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Hepatocyte Growth Factor in Human Placenta and Trophoblastic Disease

Helmut K. Wolf, Reza Zarnegar, Letealia Oliver, and George K. Michalopoulos

From the Department of Pathology, Duke University Medical Center, Durham, North Carolina

The hepatocyte growth factor (HGF) is an acidic protein with a strong mitogenic effect on hepatocytes. Hepatocyte growth factor mRNA recently was cloned from a placental cDNA library. Here we demonstrate the purification of HGF from human placenta with heparin-agarose chromatography and TSK-heparin high-pressure liquid chromatography and describe the distribution of placental HGF by immunohistochemistry using a polyclonal antibody to HGF. The yield of HGF from the placenta was approximately 100 to 200 times greater than that previously obtained from human plasma. Placental HGF was expressed strongly in the villous syncytium, extravillous trophoblast, and amnionic epithelium, and, to a lesser degree, in endothelial cells and villous mesenchyme. Hepatocyte growth factor also was identified in the trophoblast of complete hydatidiform moles, choriocarcinomas, and a case of blighted ovum. The presence of HGF in an organ characterized by rapid cell proliferation during gestation and in trophoblastic tumors strongly suggests that the growth-regulating effect of HGF is not limited to hepatocytes. (Am J Pathol 1991, 138:1035–1043)

Since the discovery of human chorionic gonadotropin by Aschheim and Zondek in 1927,[1] the human placenta has been shown to produce and contain many hormones and growth factors.[2,3] In the present study we describe the extraction and purification of hepatocyte growth factor (HGF) from human placenta tissue and examine the distribution of HGF within the normal placenta and in trophoblastic tumors.

Hepatocyte growth factor is an acidic protein growth factor with a potent mitogenic effect on hepatocytes.[4] In 1983, HGF was isolated in this laboratory from the serum of rats following partial hepatectomy and initially called Hepatopoietin A.[5] Subsequently the growth factor was isolated from rat platelets,[6] rabbit serum,[7] human serum,[8] and human plasma.[7,9] Hepatocyte growth factor is a heterodimer consisting of a heavy chain with a molecular weight of 70 kd and a light chain with a molecular weight of 35 kd held together by disulfide bonds.[7] Recently HGF mRNA was cloned and sequenced from human placenta[10] and liver[11] cDNA libraries and the entire primary structure of the protein was deduced from the sequence. The amino acid sequence of HGF has no homology to other known growth factors.[12] The primary structures of human, rat, and rabbit HGF are highly homologous.[4,12] Hepatocyte growth factor was demonstrated by immunohistochemistry and by extraction to be present in many organs of the rabbit[13] and HGF-mRNA was isolated from various organs of the rat.[12] The high degree of homology of the HGF in different species and its wide distribution in various organs suggest that HGF has important metabolic functions that may extend beyond the stimulation of hepatocyte proliferation. Knowledge of the tissue distribution of HGF on a cellular level is necessary to understand fully its role *in vivo*.

The isolation of human HGF mRNA from a placental cDNA library[10] indicates that HGF is produced in human placenta tissue and prompted us to attempt the extraction and purification of HGF from placental tissue and to characterize its distribution immunohistochemically at different stages of gestation. To determine if the expression of placental HGF depends on the presence of a fetus, we also examined the distribution of HGF in cases of blighted ovum, hydatidiform mole, and gestational choriocarcinoma.

Materials and Methods

Antibodies

Polyclonal antibodies against HGF were raised in a chicken by multiple intramuscular injections of purified rabbit-HGF in Freund's adjuvant. Serum obtained from

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Address reprint requests to George K. Michalopoulos MD, PhD, Department of Pathology, Box 3432, Duke University Medical Center, Durham, NC 27710.

blood 2 weeks after the last immunization was used in the following immunohistochemical studies. The immunoreactivities of the antiserum with human and rabbit HGF and human plasminogen were determined and compared by enzyme-linked immunosorbent assay (ELISA). The details of the immunization procedure and the ELISA have been reported previously.[13]

