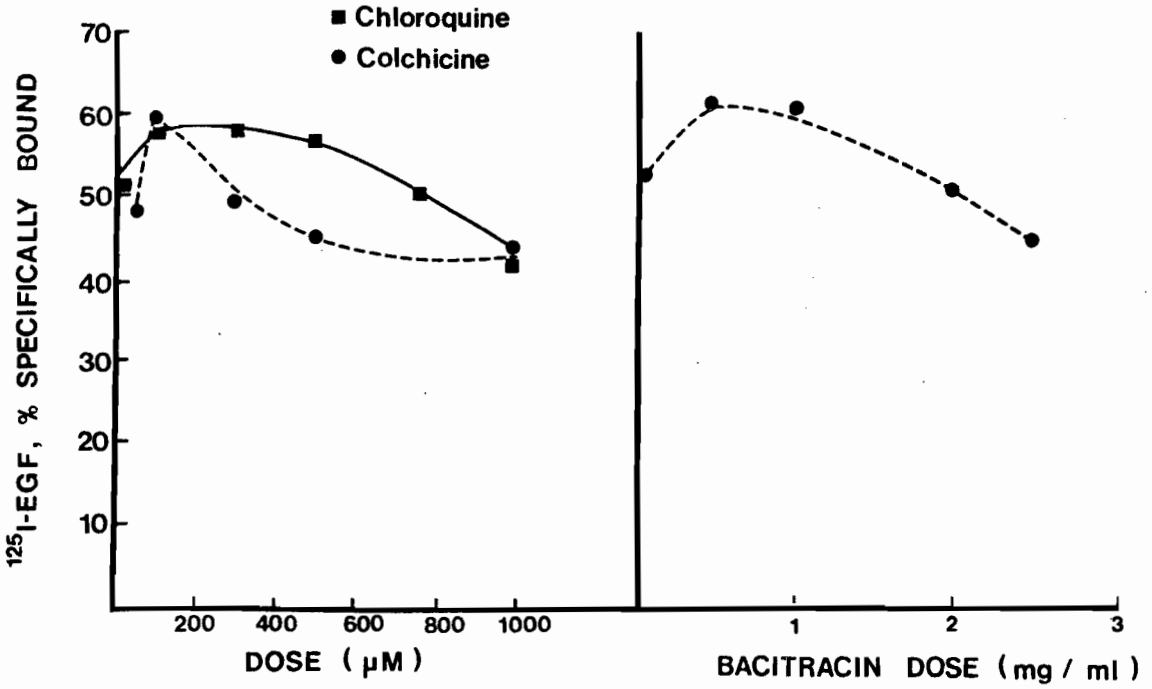

■ Chloroquine
● Colchicine
$^{125}$I-EGF, % SPECIFICALLY BOUND
70
60
50
40
30
20
10
200
400
600
800
1000
DOSE ( μM )
1
2
3
BACITRACIN DOSE ( mg / ml )

Fig. V.3 Effect of chloroquine, colchicine and bacitracin on the specific binding of [$^{125}$I]-insulin to human term placental cells. Monolayers were incubated at 37 C for the indicated time intervals, and the cell associated radioactivity was determined as described in "Materials and Methods". Each point represents the mean of triplicate determination from one representative experiment.

