Identification of Somatomedin/Insulin-Like Growth Factor Immunoreactive Cells in the Human Fetus

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ABSTRACT. Somatomedins/insulin-like growth factors (Sm/IGFs) are present in blood and in extracts from multiple tissues of the human fetus and induce the proliferation of cultured human fetal cells. To identify the cellular location of immunoreactive Sm/IGF in human fetal tissues, we have performed immunocytochemistry in tissues from prostaglandin-induced human fetal abortuses of 12 to 20 wk in gestation. Every tissue studied except the cerebral cortex contains Sm/IGF immunoreactive cells. Cells staining positively include hepatocytes, hepatic hemopoietic cells, columnar epithelia of the pulmonary airways, intestine and kidney tubules, adrenal cortical cells, dermal cells, skeletal and cardiac muscle fibers, and pancreatic islet and acinar cells. Immunostaining was specific for Sm/IGFs, but because of the cross-reactivity of the antibodies it was not possible to determine whether the immunoreactivity represented Sm-C/IGF I, IGF II, or both. Liver contained the greatest proportion of immunoreactive cells, while the thymus and spleen had only a few immunostained cells. With the possible exception of dermal and some adrenal cortical cells, the immunoreactive cells do not appear to be the primary sites of Sm/IGF synthesis, because parallel *in situ* hybridization histochemical studies using Sm/IGF oligodeoxyribonucleotide probes show that Sm/IGF mRNAs are localized predominantly to fibroblasts and mesenchymal cells. Therefore the immunoreactive cells identified in this study may define sites of action of Sm/IGFs. (*Pediatr Res* 22: 245–249, 1987)

Abbreviations

Sm, somatomedin
IGF, insulin-like growth factor
Sm-C/IGF I, somatomedin-C/insulin-like growth factor I
IGF II, insulin-like growth factor II
GH, growth hormone
NGF, nerve growth factor
PBS, phosphate-buffered saline
BSA, bovine serum albumin
RBC, red blood cells

Sm/IGFs (Sm-C/IGF I and IGF II) appear to play an important role in the stimulation of human fetal growth (1, 2). Both peptides can be detected in human fetal serum from as early as 13 wk gestation (1–5), specific receptors for each Sm/IGF are distributed widely in human fetal tissues (1, 2), and both are mitogens for cultured human fetal cells (1, 2, 6, 7). Because Sm/IGFs appear to be synthesized in many tissues, they are thought to act at or near their sites of synthesis as paracrine or autocrine growth factors (8). Support for this in man comes from the finding that multiple human fetal tissues of 9 to 19 wk gestation contain immunoreactive Sm-C/IGF I in far greater concentrations than can be accounted for by blood entrapment (9), and that multiple human fetal tissues of 16 to 20 wk gestation contain mRNAs for both Sm-C/IGF I and IGF II (20).

To identify those cells where Sm/IGFs are localized in the human fetus, we have performed immunocytochemistry on human fetal tissues and have identified cells showing positive immunoreactivity to Sm/IGFs that are of epithelial or mesodermal origin and, in general, are differentiated.

MATERIALS AND METHODS

Collection and preparation of tissues. Prostaglandin-induced human fetal abortuses of 12 to 20 wk in gestation were collected within 30 min of delivery after obtaining permission from the ethics committees of the respective institutions. Tissue samples (1 cm³) were fixed by immersion in 2% paraformaldehyde and 2% glutaraldehyde in 70 mM phosphate buffer, pH 7.0, at 4° C for 18 to 24 h. The tissues were washed in several rinses of 0.01 M PBS, pH 7.4, over a period of 2 days, dehydrated in an ascending ethanol series (70, 90, and 100%) and toluene, and embedded in paraffin. Tissue sections of 10-μm thickness were prepared by standard techniques, mounted on slides, baked at 45° C for 48 h, and stored at room temperature until processing for immunocytochemistry.

Immunocytochemistry. Immunocytochemistry was performed by the avidin-biotin-peroxidase technique (10) as modified by Towle *et al.* (11). Briefly, tissue sections were deparaffinized in toluene, rehydrated in descending ethanol series (100, 90, and 70%) into PBS and incubated in 1% hydrogen peroxide in PBS for 15 min to eliminate endogenous peroxidase activity. After three additional PBS rinses, tissue sections were incubated in 0.12 mg/ml trypsin (Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS for 5 min followed by three more PBS rinses.