Isolation and Purification of HGF from Human Placenta Tissue

Fresh normal mature placentas with an average weight of approximately 500 g were obtained after delivery. Each placenta was minced and homogenized in 1 l of 1 mol/l (molar) NaCl solution using a blender and a polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was diluted with water to 0.5 mol/l NaCl and clarified by centrifugation at 15,000*g* for 30 minutes. The extract then was applied to a column of heparin-agarose (bed volume, 200 ml), equilibrated in 0.5 mol/l NaCl in phosphate-buffered saline (PBS), and eluted with 1 l of 1.3 mol/l NaCl in PBS. The volume of eluate was reduced to 30 ml by ultrafiltration (Ym-10 ultrafilter, Amicon, Beverly, MA), adjusted to 0.3 mol/l NaCl with distilled water, and applied to a TSK-heparin column (75 mm × 7.5 mm, Supelco, Bellefonte, PA) attached to a high-pressure liquid chromatography (HPLC) pump (8800 series, Du Pont Instruments, Wilmington, DE) at a flow rate of 1 ml/min. Finally HGF was eluted from the column with 20 ml of 1.3 mol/l NaCl. The purity of the HGF in this preparation was confirmed by analysis of the eluate with sodium dodecyl sulfate polyacrylamide–gel electrophoresis (SDS-PAGE) followed by silver nitrate staining. The concentration of HGF in the eluate was determined by ELISA using the polyclonal chicken anti-HGF serum, as described previously.[13] The bioactivity of the extracted HGF was confirmed in primary rat hepatocyte cultures by demonstrating a strong mitogenic effect, as described elsewhere.[7]

Immunohistochemistry

Normal mature placentas were obtained immediately after delivery in cases of uncomplicated pregnancies. Tissue blocks from these were immersed in 4% buffered formaldehyde within 30 minutes after delivery, fixed for 6 to 10 hours, and processed routinely through graded alcohols and xylene to paraffin wax. In addition, paraffin-embedded tissue blocks of histologically normal mature placentas, umbilical cords, fetal membranes, and placental tissue from first-trimester abortions were retrieved from the surgical pathology files. To study the HGF expression in the absence of a fetus, we retrieved paraffin-embedded tissue blocks of clinically and pathologically well-documented cases of blighted ovum (1 case), hydatidiform mole (7 cases), and gestational choriocarcinoma (8 cases) from our files.

Sections were cut at 4 μ, taken up on slides coated with Histostik (Accurate Chemical & Scientific Corp., Westbury, NY), air dried, deparaffinized in xylene, cleared in 100% and 95% ethanol, incubated in 2% hydrogen peroxide for 30 minutes, rehydrated through graded alcohols, and incubated in a 0.1% solution of trypsin (Sigma Chemical Co., St. Louis, MO) and calcium chloride at pH 7.8 and 37°C for 30 minutes. The slides then were washed in PBS, preincubated with 5% normal goat serum diluted in PBS for 30 minutes, and then incubated with serial dilutions of anti-HGF immune serum or control serum obtained from nonimmunized chickens. In some sections, the diluted anti-HGF immune serum was applied after preincubation with purified placental HGF for 2 hours at 37°C. After 90 minutes, the sections were washed three times in PBS and incubated for 45 minutes with a biotinylated goat–anti-chicken IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS with 1% bovine serum albumin for 30 minutes. This was followed by three washes in PBS and incubation with a 1:160 dilution of avidin DH (Vector Laboratories) and biotinylated horseradish peroxidase H (Vector Laboratories) in 0.05 mol/l TRIS-HCL, pH 7.6. After three additional PBS washes, the slides were incubated in the substrate solution of 0.05% diaminobenzidine tetrahydrochloride (Sigma) and 0.01% hydrogen peroxide in 0.05 mol/l TRIS-HCL, pH 7.6 for 2 minutes, washed, stained with hematoxylin, dehydrated in ethanol, and embedded in Permount (Fisher Scientific, Pittsburgh, PA). In addition, hematoxylin and eosin (H&E)-stained sections were examined on all blocks. Masson's trichrome and Periodic-acid Schiff (PAS) stains were performed on sections that included the basal plate of the placenta.

Results

Placenta

On average, 100 to 150 μg HGF were extracted and purified from a normal term placenta by the procedure described in Materials and Methods. The purity of the HGF preparation was greater than 90% as determined by SDS-PAGE (Figure 1). Additional purification can be achieved by reverse-phase HPLC; however this is accompanied by a substantial loss of active HGF (data not shown). Human placental HGF had a potent mitogenic effect on rat hepatocytes in primary culture as demonstrated by incorporation of [^{3}H] thymidine (Figure 2). The antiserum raised against rabbit HGF reacted with human

