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Insulin-Like Growth Factor-I (IGF-I) Colocalizes with IGF-Binding Proteins (IGFBPs) in Mouse and Rat Ovary

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The cellular localization of insulin-like growth factor (IGF)-I mRNA, IGF-I peptide, and IGF-binding proteins (IGFBPs) was examined in mouse and rat ovaries through use of *in situ* hybridization and immunohistochemical methods. IGF-I mRNA was found to be most abundant in granulosa cells, although lower levels were also detected in cells of the theca interna, stroma, and corpus luteum. In contrast, IGF-I immunoreactivity was undetectable or low in granulosa cells, weak and variable in oocytes, high in theca interna and the corpus luteum, and highest in the stroma. Antibodies directed against IGFBP-2, 3, and 5 yielded similar patterns of immunoreactivity to that observed for IGF-I peptide. The results indicate that IGF-I is synthesized in ovarian follicles, and that IGF-I of ovarian or systemic origin becomes localized to sites containing IGFBPs in the ovary.

Key Words: Ovarian follicle; *in situ* hybridization; immunohistochemistry.

Introduction

An element important for the regulation of ovarian follicular development is insulin-like growth factor (IGF)-I. In the rat, the ovary contains high levels of IGF-I gene transcripts (Murphy et al., 1987) that are primarily localized to granulosa cells of growing follicles (Oliver et al., 1989; Zhou et al., 1991). Because IGF-I mRNA levels decrease in degenerate follicles and because IGF-I has been shown to amplify the actions of gonadotrophins in the rat ovary, it has been implicated in the process of follicular development (*see* Guidice, 1992 and Adashi, 1993 for review). However, the action of IGF-I appears to be regulated at several levels, including multiple processing patterns of the gene transcript (Lund, 1994) and interaction of IGF-I with IGF-binding (IGFBPs) proteins (Adashi, 1993). In the latter case, it has been shown that IGFBP-1 (Adashi et al., 1993), IGFBP-2 (Bicsak et al., 1990), and IGFBP-3 (Ui et al., 1989; Adashi et al., 1990) can all inhibit FSH-induced estradiol and progesterone synthesis in rat granulosa cells. The mechanism by which IGFBPs inhibit responses of granulosa cells to FSH could be through sequestration of IGF-I, since excess exogenous IGF-I can abolish IGFBP inhibition of FSH activity (Adashi, 1993). The IGFBP activity itself may be regulated by specific proteases (Liu et al., 1993). Clearly, the factors that modulate IGF-I activity are complex and not well understood.

To date, a number of investigations have been carried out on various components of the IGF system in relation to ovarian function. However, many of the studies have been conducted on freshly isolated and cultured granulosa cells from ovaries of a number of different species. Few studies have simultaneously investigated multiple components of the IGF-I system that include gene transcription, IGF-I peptide localization, and IGFBP distribution in one system or tissue. Even in important and widely used models like the mouse and rat, investigations at the level of the ovary are incomplete. For this reason, we initiated a study to test the hypothesis that there was IGF-I gene expression in granulosa cells of growing follicles in the rat and mouse ovary, and consequent accumulation of IGF-I peptide, providing for a paracrine role of IGF-I in enhancement of FSH-induced synthesis of estradiol and progesterone. The investigations employed a combination of *in situ* hybridization to localize IGF-I mRNA in mouse and rat ovaries and immunohistochemical methods to localize IGF-I peptide and IGFBPs. Interestingly, we observed that IGF-I immunoreactivity was not abundant in granulosa cells that contained high levels of IGF-I mRNA. Rather, IGF-I immunoreactivity was detected at the highest levels in the stroma, theca, and corpus luteum, the same sites in the ovary in which high levels of immunoreactivity for IGFBP-2, 3, and 5 were observed. Details of the sites of localization of IGF-I and IGFBPs in healthy and degenerating follicles, and in ovaries from untreated and hormonally stimulated animals are described herein.

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Results

IGF-I mRNA Localization

Prior to use for *in situ* hybridization experiments, the IGF-I cDNA probe was annealed to a Northern blot of poly(A) + RNA from rat ovary. It hybridized primarily to RNA molecules approx 1 kb in length, a result virtually identical to that described previously by Murphy et al., (1987a,b). When used for *in situ* hybridization experiments on sections from mouse ovaries, the IGF-I antisense riboprobe hybridized predominantly to granulosa cells. This is shown as a dark (black) signal in the photomicrograph in Fig. 1 (panels C, D, and E). Weaker signals for IGF-I mRNA abundance appear as dark gray in Fig. 1. In color photographs, the signal obtained with the staining procedure is dark blue on a background of unstained cells (Fig. 2). Again, strong signals are associated with the granulosa cell compartment. Virtually identical results were obtained on hybridization of sections of rat ovary (data not presented).