One of the following three primary antibodies were used: (a) a mouse ascitic fluid-derived monoclonal antibody against Sm-C/IGF I (13, 14) was used at a dilution of 1:1000, (b) a rat antiserum raised against glutaraldehyde conjugates of human IGF II c-

peptide and keyhole limpet hemocyanin was used at a dilution of 1:3000, and (c) a rabbit antiserum against MSA (15) was used at a dilution of 1:1000. Sections were incubated with the antibodies in humidified chambers for 48 h at 4° C. Although the monoclonal antibody raised against SM-C/IGF I was originally reported to have only 5% cross-reactivity with IGF II (13, 14), subsequent studies with different preparations of purified IGF II indicate that cross-reactivity is approximately 50%. Cross-reactivity studies of the antiserum to MSA also indicates that it recognizes both human IGF II and Sm-C/IGF I (15). Immunocytochemistry with rabbit antisera that are highly specific for human SM-c/IGF I in radioimmunoassay (16) resulted in a negative immunostaining. Therefore, these antisera were not used in the study.

Following incubation in primary antibody, the tissue sections were rinsed in PBS, then incubated in appropriate biotinylated second antibody (Vector Laboratories, Burlingame, CA; 1:500 dilution in PBS containing 0.1% BSA) at room temperature for 2 h. Following additional PBS rinses, tissue sections were incubated in avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame CA) at room temperature for 2 h, then rinsed again in PBS. The antibody bound peroxidase was visualized by reacting with 0.075% diaminobenzidine (Aldrich Chemical Inc., Milwaukee, WI) in Tris-HCl buffer, pH 7.6, containing 0.002% (v/v) hydrogen peroxide. Tissue sections were then counterstained with 0.05% toluidine blue, dehydrated in ascending ethanol series and xylene, and mounted with coverslips using Permount (Fisher Scientific, Springfield, NJ).

Immunospecificity studies. Several experiments were performed to verify that the antibodies used recognized Sm/IGFs specifically. There was an absence of immunostaining when preimmune or nonimmune rat or rabbit sera or nonimmune mouse ascitic fluid were substituted for the primary antibodies. Immunostaining also was not observed when antisera or immune ascitic fluid were immunoabsorbed with 1 to 10 μM of partially purified preparations of either Sm-C/IGF I and IGF II (17) prior to immunostaining. Immunostaining was not altered, however, when antisera or immune ascitic fluid were incubated with 1 to 10 μM concentrations of insulin, epidermal growth factor, platelet-derived growth factor and nerve growth factor. Because the antibodies were known to cross-react with both Sm/IGFs, immunoblot studies were performed with pure Sm-C/IGF I and IGF II immobilized on nitrocellulose paper (18) to determine if cross-reactivity was significant under conditions similar to those used in immunocytochemistry. At the dilutions used for immunocytochemistry, each antibody gave similar intensity of immunostaining against both peptides. Therefore, while the antibodies identified Sm/IGFs specifically, none was capable of distinguishing between Sm-C/IGF I and IGF II.

RESULTS

Identical patterns of immunostaining were observed with each antibody in every tissue studied. Immunostained cells of at least one type were seen in all the tissues except the cerebral cortex. The proportion of immunostained cells varied among the tissues studied; the greatest proportion was observed in the liver and adrenal gland and the least in spleen and thymus. Although considerable variation in the intensity of immunostaining was observed among different cell types within the same tissue, the intensity of staining was not pronounced, suggesting that the abundance of Sm/IGFs within the immunopositive cells was not high.

Liver. Moderate staining was observed in about 70% of the hepatocytes (Fig. 1 *A* and *B*). The stained and unstained hepatocytes had the same morphology, and the reason for the difference in immunoreactivity is not known. Fetal liver also contained intensely immunostained small, round nucleated hemopoietic cells with deeply basophilic nuclei. These cells, the identity of which is unclear, were observed in groups in the walls and within

the lumina of the hepatic blood vessels. Similar intensely immunoreactive cells also were observed in other tissues. The mature RBCs within the blood vessels and sinusoids were unstained.