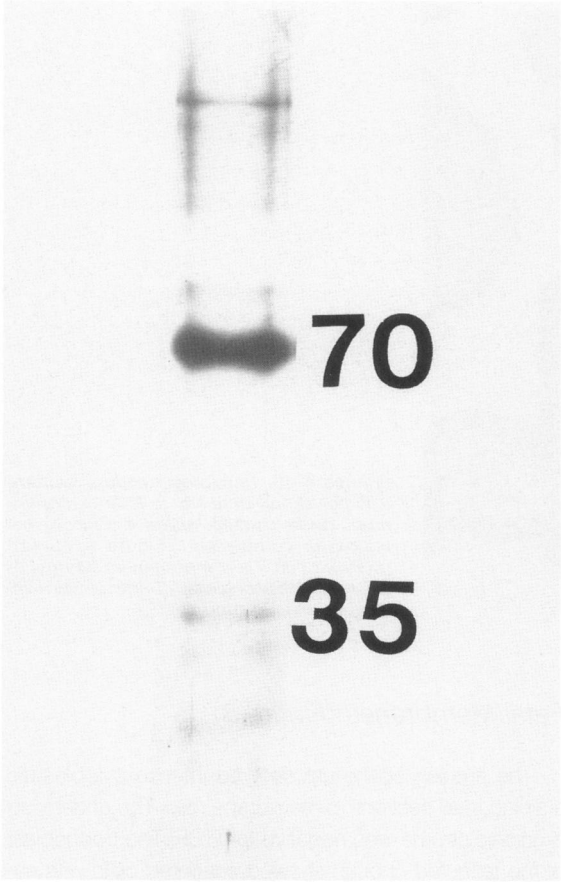


Figure 1. *SDS-PAGE analysis of active human placental HGF (100 ng protein) purified by TSK-heparin HPLC under reducing conditions (silver stain). The positions of the heavy chain (70 kd) and light chain (35 kd) are indicated.*

placental HGF even at high dilutions, although human HGF was slightly less immunoreactive than rabbit HGF (Figure 3). The antiserum had no significant reactivity with human plasminogen, despite a 38% sequence homology between HGF and plasminogen.[12]

In all specimens of normal placental tissue, the syncytiotrophoblast layer of the chorionic villi showed intense cytoplasmic immunoreactivity for HGF (Figures 4 and 5). Syncytial knots and syncytial sprouts showed intense cytoplasmic staining. In marked contrast, there was no or only minimal staining of the Langhans' cell layer (Figure 5). Cytoplasmic staining was present in some of the mesenchymal cells within the chorionic villi, particularly during the first trimester (Figure 5). Varying degrees of positive staining were present in the endothelium of the small vessels in the terminal villi. There was strong immunoreactivity for HGF in the endothelium of larger vessels in the stem villi. None of the control sections treated with nonimmune chicken serum revealed significant staining. The cytoplasmic immunoreactivity of trophoblastic, mesenchymal, and endothelial cells was removed by preincubation of the diluted anti-HGF serum with purified placental HGF. The basal plate of the placenta contained many large polygonal or elongated cells with an intense granular cytoplasmic staining for HGF (Figure 6) adjacent to similar cells that did not stain for HGF. On H&E-stained sections, it was difficult to distinguish the HGF-positive and HGF-negative cell populations. In general, the HGF-positive cells often had abundant amphophilic, slightly granular cytoplasm, and large, slightly irregular or lobulated nuclei with occasional prominent nucleoli. The HGF-negative cells generally were smaller and had homogeneous eosinophilic cytoplasm and round uniform nuclei. On sections stained with the Masson's trichrome technique, the HGF-positive cell population displayed a dark brown granular cytoplasmic staining, whereas the HGF-

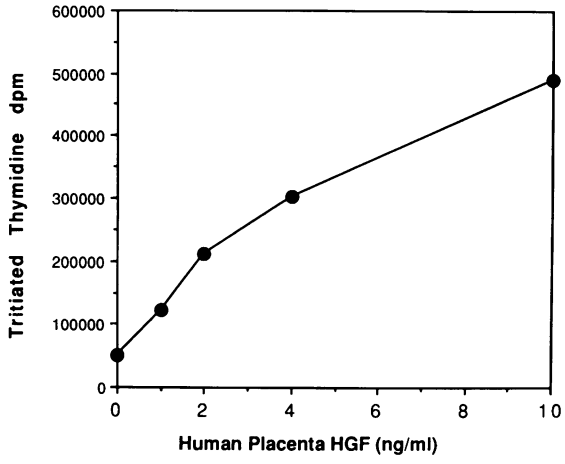


Figure 2. *Dose-response curve for the stimulation of the DNA synthesis in cultured rat hepatocytes by human placental HGF. The [^{3}H] thymidine uptake per culture is expressed as disintigrations per minute (dpm). The experimental conditions for the hepatocyte culture and the bioactivity assay were described previously.*[7]