A number of experiments were carried out to validate the accuracy of the *in situ* hybridization procedures we used for localization of IGF-I mRNA. Perhaps the most convincing was our ability to repeat and confirm the predominant localization of IGF-I mRNA to granulosa cells of ovarian follicles described in earlier *in situ* hybridization experiments on rat ovary by Oliver et al. (1989) and Zhou et al. (1991). In addition, when we used an antisense cRNA probe for the U2 snRNA gene product involved in processing of primary transcripts, the signal was localized to nuclei as predicted (Fig. 2E), whereas the IGF-I antisense cRNA probe hybridized primarily, as expected, to the cytoplasm (Fig. 2F). Signals were not detectable in RNase-treated sections (Fig. 1A), and only uniform background staining was obtained when DIG-labeled sense probes were used (Fig. 1B).

The nonradioactive detection system employed in these studies has provided improved resolution for localization of IGF-I transcripts in the ovary. Although it is clear that IGF-I mRNA levels are highest in granulosa cells, careful examination of hybridized sections reveals several other interesting features of IGF-I mRNA distribution. In most follicles, IGF-I mRNAs were evenly distributed across the granulosa cell compartment (Fig. 2F), but levels varied between follicles. In primary follicles, granulosa cells stained positively, although the signal was weaker than in small secondary follicles. The strongest signals for IGF-I mRNA were found in large secondary and early antral follicles (Figs. 1C, 2B, and 2F). In late antral follicles, a gradient of expression of the IGF-I gene was observed. The signal was weakest in granulosa cells lining the basement membrane and strongest in cells lining the antrum and in cumulus cells (examples are present in Fig. 1D and E). This gradient, however, was observed only in approximately half of the late antral follicles, and was independent of the presence or absence of visible signs of degeneration in the follicle. Levels of IGF-I mRNA were lower in follicles showing signs of morphological degeneration (Fig. 2B). In some degenerating follicles, indicated by advanced degeneration of the oocyte and disruption of the basement membrane (*see* morphology in Fig 3B), a gradient of expression was present with a strong signal in the cells closest to the remnants of the oocyte, whereas cells furthest away from the oocyte were stained weakly or not at all.

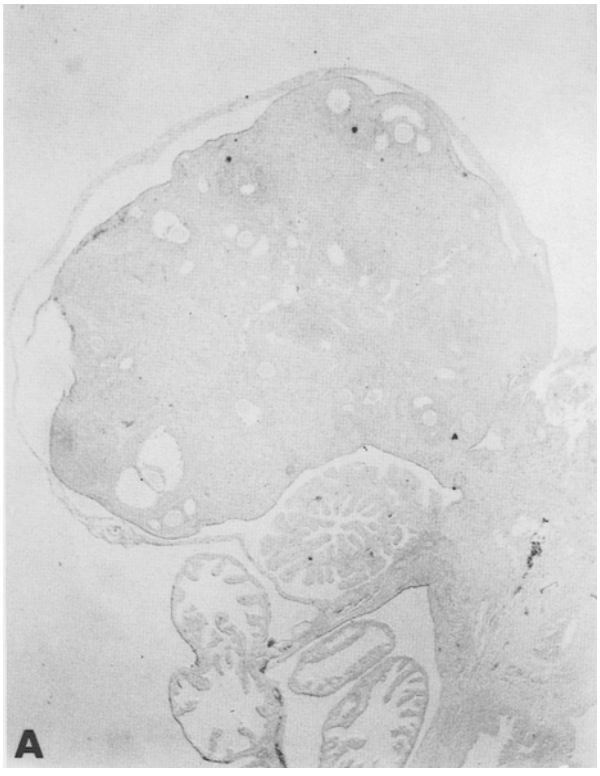
Positive staining for IGF-I mRNA was also observed in cells of the theca interna, whereas those of the theca externa were negative (Fig. 2A, D). Staining intensity was variable and was not correlated with the stage of development of the follicle. Moderate staining of oocytes was observed in some primordial, in primary, and in small secondary follicles (Fig. 2C). At further stages of development, oocyte staining was virtually absent (Fig. 2B). Staining of the corpus luteum was positive, but weak, and was often unevenly distributed across this tissue. Staining in stroma was generally weak and was heterogenous in some areas where groups of large, round cells were unstained, but surrounded by cords of weakly stained cells.

No differences were observed in the distribution and intensity of the signal for IGF-I gene transcripts between untreated ovaries and ovaries of mice treated with eCG (Fig. 1C, D), even though the signal was several-fold stronger in the uteri of mice treated with eCG compared to untreated mice (data not shown). In ovaries of mice treated with eCG + hCG, the distribution of the signal was the same, but by visual inspection, staining was enhanced in the corpus tuteum, theca interna, and stroma. No difference was observed between the mouse and rat in terms of the distribution of IGF-I gene transcript in the ovary.