Lung. Moderate immunostaining was observed in the columnar epithelium of the primitive airways and lung fluid within the airways (Fig. 1*D*). The columnar epithelium of both large and small bronchioles were stained, but the intensity of staining varied among the bronchioles. No immunostaining was observed in the pleural lining and the connective tissue of the lung parenchyma.

Kidney. Moderate immunostaining was seen in the lining columnar epithelium of the proximal and distal convoluted tubules, and weak staining was observed in the collecting ducts (Fig. 1*F*). No immunostaining was observed in the capsule, tubules of the nephrogenic zone, loops of Henle, connective tissue parenchyma, or glomeruli.

Adrenal. About 60% of the parenchymal cells in the mature adrenal cortex were immunostained, but the fetal zone of the cortex, and the medulla contained no immunoreactive cells (Fig. 1*H*).

Pancreas. In 12- to 15-wk fetuses (when the islet cells have not yet organized), positive immunostaining was observed in the epithelial cells, the presumptive progenitor cells of the exocrine, or endocrine pancreas. In the older fetuses, staining predominated in the islets and was minimal in the exocrine pancreas.

Stomach and small intestine. Throughout the gastrointestinal tract, only the epithelial cells of the mucosa showed positive staining (Fig. 1*G*). The columnar cells that lined the villi of the intestine stained more intensely than cells lining the crypts.

Thymus and spleen. Groups of immunostained cells having the morphology of immature hemopoietic precursors were observed in the corpuscles and interlobular septa of the thymus and were distributed widely in the parenchyma of the spleen. Most lymphoid cells in the thymic lobules and splenic corpuscles showed no immunostaining.

Skeletal and cardiac muscle. Moderate immunostaining was observed in the fibers of both skeletal (Fig. 1*E*) and cardiac muscles, but none was seen in the fibrous sheaths and septa.

Skin. Skin from the anterior abdominal wall showed minimal immunostaining in the stratum granulosum of the epidermis and moderate staining of the deep layers of the dermis (Fig. 1*I*). No immunostaining was observed in the adnexae of the skin, including hair follicles and sweat glands.

DISCUSSION

Using immunocytochemistry, we have shown that multiple tissues from 12- to 20-wk human fetuses contain cells with Sm/IGF immunoreactivity. Because the antibodies used in this study recognize both Sm-C/IGF I and IGF II, identification of specific Sm/IGFs was precluded. Negative immunostaining with an antiserum which is highly specific for Sm-C/IGF I in radioimmunoassay (15), however, suggests that the immunoreactivity may be attributed predominantly to IGF II. Both SM-C/IGF I and IGF II peptides are probably present in multiple fetal tissues, however, because (a) immunoassayable Sm-C/IGF I is readily detected with a highly specific antibody in extracts of many tissues obtained from human fetuses during the first half of pregnancy (9), (b) specific Sm-C/IGF I and IGF II mRNAs are present in many human fetal tissues of similar gestation (20) (Han VKM, Lund PK, D'Ercole AJ, unpublished observations), and (c) Sm/IGF mRNAs can be localized to fibroblasts and mesenchymal cells by *in situ* hybridization histochemistry using specific oligodeoxyribonucleotide probes to non-homologous regions of both Sm-C/IGF I and IGF II mRNAs (20).