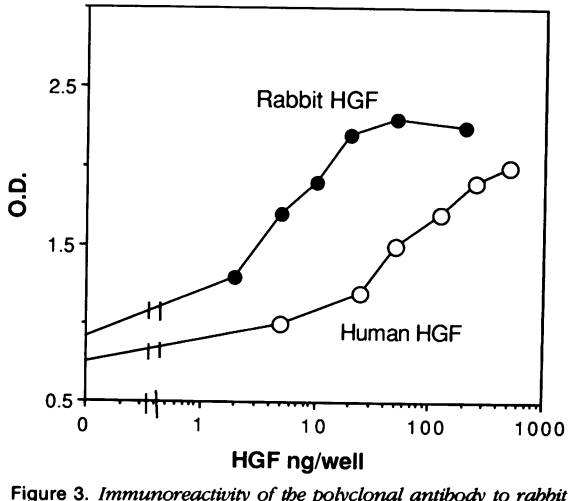
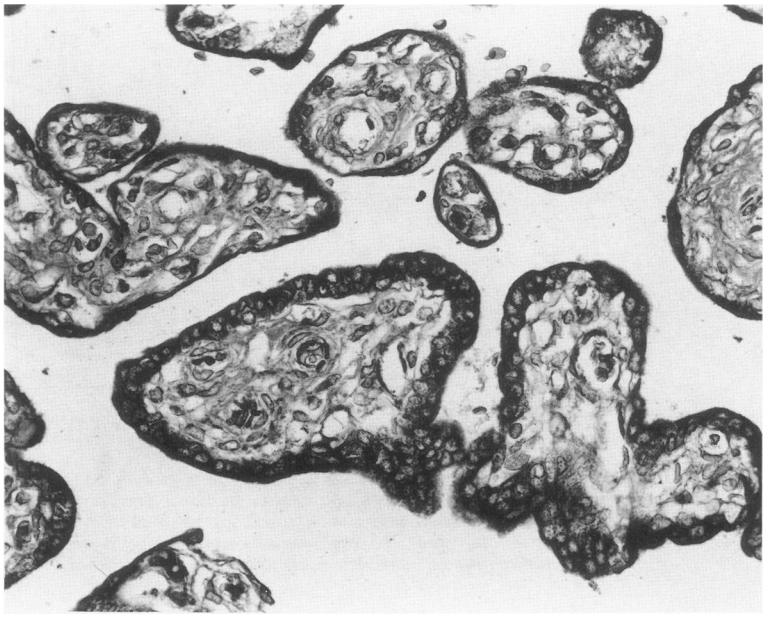


Figure 3. *Immunoreactivity of the polyclonal antibody to rabbit HGF with HGF obtained from rabbit serum and human placenta as determined by ELISA (OD = optical density at 492 nm).*



Figures 4–10. *Immunoperoxidase staining with polyclonal antibody to HGF or nonimmune chicken serum (negative controls); hematoxylin counterstain.* **Figure 4.** *Mature chorionic villi. There is prominent staining of the syncytiotrophoblastic lining of the villi. Original magnification, 325×.*

negative cells showed a pale homogeneous gray hue. On a PAS stain, many of the HGF-positive cells contained minute PAS-positive cytoplasmic granules that were not present in HGF-negative cells.

Umbilical Cord

We found intense cytoplasmic staining of the endothelium of the umbilical vessels (Figure 7) and of the amnionic epithelium at the surface of the cord. Some of the stromal cells of the cord demonstrated weak staining.

Fetal Membranes (Figure 8)

The amnion epithelium showed intense cytoplasmic staining in all sections of membrane rolls. The underlying amnionic stroma was negative for HGF. The trophoblast of the reflected chorion showed scattered cells with cytoplasmic immunoreactivity for HGF. The staining intensity of these cells, however, was not as intense as that of the amnionic epithelium. In the outer layer of the reflected membranes, scattered groups of HGF-positive cells were present. However we could not distinguish with certainty between decidual and trophoblastic cells.

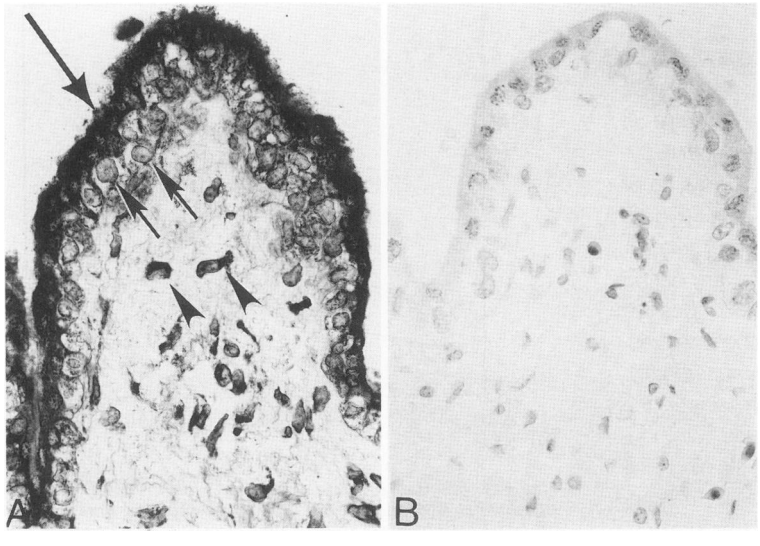


Figure 5. *First trimester villus.* **A:** *There is intense staining of the syncytiotrophoblast* **(long arrow).** *No staining is present in cells of the cytotrophoblast* **(short arrows).** *Staining is also present in some mesenchymal cells (arrow heads).* **B:** *Nonimmune serum control. Original magnification, 325×.*