Immunohistochemistry

The positive immunohistochemical procedure reaction with each protein (IGF-I, IGFBP-2, 3, and -5) was detected by brown cytoplasmic staining. Blue nuclei represent counterstaining with hematoxylin. No background signal was generated by the biotinylated antibody, the avidin–biotinylated peroxidase complex, or by residual endogenous peroxidase activity. In addition, control sections to which nonimmune rabbit serum was applied at a dilution of 1:500 showed no staining (Fig. 3G). A further assessment of the specificity of the IGF-I peptide signal was accomplished by

Fig. 1. *(opposite page)* Localization of IGF-I mRNA in mouse ovary by *in situ* hybridization. **(A)** IGF-I antisense probe hybridized to ovary section from untreated mouse that was incubated with RNase prior to hybridization, showing no staining. **(B)** U2b snRNA sense probe hybridized to ovary section from untreated mouse showing no staining. **(C, D, E)** IGF-I antisense probe hybridized to ovary section from untreated mouse (C), ovary section from a mouse treated with eCG (D) or with eCG + hCG (E) showing dark gray to black staining over light gray background. 20X Magnification.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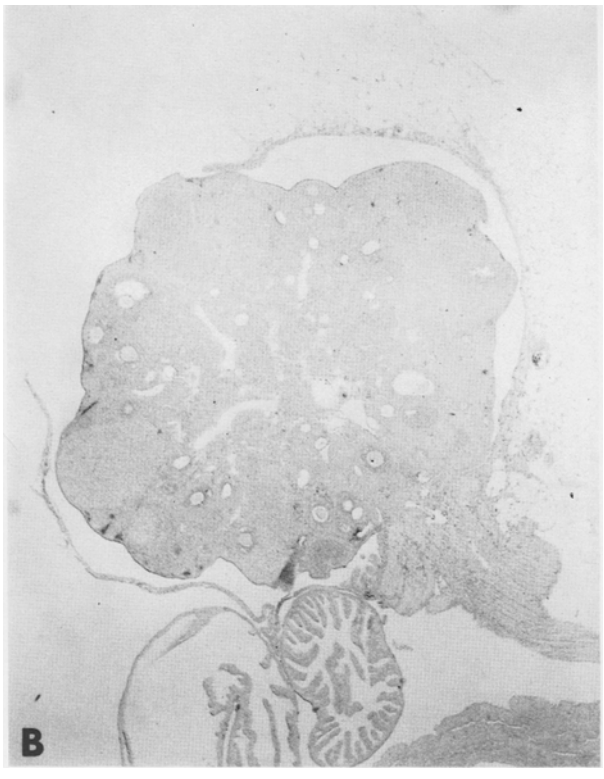
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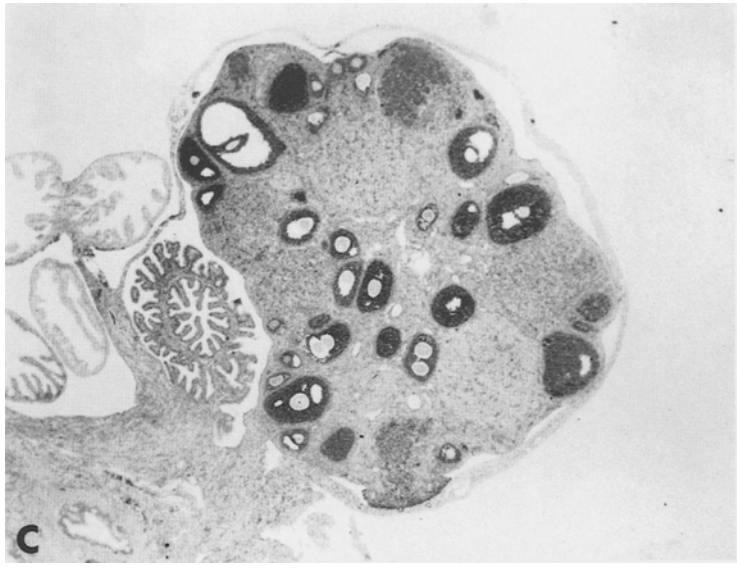
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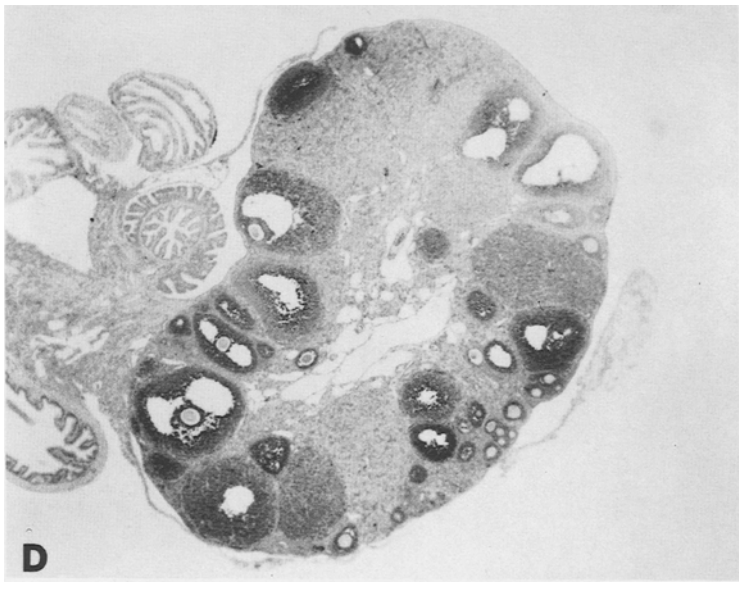
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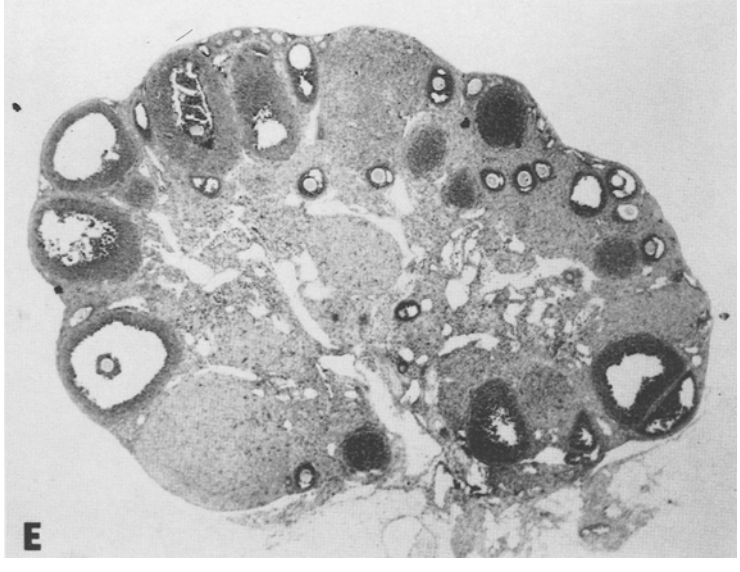
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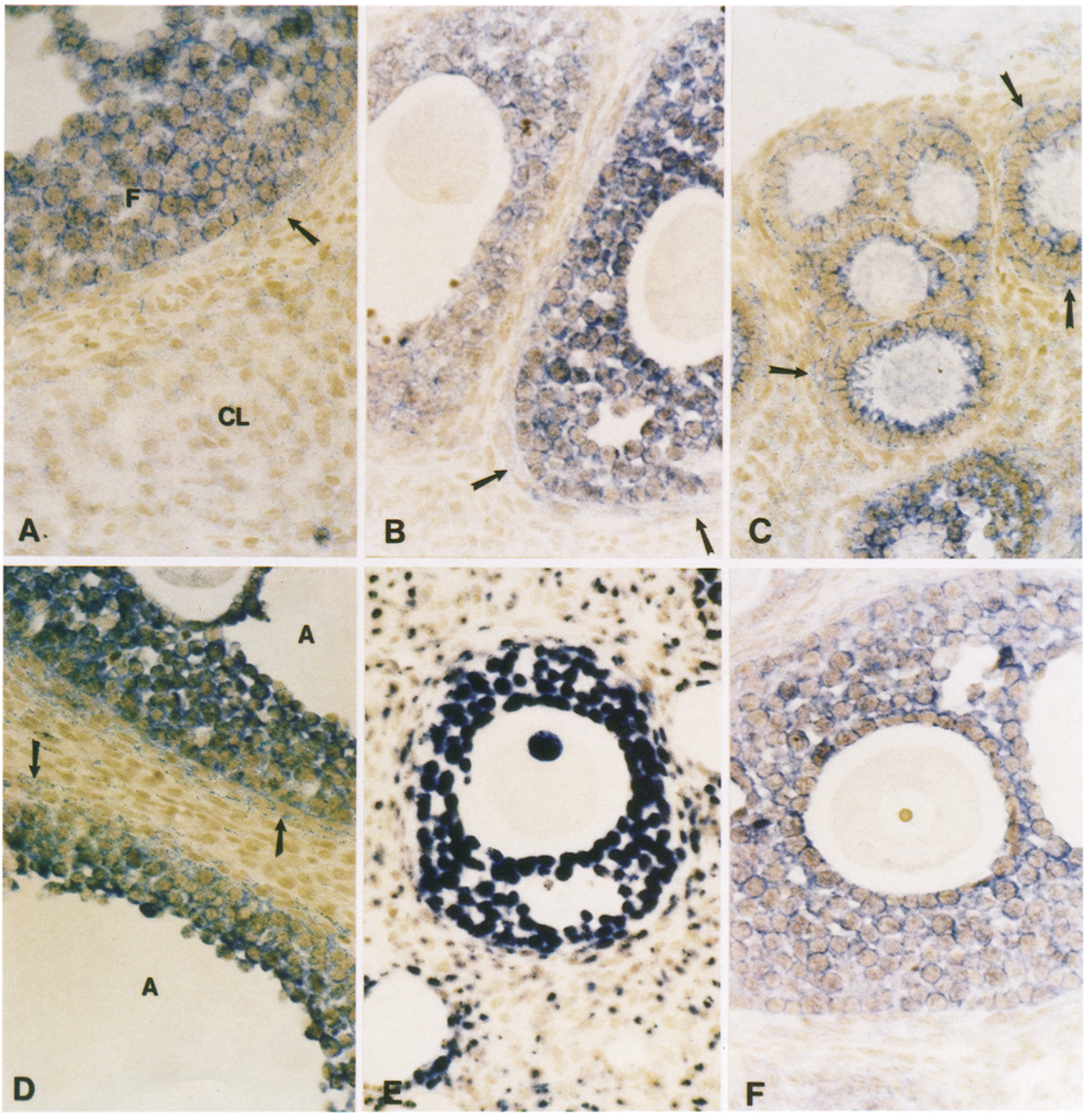
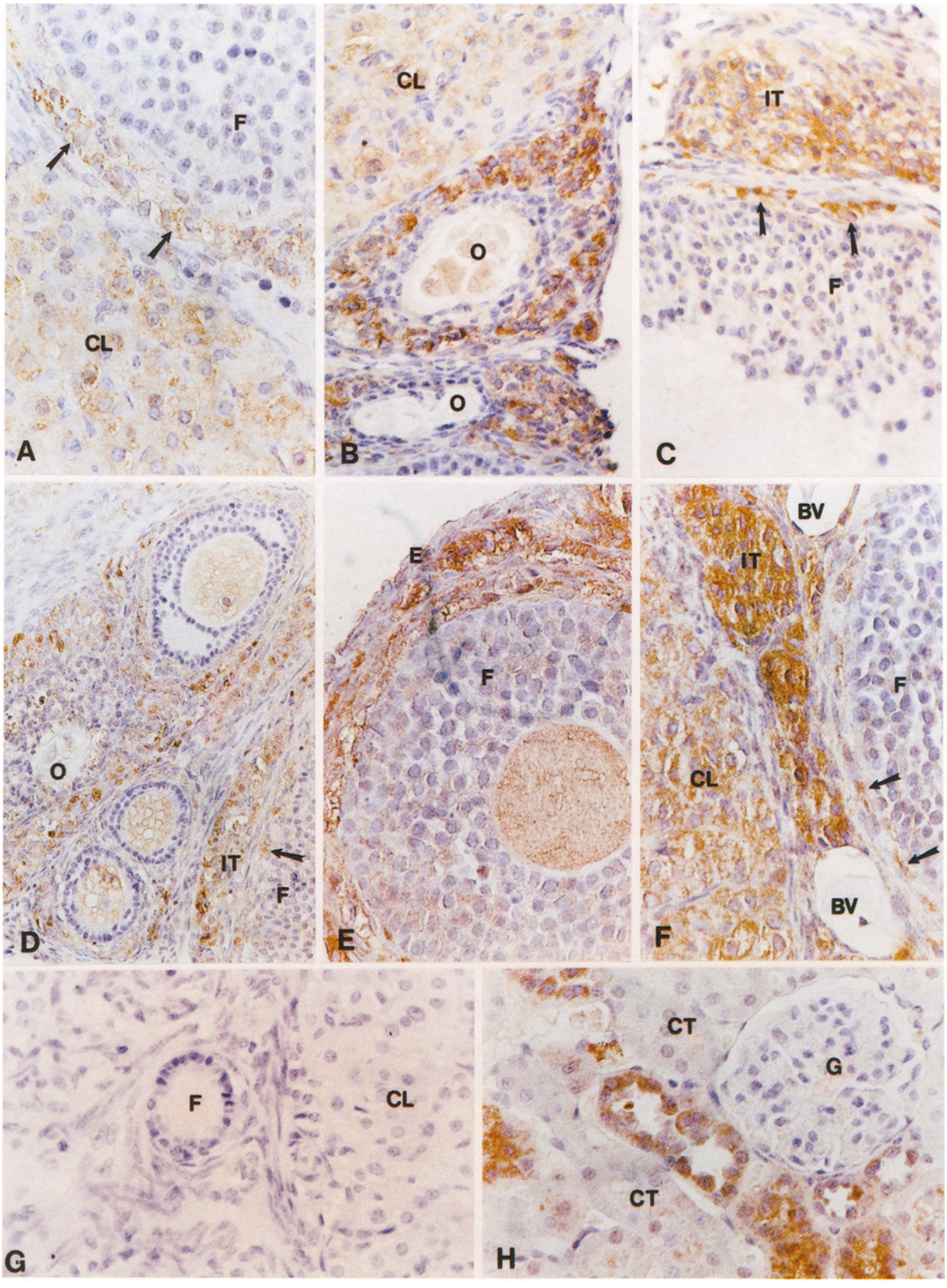