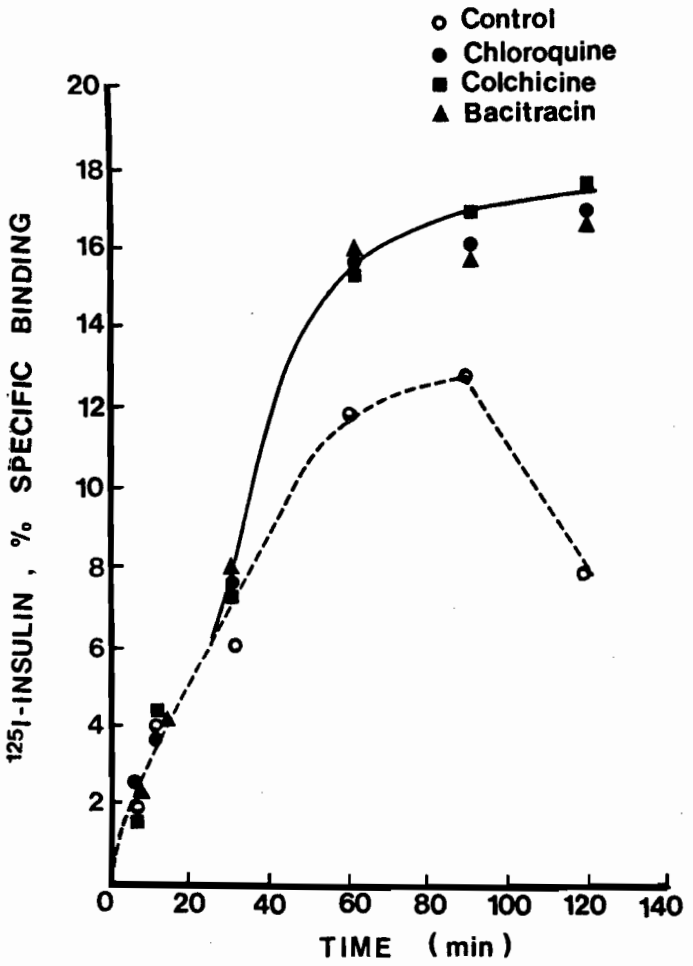
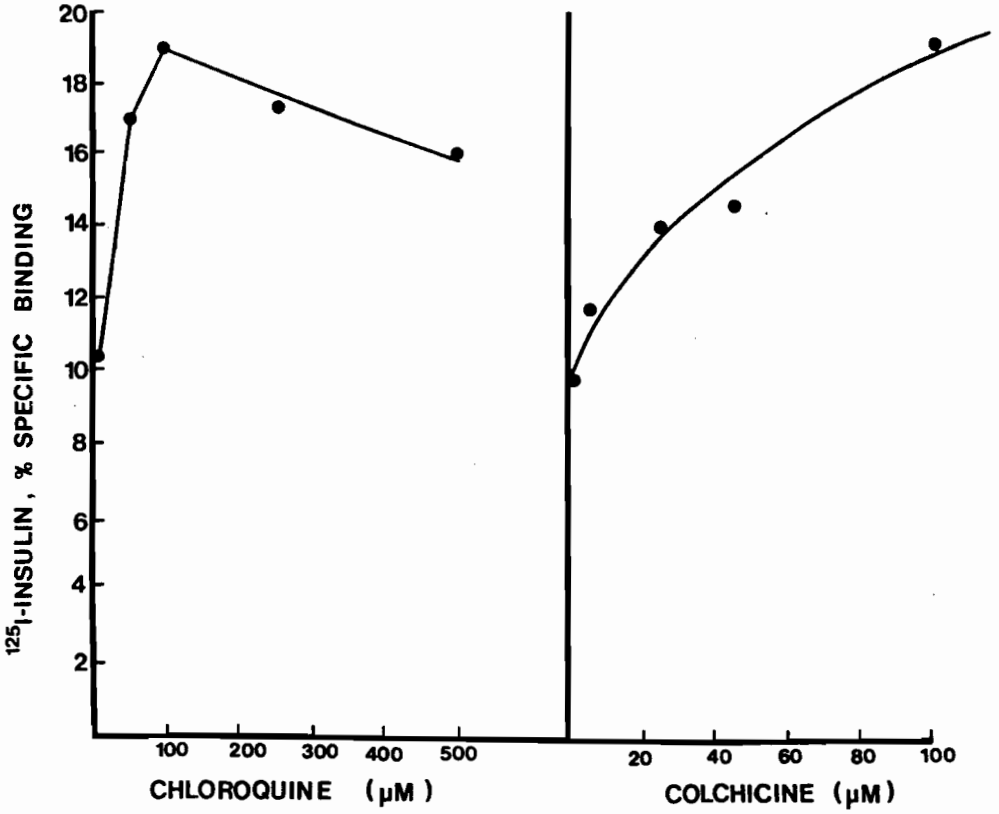

Control
Chloroquine
Colchicine
Bacitracin
$^{125}$I-INSULIN , % SPECIFIC BINDING
20
18
16
14
12
10
8
6
4
2
0
20
40
60
80
100
120
140
TIME ( min )

Fig. V.4 Effect of chloroquine and colchicine on the binding of [$^{125}$I]-insulin to human term placental cells as a function of chloroquine and colchicine concentration. Monolayers were incubated for 1 h at 37 C in 0.1 M Hepes-BSA assay buffer, pH 7.5, containing various concentrations of chloroquine and colchicine. Binding was determined as described in "Materials and Methods", and corrected for nonspecific binding by examing parallel samples in which the cells were incubated with 100 ug/ml of native insulin.

Fig. V.5 Effect of bacitracin on the binding of labeled insulin to human term placental cells as a function of bacitracin concentration. Monolayers were incubated for 1 h at 37 C in 0.1 M Hepes-BSA assay buffer, pH 7.5, containing various concentrations of bacitracin.