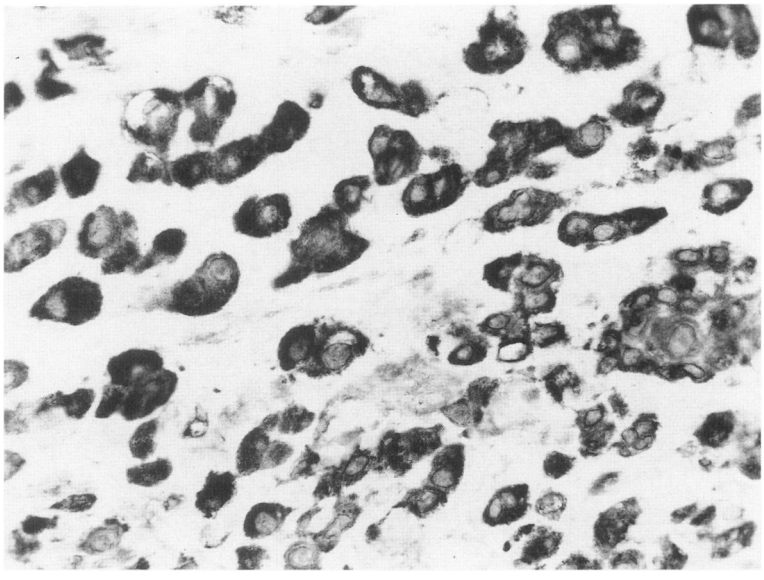


Figure 6. *Basal plate of mature placenta. There are single cells and small groups of extravillous trophoblastic cells with intense granular cytoplasmic staining. Original magnification, 400×.*

Blighted Ovum

The material examined consisted of spontaneously aborted products of conception at 9 weeks of gestation. The chorionic sac had been expelled *in toto* and was received intact in the laboratory. No fetus and amnion were identified on gross and microscopic examination of the entire material. The chorionic villi were filled with loose, nonedematous, avascular mesenchyme. The trophoblastic epithelium covering the villi displayed two cell layers. In contrast to the normal appearance of the cytotrophoblast with oval or cuboidal cells and clear cytoplasm, the cells of the inner layer were mostly flat and their cytoplasmic staining properties were identical to those of the syncytium. Both layers showed a strong immunoreactivity for HGF. Many mesenchymal cells of the villous cores also showed cytoplasmic staining for HGF.

Hydatidiform Mole

All seven cases examined by immunohistochemistry for the presence of HGF had the typical morphologic appearance of complete hydatidiform moles, with markedly hydropic villi, avascular villous mesenchyme, and absent fetal remnants and membranes. Varying degrees of tro-

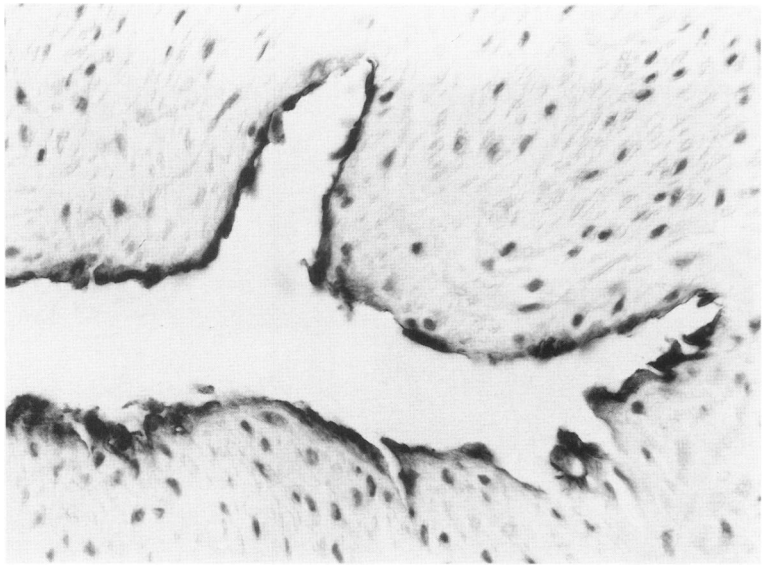


Figure 7. *Umbilical artery. There is strong staining of endothelial cells. Cells of the muscularis show no reaction. Original magnification, 520×.*