Fig. 2. Localization of IGF-I mRNA in cross-sections of mouse ovary by *in situ* hybridization. The signal generated from DIG-labeled cRNA probes is blue on unstained (flesh tone) cells. **(A)** Ovary from untreated mouse; detail of early antral follicle and corpus luteum. **(B)** Ovary from untreated mouse; degenerating follicle on the left and apparently healthy follicle on the right. **(C)** Ovary from mouse treated with eCG; cluster of primary and early secondary follicles. **(D)** Ovary from mouse treated with eCG; detail of two large antral follicles. **(E)** Control section: secondary follicle from ovary of untreated mouse with nuclear localization of U2b snRNA antisense signal. **(F)** Early antral follicle from ovary of untreated mouse with cytoplasmic localization of IGF-I antisense signal. A = antrum; F = follicle; CL = corpus luteum; arrows point to signal in cells of the theca interna. 250X Magnification.

Fig. 3. *(opposite page)* IGF-I, IGFBP-2, and IGFBP-5 immunoreactivity in mouse ovary and rat kidney. Antirat IGF-I antiserum 878/4 diluted 1:2500 was employed to localize IGF-I **(A, B, C, D)**. A positive signal is brown owing to DAB staining, and cell nuclei are blue due to hematoxylin counterstaining. (A) Ovary from untreated mouse; detail of secondary follicle and corpus luteum (250X magnification). (B) Ovary from untreated mouse; two degenerating follicles with remnants of oocyte and detail of corpus luteum (250X magnification). (C) Ovary from untreated mouse; detail of late antral follicle and interstitial tissue (250X magnification). (D) Ovary from mouse treated with eCG; the follicle at the bottom right is at the late antral stage (140X magnification). **(E)** IGFBP-2 immunoreactivity in ovary from untreated mouse using antirat IGFBP-2 antiserum diluted 1:500; secondary follicle and ovarian epithelium (280X magnification). **(F)** IGFBP-5 immunoreactivity in ovary from untreated mouse using antihuman IGFBP-5 antiserum diluted 1:500; detail of corpus luteum and of secondary follicle (280X magnification). **(G)** Control section of ovary from untreated mouse incubated with nonimmune serum diluted



1:500, showing no staining (250X magnification). **(H)** IGF-I immunoreactivity in rat kidney cortex using IGF-I antiserum 878/4 diluted 1:2500; the darkly stained cells are cells of the collecting ducts (280X magnification). Kidney: G = glomerulus, CT = convoluted tubules. Ovary: BV = blood vessels; E = epithelium; F = follicle; CL = corpus luteum; IT = interstitial tissue; O = remnants of oocyte; arrows point to staining of cells of the theca interna.

preabsorbing the IGF-I 878/4 antiserum used herein with 2.5×10^{-1} μg/mL target antigen, which virtually eliminated the signal (data not shown). The localization of the signal generated with the IGF-I antiserum in the cortex of rat kidney was also an indication of the specifity of the antiserum used in these experiments. Strong staining was observed in collecting ducts, weak staining in convoluted tubules, and no staining in glomeruli (Fig. 3H) in agreement with the distribution reported by other laboratories (Bortz et al., 1988; Matjeka and Jennische, 1992). Finally, the same results were obtained with two IGF-I antisera, although only data with the 878/4 antiserum are shown here.

The distribution of the IGF-I peptide differed dramatically from the distribution of IGF-I mRNA. IGF-I immunoreactivity was found mainly outside granulosa cells and was highest in stroma, particularly in areas adjacent to clusters of growing follicles (Fig. 3C, D). Connective tissue did not stain (Fig. 3D). IGF-I immunoreactivity was also elevated in the corpus luteum, although lower than in stroma (Fig. 3B) and, as in the stroma, was heterogeneous. Cells of the theca interna were also strongly positive for the IGF-I peptide (arrows Fig. 3A, C, and D), whereas cells of the theca externa did not stain.

IGF-I immunoreactivity in oocytes was variable. In primary follicles, staining of the oocyte was weak, but increased in larger oocytes and remained strong in oocytes of secondary follicles (Fig. 3D). In later stages of follicular development and in degenerating follicles, staining of the oocyte was decreased. IGF-I immunoreactivity was undetectable in granulosa cells of primary and small secondary follicles (Fig. 3D), but weak staining of granulosa cells was observed in follicles at later stages of development (Fig. 3A). Weak IGF-I immunoreactivity was present in granulosa cells of most, but not all, degenerating follicles and from healthy late antral follicles (Fig. 3C). In some degenerating follicles, immunoreactivity was undetectable in the cells closest to the remnants of the oocyte and was very strong in cells furthest away from the oocyte (Fig. 3B), opposite to the gradient of expression of IGF-I mRNA.

Experiments were also conducted to evaluate the distribution of IGF-I immunoreactive material in hormonally stimulated animals. No differences were observed in the intensity and distribution of IGF-I immunoreactivity between ovaries of untreated and eCG-treated animals. However, treatment with eCG + hCG reduced IGF-I immunoreactivity in stroma and in oocytes. No differences were observed in the distribution of the signal between species (rat and mouse).