$^{125}$I-INSULIN, % SPECIFIC BINDING
20
18
16
14
12
10
8
6
4
2
100
200
300
400
500
CHLOROQUINE (μM)
20
40
60
80
100
COLCHICINE (μM)

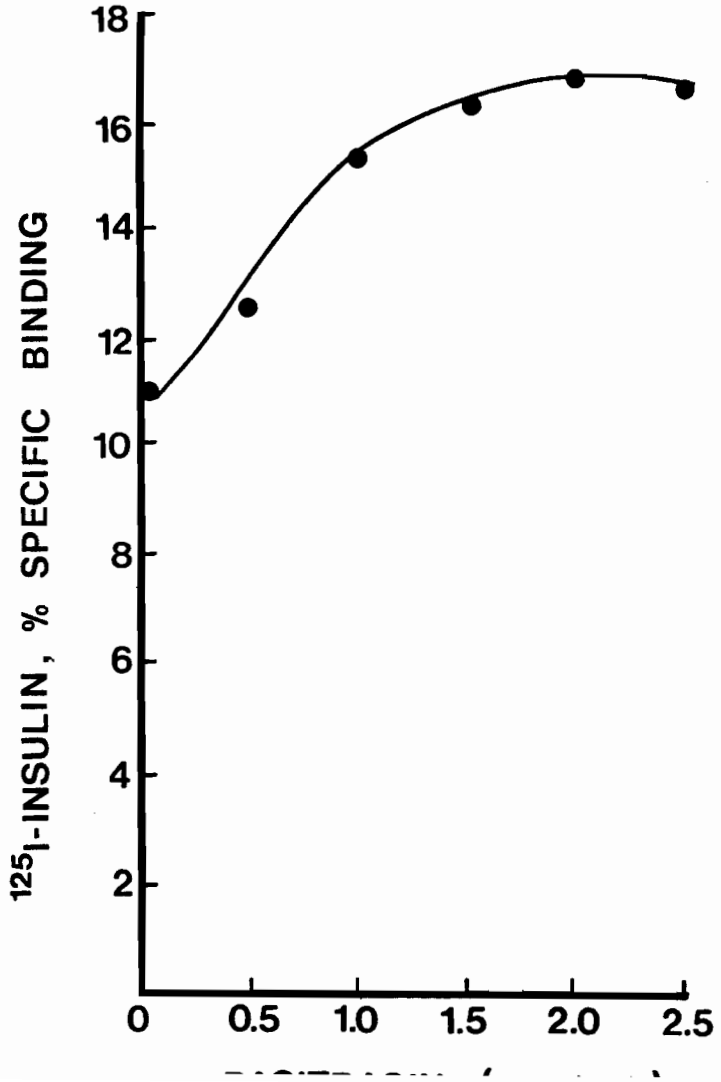

$^{125}$I-INSULIN, % SPECIFIC BINDING
18
16
14
12
10
8
6
4
2
0
0.5
1.0
1.5
2.0
2.5

Fig. V. 6 Effect of EGF and various inhibitors on stimulation of [$^{14}$C]-3-O-methyl-glucose and AIB uptake in human term placental cells. Term placental cells were incubated for 30 min with the inhibitors in the absence (open bars) or presence (cross-hatched bars) of EGF (200 ng/ml) and the amount of [$^{14}$C]-3-O-methyl-glucose and AIB uptake was determined as described under "Materials and Methods". Values shown are mean $\pm$ S.E. of triplicate determinations from three separate placentae. Statistical analysis showed a significant increase of 3-O-methyl-glucose uptake by EGF whether in the absence or presence of inhibitors ($p<0.05$). Only chloroquine-treated samples showed a significant increase as compared to samples incubated in EGF alone ($p<0.05$). No statistically significant effect of EGF on AIB uptake was observed.