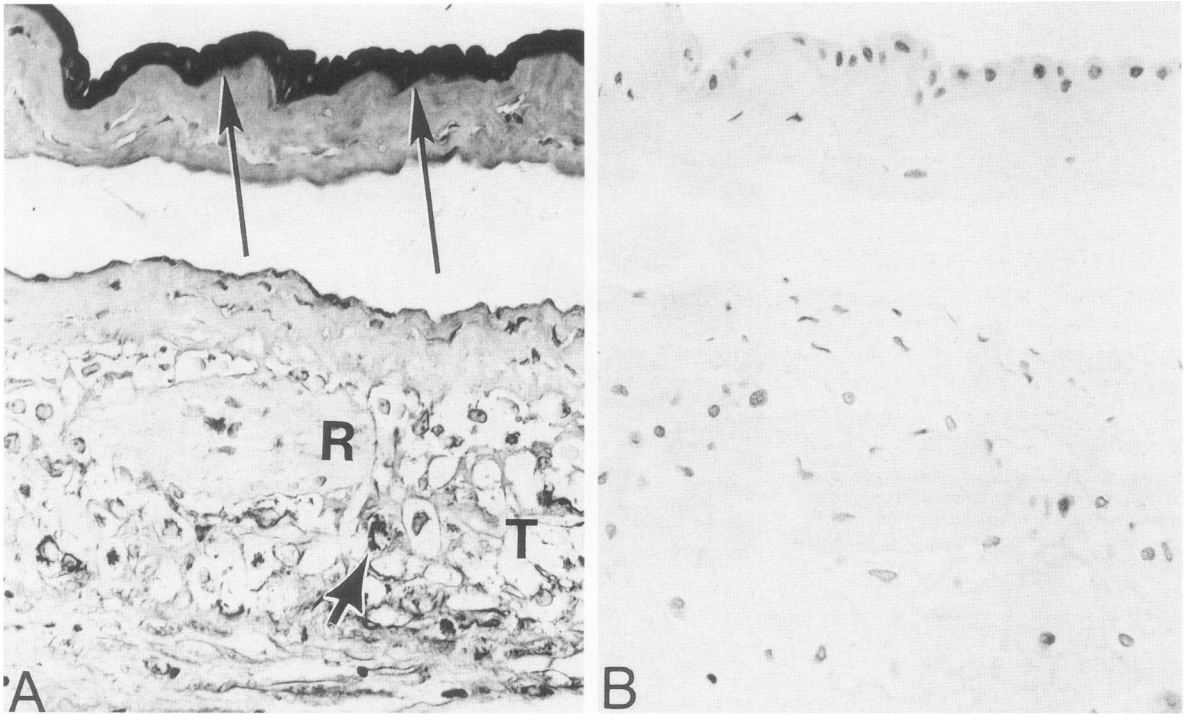


Figure 8. *Reflected fetal membranes.* **A:** *The amnion epithelium* **(long arrows)** *shows intense staining. In the trophoblast layer* **(T)**, *scattered cells reveal cytoplasmic staining* **(short arrow).** *(***R**, *residual villus).* **B:** *Nonimmune serum control. Original magnification, 250×.*

phoblastic proliferation were present. Clinically in all cases there was a history of markedly elevated levels of chorionic gonadotropin (hCG) in the maternal serum. In all cases the trophoblastic epithelium showed a strong cytoplasmic immunoreactivity for HGF that included sheets of proliferating trophoblastic cells (Figure 9). In some areas, the cytotrophoblast stained weaker as compared to syncytium and intermediate cells, whereas in most areas the staining of the cytotrophoblast and the syncytium was uniformly positive. Many of the villous mesenchymal cells stained positive for HGF.

Choriocarcinoma

In all eight cases of choriocarcinoma, neoplastic cells with a strong cytoplasmic immunoreactivity for HGF were present (Figure 10). However the staining pattern was less uniform as compared to normal trophoblast and hy-

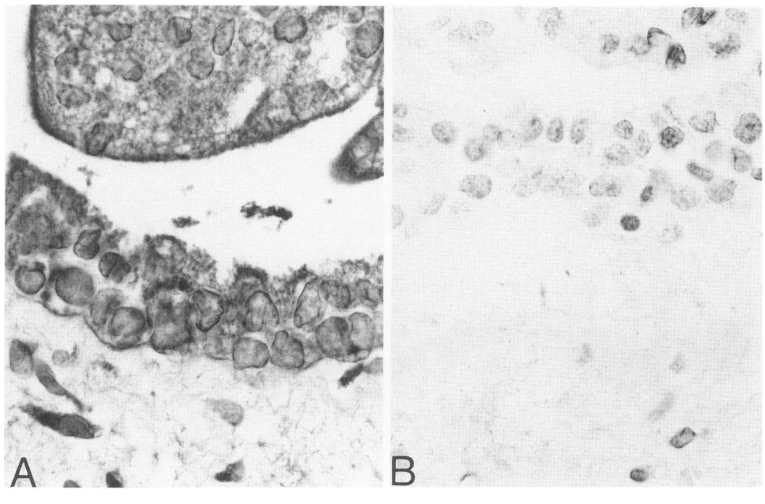


Figure 9. *Complete hydatidiform mole.* **A:** *The trophoblastic epithelium is hyperplastic and shows strong expression of HGF in its entire thickness. Some stromal cells are also positive for HGF.* **B:** *Nonimmune serum control. Original magnification, 680×.*