The cellular localization of IGFBPs was remarkably similar to that of the IGF-I peptide. Overall, staining for IGFBPs was very low or undetectable in granulosa cells, and stronger in other cell types. Also the distribution of immunoreactive material was very similar whether antisera for IGFBP-2, 3, or 5 were used. IGFBP-2 immunoreactivity in granulosa cells was weak and increased marginally in late antral follicles. No differences in staining of the granulosa cells were observed between healthy looking and degenerating follicles at any stage of development. In some degenerating follicles, a similar pattern of expression of IGFBP-2 was observed as for the IGF-I peptide shown in Fig. 3B. Cells closest to the oocyte stained weakly, whereas cells furthest away from the oocyte stained more strongly. As with the IGF-I peptide, IGFBP-2 immunostaining of the theca interna varied from medium to strong, but no consistent patterns of staining intensity could be observed (Fig. 3E) Also, unlike the IGF-I peptide, the theca externa appeared stained for IGFBP-2 in growing follicles, but not in late antral follicles or in corpus luteum (Fig. 3E). Oocytes showed strong immunoreactivity for IGFBP-2 only, and this was observed in approximately half of the apparently healthy secondary follicles. The signal was invariably weak when the follicles showed signs of degeneration. No differences were observed in the distribution of IGFBP immunoreactivity between ovaries from untreated animals and eCG-treated animals. In gonadotrophin-stimulated animals, addition of hCG to the treatment regimen also did not alter the distribution of IGFBP immunoreactivity, but the relative intensity of IGFBP-2 staining was reduced, whereas that for IGFBP-3 was enhanced. Although other minor differences among IGFBP-2 immunoreactivity, IGFBP-3 immunoreactivity, and IGFBP-5 immunoreactivity were detected, the striking observation was the overall similarity of the distribution of these three binding proteins in the ovary, and the virtual overlap of sites of localization of IGF-I and IGFBPs (Fig. 3).

Discussion

The major finding of the present study is that the localization of IGF-I peptide does not coincide with the cellular distribution of IGF-I mRNA in mouse and rat ovaries. Rather, it is localized in sites that contain IGFBPs in close juxtaposition to cells expressing the IGF-I gene. In several mammalian tissues, the site of IGF-I immunoreactivity is the same as the site of mRNA localization (Matjeka and Jennische, 1992; El-Roeiy et al., 1994). However, there are many exceptions. These include liver (Bestetti et al., 1992), testis (Dombrowicz et al., 1992), and human and rat fetal tissues where both IGF-I or II proteins are localized at sites different from their messenger RNAs (Han et al., 1987a,b; Suilivan and Feldman, 1994). Colocalization of IGF with IGFBPs has also been observed in studies of rat kidney (Kobayashi et al., 1991) and human reproductive tissues (Hill et al., 1993; Pfeiffer and Chegini, 1994; Tang et al., 1994).

The fact that granulosa cells contain abundant amounts of IGF-I mRNA, but stain only weakly for the IGF-I peptide leads to the possibility that IGF-I peptide may be actively synthesized in these cells, rapidly exported, and then bound by IGFBPs in adjacent regions. Alternatively, the IGF-I mRNA in granulosa cells may be inefficiently translated. Absence of translation has been reported for Vgr1 gene transcripts in mouse oocytes (Wall et al., 1993)

and IGF-II in Wilm's tumors (Baccarani et al., 1993). In the case of limited translation, systemic IGF-I might be the major source of ovarian IGF-I. Regardless of its origin, the localization of IGF-I appears to be determined by the localization of IGFBPs. In this context, the distribution of IGFBP mRNA described by Nakatini et al. (1991) and Erickson et al. (1992) in rat ovary is also different from the pattern of IGFBP protein localization demonstrated in our study. Thus, similar to IGF-I, the binding proteins appear to be produced at one site and transported to another site., The mechanisms for transport exist, since examples of transport of IGFBPs across capillary boundaries into subendothelial tissues have been described previously (Bar et al., 1990).

Levels of IGF-I mRNA have been reported to decrease in granulosa cells of degenerating follicles in the rat (Oliver et al., 1989; Zhou et al., 1991). For the most part, this was confirmed in the present study, although some follicles with clear signs of degeneration were observed that did not show the concurrent decline in messenger RNA. This implies that the loss of IGF-I mRNA may be a late event in the process of degeneration. It is possible that an alteration in communication between granulosa and thecal cells is involved in the degeneration process, since cultured isolated granulosa cells quickly lose IGF-I gene transcripts (Botero et al., 1993). In our study, there was a gradient of expression of the IGF-I gene in some degenerating follicles. Their morphological appearance suggests that they may be differentiating to form secondary interstitial tissue, which produces androgens (Guraya and Greenwald, 1968). In this process, the granulosa cell mass gradually undergoes atrophy as the cells originating from the theca interna invade the structure. Thus, the cells staining strongly for IGF-I mRNA may have been remaining granulosa cells, whereas the cells that surrounded them and stained weakly may have been cells originating from the theca interna. In the present study, the pattern of expression of the IGF-I gene was homogenous across the granulosa cell compartment, except in late antral follicles, where a gradient of expression was observed. The signal became weaker in granulosa cells lining the basement membrane, and remained strong in cells lining the antrum and in cumulus cells. Because the gradient was not always present and was independent of visible signs of degeneration, it is possible that the gradient reflects differentiation rather than degeneration. Alternatively, degeneration might have been under way in follicles, at a level not detectable by the morphological criteria employed.