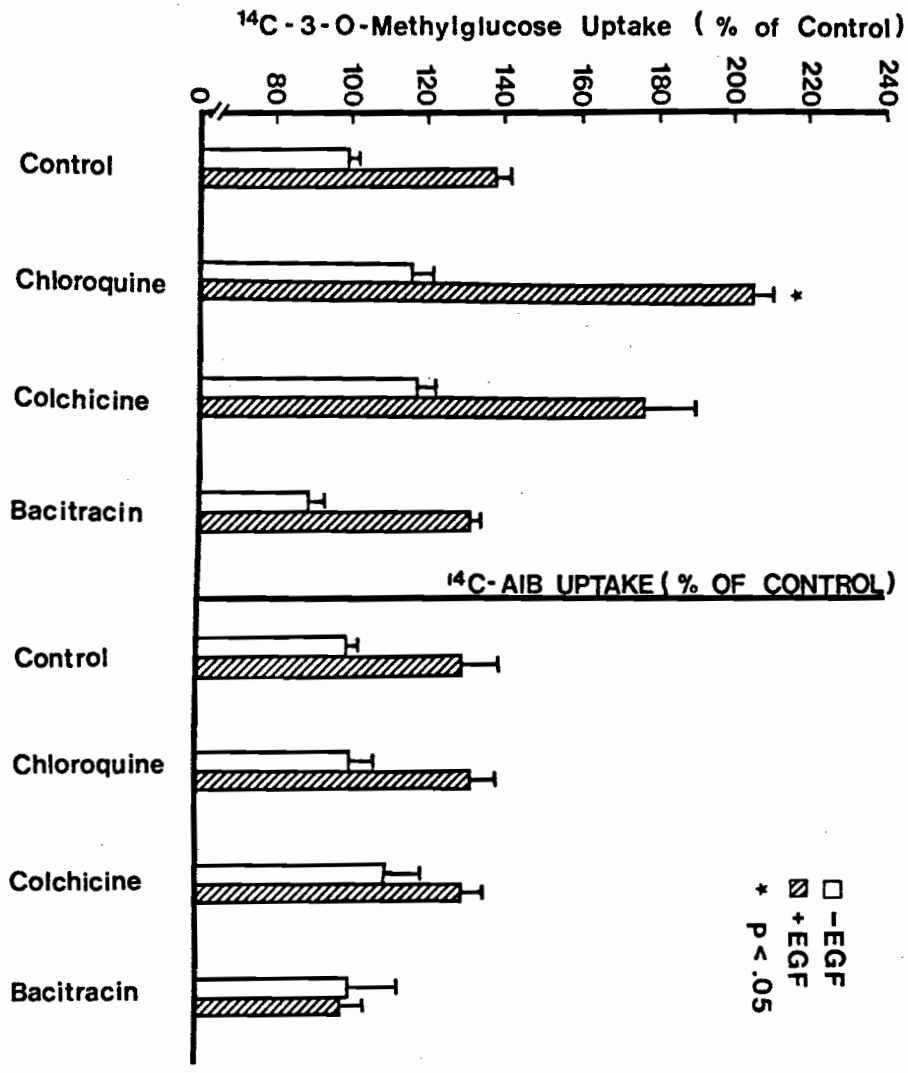

$^{14}$C - 3 - O - Methylglucose Uptake ( % of Control)
0
80
100
120
140
160
180
200
220
240
Control
Chloroquine
*
Colchicine
Bacitracin
$^{14}$C - AIB UPTAKE ( % OF CONTROL)
Control
Chloroquine
Colchicine
Bacitracin
□ - EGF
▨ + EGF
* P < .05

Table V.1 Effect of Various Inhibitors on TCA Precipitability of $^{125}$I-EGF Exposed to Human Term Placental Cells

| Addition (concentrations) | $^{125}$I-EGF integrity | | | |
|---|---|---|---|---|
| | $^{125}$I-EGF in incubation medium | | $^{125}$I-EGF eluted from cell | |
| | TCA precipitability | % of control | TCA precipitability | % of control |
| No addition | 82.3±0.4 | 91.1 | 84.5±0.1 | 93.6 |
| Chloroquine (100 uM) | 86.7±0.1 | 95.9 | 90.0±0.3 | 100.6 |
| Colchicine (100 uM) | 83.8±0.1 | 92.7 | 85.3±0.2 | 94.4 |
| Bacitracin (1 mg/ml) | 80.4±0.1 | 88.9 | 86.7±0.1 | 95.2 |

Mean ± S.E. of triplicate determinations (placenta n=1).

Table V.2 Effect of Various Inhibitors on TCA Precipitability of $^{125}$I-Insulin Exposed to Human Term Placental Cells

| Addition (concentrations) | $^{125}$I-insulin integrity | | | |
|---|---|---|---|---|
| | $^{125}$I-insulin in incubation medium | | $^{125}$I-insulin eluted from cell | |
| | TCA precipitability | % of control | TCA precipitability | % of control |
| No addition | 68.0±0.1 | 72.4 | 89.7±0.1 | 95.5 |
| Chloroquine (100 uM) | 67.8±0.1 | 72.2 | 92.6±0.1 | 98.6 |
| Colchicine (100 uM) | 68.3±0.3 | 72.7 | 88.4±0.1 | 94.2 |
| Bacitracin (1 mg/ml) | 79.5±0.0 | 84.6 | 92.4±0.6 | 98.5 |

Mean ± S.E. of triplicate determinations (placenta n=1).