Bennington *et al.* (18) have reported the localization of Sm-C/IGF I in human fetal and adult tissues by an immunoperoxidase technique using a rabbit antiserum to human Sm-C/IGF I. Although a detailed description is lacking, the distribution of
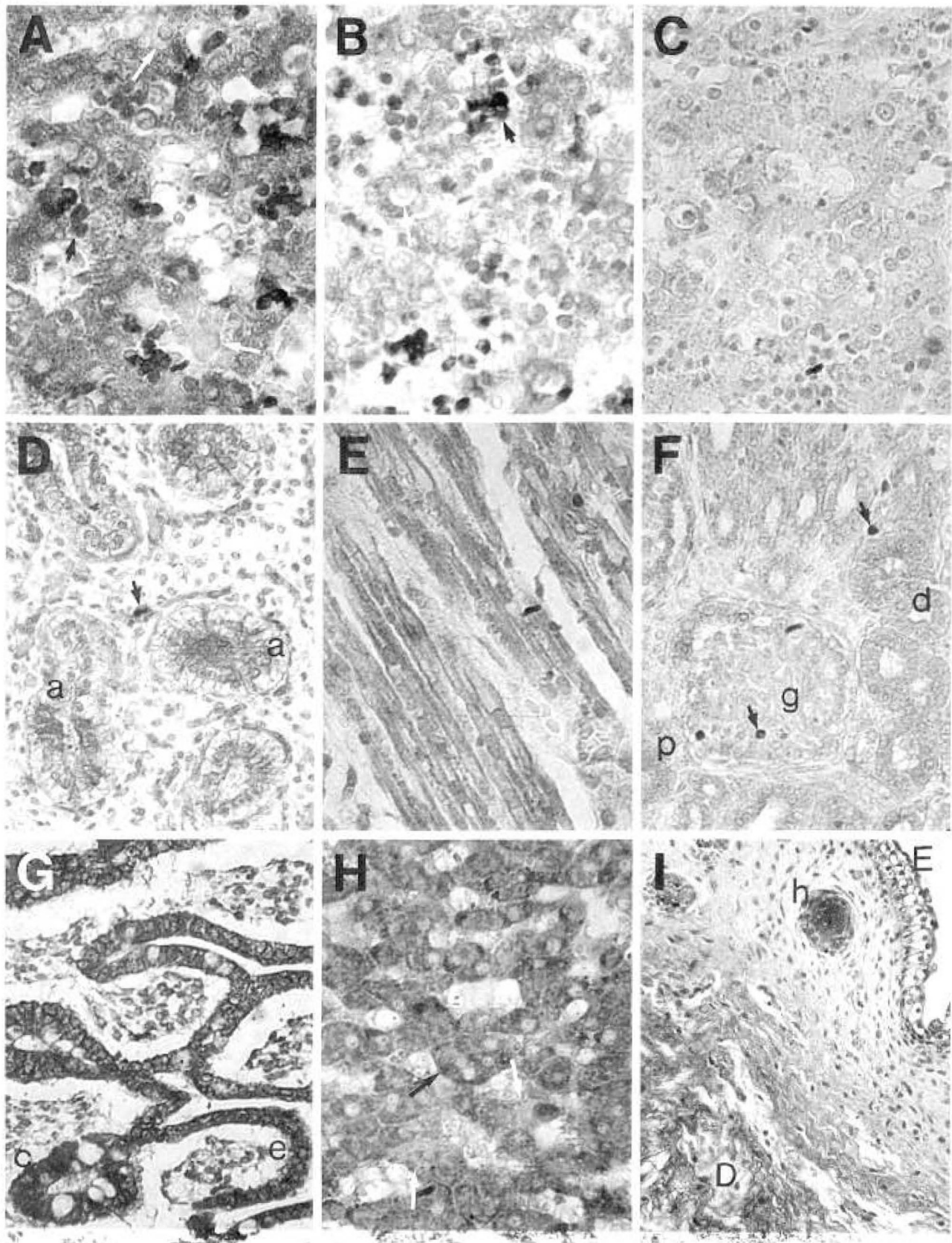
Fig. 1. Photomicrographs of human fetal tissues immunostained with antibodies against Sm/IGFs by the avidin-biotin peroxidase technique, using diaminobenzidine as a color agent. *A*, human fetal liver immunostained with Sm 1.2, a monoclonal antibody raised against Sm-C/IGF I; the *white arrows* point to unstained hepatocytes, the *small dark arrow* points to an intensely stained hemopoietic cell. *B*, human fetal liver, immunostained with an antiserum against IGF II c-peptide; arrows, same as *A*. *C*, human fetal liver, immunostained with a preimmune nude mouse ascitic fluid (similar results were obtained with preimmune and nonimmune rabbit and rat antisera, and following immunoabsorption of antibodies with 1–10 μM Sm/IGFs). *D* to *G*, human fetal tissues immunostained with Sm 1.2. *D*, Lung. *a*, primitive airway filled with fetal lung fluid; the arrow points to an immunopositive hemopoietic cell. *E*, Skeletal muscle. *F*, Kidney. *g*, glomerulus; *p*, proximal convoluted tubule; *d*, distal convoluted tubule; the arrows point to hemopoietic cells. *G*, intestine. *e*, columnar epithelium of a villous; *c*, columnar epithelium of a crypt of Lieberkuhn. *H*, adrenal. *C*, adrenal cortical cells; the *dark arrow* points to a stained cell and the *white arrows* point to unstained cells. *I*, Skin. *E*, epidermis; *D*, dermis; *h*, hair follicle.