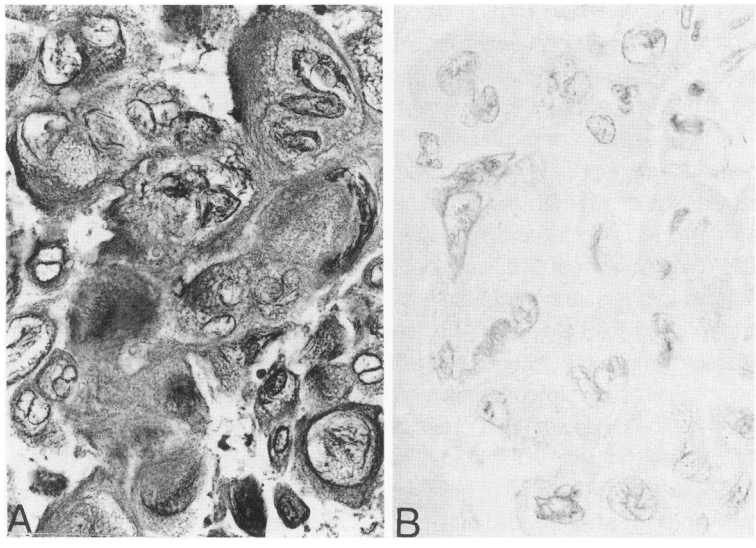


Figure 10. *Choriocarcinoma.* **A:** *The cytoplasm of the tumor cells strongly expresses HGF.* **B:** *Nonimmune serum control. Original magnification, 680×.*

datidiform moles. The proportion of immunoreactive cells varied considerably. Immunoreactivity for HGF was more intense and more frequent in syncytiotrophoblast and intermediate trophoblast as compared to the cytotrophoblast.

Discussion

Hepatocyte growth factor is a protein growth factor the primary structure of which has only recently been completely characterized.[10,11] The tissue distribution of HGF in the human has not yet been described.

Until now, plasma[7,9] and serum[8] were the only reported sources of human HGF. Increased serum levels of HGF were found in patients with severe liver injury.[9] In this study we demonstrate for the first time that the human placenta is a relatively rich source of HGF. The yield of HGF obtained from mature placentas (approximately 100 to 150 μg per placenta, or 200 to 300 μg/1000 g of fresh placental tissue) was approximately 200 times greater than that from human plasma (1 to 2.5 μg/l) described earlier.[7] These results, together with the fact that placenta tissue is readily available in many hospitals, render the placenta a suitable source for the purification of human HGF. The detection of high quantities of HGF mRNA in the placenta and the cloning of HGF mRNA from a cDNA library of human placenta[10] provides further evidence of HGF synthesis in the placenta.

Because the placenta is composed of a great variety of different cell types that are derived in part from the conceptus and in part from the mother, it was of interest to determine the localization of HGF by immunohistochemistry. We found that the entire cytoplasm of the syncytiotrophoblast was strongly positive for HGF in all specimens examined. A similar cytoplasmic immunoreactivity for protein hormones and other proteinaceous signal molecules has been demonstrated in endocrine cells of various organs and usually is considered to indicate that the protein is synthesized in the cell in question.[14] A different staining pattern, with highlighting of plasma membranes, is seen when receptor-bound proteins are visualized. Therefore it is reasonable to assume that the syncytium is the major site of HGF production in the placenta. Further evidence is provided by the presence of abundant rough endoplasmic reticulum, many polyribosomes, and Golgi complexes within the syncytium that are the ultrastructural correlates of active protein synthesis.[15]

Langhans' cells contained no significant amount of HGF, which is consistent with their paucity of cytoplasmic organelles and their function as stem cells. The weak staining for HGF in some mononuclear cells of the villous trophoblast may reflect the presence of transitional or intermediate cells between Langhans' cells and syncytium.[16] These transitional cells have already acquired some of the cytoplasmic characteristics of the syncytium but are still mononuclear.

The distribution of HGF within the villous trophoblast is similar to that of other placental proteohormones and growth factors. Chorionic gonadotropin,[17,18] human placental lactogen (hPL),[18] adrenocorticotropic hormone,[14] growth hormone,[14] β-endorphin,[19] interleukin-2,[20] and insulinlike growth factor-1[21] have been shown to be expressed predominantly by the syncytium rather than in the villous cytotrophoblast.