## PART THREE: GENERAL DISCUSSION AND CONCLUSIONS

### I. General Discussion

We have characterized the binding of several growth promoting peptides (insulin, EGF, IGF-I and IGF-II) to human placental cells in monolayer culture. The binding studies were undertaken using whole cell radioreceptor assays in a Hepes assay buffer. Specific binding of labeled ligands to term placental cells was found to be a saturable process, time- and temperature- dependent and proportional to cell protein and ligand concentrations. Steady state binding was maximal at lower temperatures (4 and 22 C) and exhibited a high degree of specificity. During these investigations, a number of important observations were made.

#### 1. Conditions for the Radioreceptor Assay

Physical and chemical factors had significant influences on peptide hormone binding to placental cells *in vitro*. For example, maximal specific binding of both IGF-I and IGF-II occurred with a broad pH range of 6.5 to 8.5, in contrast to the very sharp pH optimum for insulin and EGF (pH 7.5) under similar conditions. In addition, temperature had important effects on the placental assay system. In most systems, as in our placental cell assay, maximal binding is observed at lower temperatures (133). With many cell types, including the placenta, this is due to the considerable degradation of labeled ligand which is more significant at higher temperatures (136,239,250). Furthermore, it has been recognized that the hormone-receptor complex is internalized by the cell more rapidly and to a greater extent at 37 C than at lower temperatures (79).

Another factor which affects hormone-receptor binding is the concentration and purity of albumin used in the incubation medium. Albumin can alter the rate of degradation of insulin (251) and can influence IGF binding due to the presence of IGF binding proteins in impure albumin preparations. Finally, with some cell types, including the placenta, the choice of buffer influences the extent of internalization or down-regulation observed (252).

The placental cell suspensions utilized in our assay system were extremely rich in degrading activity for insulin and IGF, but not for EGF. In our in vitro test system bacitracin was capable of decreasing the degradation and increasing the specific binding of insulin and IGF peptides. Thus, bacitracin was found to be essential for the study of insulin and IGF receptors in human placentae, but less critical for the study of EGF receptors. The process of insulin degradation described in this cell system would appear to be both non-receptor as well as receptor-mediated. This is in agreement with studies in other cell systems by other investigators (202). It is not certain whether bacitracin acts solely at the membrane surface or also at intracellular sites. Further studies are necessary to clarify this question.

A typical Scatchard plot for insulin binding to most cell types is curvilinear, being concave upward. This could result from two or more orders of independent sites, each with fixed affinity for insulin (253). Although cells can have receptors for both insulin and IGFs, IGF receptors mediate distinct biological effects and represent distinct structural entities (254). Nevertheless, insulin may bind to both type of receptors with different affinities.

An alternative explanation for the curvilinear Scatchard plots involves negatively cooperative interactions (138). In this model, the affinity of the insulin receptors progressively decreases as more receptors sites are occupied by insulin. This hypothesis was supported by the demonstration that the initial dissociation rate of labeled insulin was increased when excess unlabeled insulin was added. Thus, at low insulin concentration most receptor sites are empty, and the receptors are in their highest affinity state. With increasing receptor occupancy by insulin the affinity of the remaining receptors decreases progressively.

In addition, Donner (255) has investigated the possibility that insulin degradation and subsequent heterogeneity of bound insulin may also result in binding kinetics suggestive of negative cooperativity or more than one class of receptor sites. He observed that hepatocytes degraded insulin following binding but that the degraded hormone fragments remained bound to the cells. The Scatchard plots became linearized when the binding data were corrected for intact [$^{125}$I]-insulin measured by gel filtration.

In our studies, [$^{125}$I]-insulin binding to cultured placental cells also demonstrated a curvilinear Scatchard plot. Any one of the three hyphotheses just presented: 1). heterogeneous receptor populations, 2). the negative cooperativity model, and 3). hormone degradation, could be possible explanations for the placental cell curvilinear results. Additional work will be required to delineate the factors involved in the complex binding kinetics observed for insulin in this cell system.

The early experiment of Carpenter et al (256) with human

fibroblasts had demonstrated a single class of EGF receptors, which differs from our findings. Although Carpenter et al (256) also measured EGF binding to many other cell types, they did not perform complete binding analysis over a large concentration range and therefore might not have observed heterogeneous receptor populations if they had existed. In addition, dissociation of [$^{125}$I]-EGF was also examined by Carpenter et al (256) in human fibroblasts in the presence and absence of unlabeled EGF. No evidence for negative cooperativity was found even though such a phenomenon has been reported for placental membranes (257). However, their dissociation experiments were carried out at 37 C, where it is difficult to demonstrate the effect of "site-site" interactions on dissociation even for insulin (258). This effect is best seen at lower temperatures. It has been suggested that the inability to detect this effect at 37 C does not prove its absence, but merely that the high temperatures facilitate "site-site" interactions such that they may occur too quickly to be detected.