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Sm-C/IGF I immunoreactive cells appears to be similar to that in our study. Similarly, Andersson *et al.* (19) recently reported identification of Sm-C/IGF I immunoreactive cells in several tissues from adult rats. Although their antibodies differ in specificity from ours, and the species and ages of the animals studied are also different, the findings in the two studies are similar.

One interpretation of our findings and those of others is that immunostaining identifies Sm/IGF synthesizing cells that are dispersed widely in many tissues. We believe that such a conclusion may be erroneous, because immunocytochemical techniques only identify the cellular location of peptides possessing specific antigenic determinants; such techniques do not indicate whether an immunostained cell is a site of synthesis of the antigen or whether it has accumulated the antigen from another source. Using *in situ* hybridization histochemistry with specific oligodeoxyribonucleotide probes for mRNAs encoding Sm-C/IGF I and IGF II, we have observed that Sm/IGF transcripts in human fetal tissues are localized predominantly in fibroblastic and mesenchymal cells (20). In general, these Sm/IGF mRNAs are localized to connective tissue elements such as organ capsules and septa. Furthermore, most of the immunopositive cells observed in the present study did not possess Sm/IGF mRNAs in significant abundance to be identified by *in situ* hybridization. While this lack of concordance among cells identified by the two techniques suggests that most of the immunostained cells do not synthesize Sm/IGFs, it is possible that the mRNAs of some fetal cells are unstable, are not translated, or result in production of a peptide form that is not recognized by the antibodies used. On the other hand, immunostained cell may have sequestered Sm/IGF from other sources by cell surface binding with subsequent internalization (21) and sequestration in endoplasmic reticulum, as has been shown for vascular endothelium (22). Moreover, the immunoreactive cells may be sites of degradation of the peptides. Regardless of the mechanisms involved, the immunolocalization of Sm/IGF to specific cells *in vivo* is likely to have physiologic significance.

The observation that fibroblasts, which are rich in Sm/IGF mRNA and are capable of Sm/IGF synthesis in culture (23–25), usually exhibit no immunostaining suggests that these cells have little capacity to store Sm/IGFs. A paucity of intracellular storage is supported by the delay of 4 to 8 h in a rise of Sm-C/IGF I in tissues and plasma following the injection of GH into hypophysectomized rats (8) and a similar delay when GH is administered in man (26, 27). In contrast, cultured human fetal hepatocytes appear to store Sm/IGFs since they immunostain for these peptides (28). This Sm/IGF is released from both rat and human isolated fetal hepatocytes under hormonal control (28, 29). It is not yet known whether the Sm/IGF present and released is due to sequestration or *de novo* synthesis.

In most of the tissues we examined, the connective tissue cells that possess Sm/IGF mRNAs are near the cells that exhibit Sm/IGF immunoreactivity. This juxtaposition of cells that synthesize Sm/IGFs with those that may respond to Sm/IGFs supports the paracrine hypothesis of action of these peptides. The dermis (and possibly adrenal cortex) may be an exception, because in this tissue immunostaining and *in situ* hybridization occur in the same cells. If this is the case, an autocrine mechanism of growth control could be operative (30, 31). The observation that Sm/IGF immunoreactivity is prominent in differentiated cells support the concept that these peptides are important mediators of differentiated cell functions (30–33), as well as proliferation.

The results of the present study can be interpreted in a number of ways. We believe, however, that these immunolocalization studies indicate sites where Sm/IGFs are accumulated and/or act given the evidence from *in situ* hybridization histochemistry that most of the immunostained cells are not the major sites of Sm/IGF gene expression. Large *et al.* (34) found markedly different abundances of NGF and its mRNA in different brain regions. Although these alterations in relative abundance may be explained by neuronal retrograde transport of NGF through

axons, it seems possible that other mechanisms, such as those we have postulated, may be responsible for the discrepancies in Sm/IGF immunolocalization and *in situ* hybridization histochemistry that we have observed.

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