In the basal plate of the placenta there were two populations of cells with striking differences in their expression of HGF. This finding raised the question as to the nature of the cells involved. The great difficulty in distinguishing maternal decidual cells from extravillous tropho-

blastic cells that occur in the placental basal plate by routine histology, by determination of the sex chromatin in cases of male pregnancies, and even by ultrastructural examination has been emphasized repeatedly.[16,22–24] The trophoblastic cells usually show more abundant granular amphophilic cytoplasm and larger and more irregular nuclei, whereas the decidual cells contain small uniform round nuclei and homogeneous cytoplasm.[24] The discrimination of both cell populations is greatly facilitated by their staining characteristics on Masson's trichrome and PAS stains.[22,23] On sections stained with Masson's trichrome technique, the extravillous trophoblast usually shows a dark brown granular appearance, whereas the cytoplasm of the decidual cells has a uniformly grayish hue.[23] In the trophoblastic cells, there is a finely granular PAS-positive, diastase-resistant cytoplasmic stippling, while the decidual cells show diffuse faint staining with the PAS reaction.[22] By these criteria, the HGF-positive cells in the basal plate of the placenta were identified as extravillous trophoblasts. The decidual cells did not stain for HGF beyond the background level. This distribution parallels the immunolocalization of hPL in the basal plate of the placenta[24] and provides further evidence for the apparent role of the trophoblast in the synthesis of HGF.

The presence of HGF in the trophoblast in cases of blighted ovum and hydatidiform mole clearly demonstrates that the trophoblastic expression of HGF does not depend on the presence of a fetus, as is the synthesis of placental estriol, which is known to require an intact feto-placental unit.[25] Even during malignant transformation, the trophoblast may maintain its capability to synthesize HGF, as we were able to demonstrate in cases of gestational choriocarcinoma. The distinction between the HGF expression of syncytium and cytotrophoblast was less marked in hydatidiform moles and choriocarcinomas as compared to normal first-trimester villi. A similar loss of differentiation has been observed for the expression of hCG in choriocarcinomas.[26] If these are secondary changes or if the presence of HGF in the cytotrophoblast materially contributes to the trophoblastic proliferation in these lesions remains to be determined.

Based on the overall amount of syncytium in the placenta and its intense immunoreactivity for HGF, it may be assumed that the syncytium is the main source of placental HGF. However the presence of HGF in fetal membranes, fibroblasts, and endothelial cells of the chorionic villi, and in the endothelium of umbilical vessels demonstrates that the distribution of HGF is not limited to the trophoblastic epithelium. *In situ* hybridization for HGF mRNA will be required to provide the ultimate demonstration of HGF synthesis in different subsets of cells that are immunoreactive for HGF.

Although there is evidence that the extraembryonic membranes may support fetal development by regulating the levels of growth factors in the amniotic fluid,[3] few studies mentioned the distribution of growth factors or other proteinaceous signal molecules within these membranes. In addition to HGF, the amnionic epithelium contains corticotropin-releasing factor,[27] but no evidence of hPL production in the amnion has been found by immunohistochemistry or *in situ* hybridization.[28] The finding of HGF immunoreactivity in many trophoblastic cells of the reflected chorion represents evidence of the common developmental derivation of the membranous trophoblast and the trophoblast of the placenta proper. The intimate relationship of chorionic cells and decidual cells in the outer layer of the fetal membranes[29] is similar to that in the placental basal plate and renders the distinction of trophoblastic cells from decidual cells very difficult. In analogy to findings in the placental basal plate, it may be assumed that the HGF-positive cells in the outer layer of the reflected membranes are trophoblastic rather than decidual in origin.

Given the wide distribution of HGF within placental tissues, the most interesting question as to the function of placental HGF remains. The only known function of HGF is the stimulation of mitoses and cellular enlargement in cultured hepatocytes.[4] The presence of abundant HGF in the placenta, an organ that is characterized by a rapid proliferation of cells, strongly suggests that the growth-stimulating effect of HGF may not be limited to hepatocytes. The syncytium, a major source of placental HGF, does not contain mitotic figures and has been considered a 'mitotic end stage.'[16] In the underlying cytotrophoblast, however, mitotic figures are frequent. This suggests the possibility that HGF produced in the syncytium may stimulate the proliferation of the underlying cytotrophoblast in a paracrine fashion. Because the syncytial immunoreactivity for HGF is not noticeably reduced at full term, when the proliferative activity of the villous trophoblast is greatly diminished, it is also possible that HGF may elicit its action in an autocrine manner on the syncytium itself and may be involved in the regulation of syncytial maturation. Finally, because of the close spatial relationship of HGF-containing trophoblastic and maternal cells in the placental basal plate and the fetal membranes, HGF may be involved in regulating the marked enlargement of the uterus throughout gestation or, perhaps, the decidualization of the endometrium.

Whether HGF is released in significant amounts into the fetal or maternal circulation and regulates fetal growth and differentiation and/or maternal adaptation to gestation awaits future clarification. The recent demonstration of HGF-mRNA in fetal liver, kidney, and pancreas[30] indicates that the presence of HGF during gestation is not limited to the placenta. Clearly characterization of the HGF receptor and its tissue distribution would greatly facilitate the understanding of the role of HGF in the placenta as well as in other sites.

Acknowledgments

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