2. Receptor Regulation

Although the phenomenon of down-regulation has been well documented, the mechanism is poorly understood. At least three processes could explain the decrease in insulin and EGF receptors induced by the specific hormone that we observed: 1). inhibition of receptor biogenesis, 2). accelerated endocytosis and/or degradation of receptors, or 3). a combination of these two processes (210).

If peptide hormones act by inhibiting receptor synthesis, then the rate of hormone-mediated loss should be equal to or less than the rate of receptor loss caused by inhibition of protein synthesis. In our studies, inhibition of protein synthesis with

cycloheximide resulted in almost the same rate of insulin-induced receptor loss. In contrast, EGF-induced receptor loss was inhibited in the presence of cycloheximide. Furthermore, it appeared that cycloheximide permitted recovery of EGF binding from 8 to 16 h in the presence of EGF whereas it blocked recovery upon removal of EGF. Our observations on the effect of cycloheximide are similar to Prince et al (213) and Kosmakos et al (210) who studied insulin-induced down-regulation in human fibroblasts and IM-9 lymphocytes respectively. They differ with the results of our own insulin-induced down-regulation in the placental cell system, as well as with the results of Aharonov et al (230) on EGF-induced down-regulation in 3T3 fibroblast cells, where cycloheximide had no effect.

Our studies showed EGF-induced receptor loss proceeded at a relatively rapid rate (t 1/2 = 2h). In contrast, receptor recovery proceeded over a slower period (t 1/2 = 8h). The difference in the rate of these two processes suggests that EGF receptor recovery does not merely imply the absence of ongoing receptor loss. On the other hand, insulin-induced receptor loss and recovery both proceeded in a similar fashion at a relatively rapid rate (t 1/2 = 4h).

Recovery of both insulin and EGF receptors in down-regulated placental cells did not occur in the presence of cycloheximide, supporting the thesis that protein synthesis is required for receptor recovery (212). In addition, the down-regulation of each hormone was specific for its own receptor, and each did not change the concentration of receptors for the other hormone. Furthermore,

our observations upon the hormone-induced loss of receptors indicated, that for both insulin and EGF, this was due to decreased number of receptors with no significant change in apparent affinity.

The changes in hormonal sensitivity which occur in many endocrine disorders could be partly related to alterations at the receptor level (96). For example, reduction in insulin binding in thymic lymphocytes of obese mice is accompanied by a decreased effect of insulin on amino acid transport, suggesting that the induced reduction in receptor concentration influences the metabolic action of insulin in this cell (101). In addition, certain forms of hormone deficiency have been shown to be accompanied by increased concentration of receptors in the respective target tissues (259). The extent to which hormonal regulation of tissue receptor concentration is responsible for changes in hormone sensitivity during human endocrine disorders has not been determined. However, it is clear that receptor modulation by circulating hormone concentration is probably an important physiological process *in vivo*.

3. *Ontogeny of Growth Factor Receptors*

Although the role of EGF, insulin and IGF in the growth of the fetus has not been elucidated, it has been shown that fetal tissues possess growth factor receptors. Nexo et al (167) and Adamson et al (260) report that fetal mouse tissues have detectable EGF receptors on the 11th day of gestation. Sm C receptors have been measured in a large range of fetal pig tissues throughout the gestational period (162). The binding of labeled Sm C was increased in late gestation lung and placenta compared to early gestation or

adult tissues. In the fetal pig placenta insulin binding decreased whereas in the fetal mouse placenta insulin binding increased as a function of gestational age (261). In view of the significant differences in mammalian placentation, these different results are not surprising.

We have also studied the ontogeny of growth factor receptors in human placentae. Specific binding of insulin and EGF increased as the gestational age increased, being approximately 3- fold for insulin and 2- fold for EGF in term gestation compared to early gestation placental cells. However, the specific binding of IGF-I and IGF-II at both gestational ages was similar. This suggests that the human placental complement of IGF receptors is established early, and that maximal binding capacity for the cells has been reached as early as the 12th wk of gestation. These data are in agreement with our observations using placental membrane preparations (163).

Placental insulin, EGF and IGF receptors studied by both methods (differential centrifugation to obtain plasma membrane receptors and tissue culture to study whole cell receptors) displayed essentially identical binding characteristics, indicating that our conditions of trypsin digestion and monolayer culture do not alter specific receptors. The only appreciable difference was that more IGF was specifically bound to membrane preparations. Placental cells were capable of degrading IGF peptides even at 4 C, but this was not seen as actively in membrane assay systems. Therefore the decrease in IGF binding seen with placental cells can be partly explained by a decrease in intact tracer in the

incubation medium of the cell suspension.

4. Metabolic Effects of Growth Factors

In the present study of cultured placental cells, the binding of EGF to specific membrane receptors resulted in stimulation of hCG and hPL release and in stimulation of [$^{14}$C]-3-O-methyl-glucose ([$^{14}$C]-3-OMG) uptake. It can be concluded that the binding measurements reflect "functional" placental receptors for EGF. There was a good correlation between the binding and biological dose-response curves for EGF, despite the fact that binding and biological activity were measured under different conditions of time and temperature (see Discussion section of Chapter IV).

The events following EGF binding to placental cells are not yet well characterized. Internalization and subsequent degradation of EGF appears to be an obligatory part of the sequence leading from cell binding to biological response (168). However, it has been known for some time that certain of the biological effects of EGF and other growth factors are extremely rapid. For example, increases in uptake of such substances as [$^{14}$C]-3-OMG occur within minutes following EGF binding to the membrane surface (168). Since these rapidly elicited responses occur before internalization is evident, they must be based on a mechanism which is linked to the cell surface. On the other hand, responses such as the stimulation of hCG and hPL release or DNA synthesis and cell proliferation, which take a longer period of time to occur, could reasonably be attributed to events which involve internalization and possible degradation of the ligand. Several studies suggest however that there is no clear causal relationship between the continuous internalization of hormone-receptor complexes and the

mitogenic responses (111-116).

In the present study, bacitracin was found to modify insulin binding and degradation studied at 37 C for 1 h. It is not certain whether bacitracin acts at intracellular sites as well as on the membrane surface (202). One major finding of the present study was that chloroquine and colchicine increased EGF binding to the same extent as bacitracin, and also permitted the stimulation of [$^{14}$C]-3-OMG uptake in the presence of EGF. The effect of these drugs can not be attributed to toxicity since basal [$^{14}$C]-3-OMG uptake remained unaffected. No relationship could be established between the inhibition of ligand degradation and glucose uptake.

The pleiotypic effects of insulin include not only a broad spectrum of metabolic activities, but also growth-promoting effects. IGF-I and IGF-II are polypeptides that have growth promoting and insulin-like metabolic activities, but are immunologically distinct from insulin (262). In general, insulin is more potent for metabolic effects whereas IGFs are more potent with regard to growth-promoting effects (262). Cultured placental monolayers in situ offer an excellent opportunity for the evaluation of insulin and IGF binding and action. To determine whether metabolic actions such as glucose and amino acid uptake as well as hCG and hPL release are mediated by receptors for insulin, IGFs, or both, selective blockade of receptors with specific antagonists (eg. the naturally occurring antibodies to the insulin receptor), can be employed (104). Such studies will be the object of future research in this laboratory.

## II.Conclusions

Placental growth occurs in response to many factors: growth factor receptor number, receptor affinity and receptor regulation, as well as interactions between growth factors and environmental factors such as placental nutrient uptake and metabolism, leading to further differentiation of placental cells.

Our studies using placental monolayer cell cultures have provided readily available and relatively simple methods to assess growth factor binding and biological effects. These may give insight into the possible *in vivo* effects of growth factors. However, extrapolations from such *in vitro* experiments to the *in vivo* state are confounded by the use of serum containing unknown ingredients in the culture medium and by the fact that the milieu of cells in culture is very different from that occurring *in vivo*. Furthermore, some growth factors may not act directly but mainly through the mediation of or in concert with other growth factors. Thus these studies can not be thought of as complete or as a unit by itself; instead they should be viewed as part of a ongoing integration of growth factor-placenta interactions.

Placental growth is important for fetal growth. Restriction of placental and fetal growth has been observed when maternal nutrition has been insufficient (263,264). Normal placentae increase in size almost linearly up to 36 weeks. Placental growth is influenced by hPL and hCG: low levels of hPL in the maternal serum have been associated with fetal growth retardation (265), while hCG appears to bestow maternal immunologic tolerance on the fetus. Although our studies were primarily aimed at characterizing growth factor receptors in human placental cells at various

gestational ages, we also began an examination of the possible role of these growth factors in nutrient uptake and hormone release by placental cells. We observed that EGF significantly increased nutritional supply to and hCG and hPL release from cultured placental cells. However, we are only beginning to understand the role of the placenta in fetal growth. In order to elucidate the role of growth factors, it is important to determine which specific placental metabolic effects are associated with each of the growth factors at various stages of fetal development and to what extent the growth factors interact to promote growth.

Enhanced purification of growth factors and their increased availability through synthetic means will lead to the development of more sensitive assays that will provide invaluable tools for further investigation of this fascinating field. It is hoped that the achievements of the coming years will answer many of our questions and also provide us with new clinical aids in the treatment of growth disorders involving the human fetus.

## PART FOUR: CONTRIBUTION TO KNOWLEDGE

Studies of placental monolayer cell cultures have provided readily available and relatively simple methods to assess growth factor binding and biological activity.

The work described in this thesis has yielded the following contributions to knowledge:

1. Cultured human early gestation and term placental cells possess highly specific insulin, EGF, IGF-I and IGF-II receptors.
2. In our *in vitro* test system bacitracin is capable of decreasing the degradation and increasing the specific binding of insulin and IGF peptides.
3. Insulin and EGF receptors are directly modulated by ambient concentrations of insulin and EGF respectively.
4. There is an ontogeny of growth factor receptors in human placental cells: specific binding of insulin and EGF increases as the gestational ages increases, while IGF at both gestational ages is similar.
5. The preincubation of placental cells with EGF for 24 h significantly increases the amount of hCG and hPL released from both early gestation and term placental cells.
6. EGF produces an acute stimulation of [$^{14}$C]-3-O-methyl-glucose uptake by term placental cells.

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Appendix 1 Estimation of Cell Protein Concentrations in the Presence or Absence of EGF

| Days in Culture | EGF Added (ng/ml) | | | |
|---|---|---|---|---|
| | 0 | 200 | 400 | 2000 |
| | Cell Protein Concentration (mg/ml) | | | |
| 1 | 2.1 ± 0.3 * | 2.0 ± 0.2 | 2.2 ± 0.3 | 2.1 ± 0.1 |
| 2 | 2.0 ± 0.2 | 2.1 ± 0.1 | 2.0 ± 0.2 | 1.9 ± 0.2 |
| 3 | 1.9 ± 0.3 | 2.0 ± 0.3 | 1.9 ± 0.2 | 2.2 ± 0.1 |
| 4 | 2.1 ± 0.1 | 2.0 ± 0.2 | 2.1 ± 0.2 | 1.9 ± 0.3 |
| 5 | 1.9 ± 0.1 | 1.9 ± 0.3 | 1.8 ± 0.3 | 1.9 ± 0.3 |

* Mean ± S.E. Triplicate determinations from two term placental cell cultures.

Appendix 2 Abstract

BINDING AND INTERNALIZATION OF EPIDERMAL GROWTH FACTOR (EGF) IN HUMAN TERM PLACENTAL CELLS IN CULTURE: RELATIONSHIP TO BIOLOGICAL RESPONSE. W.H. Lai, H.J. Guyda and J.J.M. Bergeron, Department of Pediatrics and Anatomy (JJMB), McGill University, Montreal, Canada

The relationship between [$^{125}$I]-EGF internalization and biologic response in primary cultures of human syncytiotrophoblast has been assessed by quantitative electron microscope radioautography. Continuous labeling of cultures with [$^{125}$I]-EGF (0.2 nM, 150 uCi/ug) at 37 C revealed a progressive increase in intracellular EGF with 5% of total label in intracellular organelles at 2 min and 88% by 60 min. Detailed analysis showed a progressive transfer for [$^{125}$I]-EGF from microvillar processes to the base of microvilli followed by uptake into vesicular and polymorphic endosomes as well as multivesicular bodies. Since incubations were carried out with subsaturating doses of EGF with minor degradation of EGF, the results suggest a temporal transfer of occupied and unoccupied EGF receptors into intracellular compartments. Chloroquine (CHL,100 uM), colchicine (COL, 100 uM) and bacitracin (BAC, 1 mg/ml) augmented EGF specific binding from 45% in control cultures to 60%, 60% and 65% respectively, with a majority of the ligand (72-83%) being observed in endosomal structures and multivesicular bodies after 60 min of incubation. Studies of EGF-stimulated glucose uptake relvealed a significant stimulation of

[$^{14}$C]-3-O-methyl-glucose uptake by chloroquine ($p<0.05$). No significant effect of the same inhibitors was observed on [$^{14}$C]-alpha-amino isobutyric acid uptake. In summary, EGF induced the internalization of occupied and possibly unoccupied EGF receptors. Agents interfering with EGF degradation resulted in greater receptor occupancy especially at intracellular sites. The results are consistent with some EGF actions being exerted at the loci of endosomes and/or multivescular bodies.