Treatment of mice with eCG alone affected neither the distribution nor the levels of IGF-I mRNA and peptide in the ovary. This is consistent with the observation that FSH has no effect on the expression of the IGF-I gene in cultured rat granulosa cells (Botero et al., 1993) or on the accumulation of immunoreactive peptide in the associated culture medium (Adashi et al., 1985). Treatment with eCG + hCG, on the other hand, increased levels of IGF-I mRNA and decreased levels of IGF-I peptide in stroma. Thus, the addition of an ovulatory stimulus to this hormonal regime altered the expression pattern, indicating that hormonal regulation of IGF-I is tissue-specific, and may be occurring at one or more stages in the process.

Weak staining for IGFBP-2 was observed in granulosa cells with patterns similar to those observed for IGF-I peptide. Since IGFBP-2 is absent from media of cultured granulosa cells (Liu et al., 1993), it is probable that this IGFBP-2 has been imported from other sources, possibly thecal and secondary interstitial tissue where levels of IGFBP-2 gene transcripts are high (Nakatini et al., 1991). Our immunohistochemical analyses showed that levels of IGFBP-2 protein also were highest in these tissues. The striking differences in IGFBP-2 staining among oocytes of secondary follicles indicate that a marked change in levels of IGFBP-2 in the oocytes may precede visible signs of degeneration in these follicles. The pattern of oocyte staining for IGFBP-2 was not the same as that observed for IGF-I and may reflect a role for IGFBP-2 distinct from IGF-I.

The localization patterns for IGFBP-2, 3, and 5 overlapped to a great extent, but some unique cellular distributions were present. This implies that each IGFBP can be regulated independently and may serve distinct functions in the ovary. Furthermore, the localization of the IGFBPs, although very similar, was not always identical to the localization of IGF-I peptide. For example, staining for IGFBPs was often strong in theca externa, whereas IGF-I peptide was undetectable. Thus, IGFBPs were not always associated with IGF-I and may have distinct roles that are unrelated to IGF-I.

In summary, these investigations demonstrate that IGF-I immunoreactivity in various cell types in the ovary is generally inversely related to levels of IGF-I mRNA, except in oocytes. The inverse relationship was most pronounced in granulosa cells, stroma and in some degenerating follicles. IGF-I immunoreactivity was undetectable or low in granulosa cells, and high in the theca interna, corpus luteum, and stroma. IGFBP-2, -3 and -5 showed a distribution similar to that of IGF-I. The principal differences were the presence of IGFBPs in the theca externa and the presence of high levels of IGFBP-2 in some oocytes of secondary follicles. These results suggest that locally produced IGF-I peptide is rapidly secreted into surrounding tissues and associates with IGFBPs, and that systemic IGF-I may contribute significantly to the ovarian IGF-I content.

Materials and Methods

Animals and Collection of Tissues

Random-bred CD1 Swiss albino mice from Charles River Breeding Laboratories (St. Constant, PQ) and mature Sprague-Dawley rats (180–200 g; Biosciences, University of Calgary, Calgary, AB) were used throughout the study. To induce follicular development, seven-wk-old females were treated with an ip injection of 7.5 IU eCG (Folligon, Intervet U.K. Ltd., Cambridge, UK). One group of mice was sacrificed by cervical dislocation 44 h after eCG stimu-

lation. Another group was further stimulated at 48 h with 7.5 IU hCG (Sigma, St. Louis, MO) and sacrificed 3 h later.

For *in situ* hybridization and immunocytochemical experiments, whole ovaries were taken from untreated, eCG-treated, and eCG + hCG-treated mice, and from untreated rats. Ovaries were sectioned into two equal parts prior to fixation. For each treatment, ovaries from three or four animals were examined.

In Situ *Hybridization*

Strand-specific riboprobes (cRNA) were generated by in vitro transcription of a 217-bp *AvaI/BamI* IGF-I cDNA fragment (*see* Murphy et al., 1987b for original cDNA clone) that was subcloned into the *SmaI/Hin*cII sites of pGEM-2 vector (Promega, Madison, WI) by blunt-end ligation. The plasmid was linearized with *Eco*RI for generation of antisense RNA using T7 RNA polymerase, and with *Hin*dIII for generation of sense RNA using SP6 RNA polymerase. The linearized plasmid was isolated as a single band from a 1% agarose gel with DNA-binding glass beads (USBioclean; USB, Cleveland, OH). The cRNA probes were labeled with digoxigenin (DIG-11-dUTP; Boehringer Mannheim, Indianapolis, IN) using the in vitro transcription protocol provided by Boehringer Mannheim with the addition of 2–5 μCi α-^{32}P-UTP (3000 Ci/mmol, Amersham, Oakville, ON, Canada) to allow calculation of the amount of probe synthesized. U2 snRNA (small nuclear RNA, which is primarily localized to the nucleus) sense and antisense probes (Dean et al., 1989) were included as additional controls to verify specificity of *in situ* hybridization experiments.

Tissues were fixed in 4% paraformaldehyde for 12 h at 4°C, dehydrated through an ethanol series (70% overnight at 4°C, 80% for 30 min, 95% for 30 min, 2X 100% for 20 min each), cleared in chloroform for 4–8 h and embedded in paraffin (Paraplast+, Oxford Labware, St. Louis, MO) at 60°C for 2 h with three changes of paraffin. Sections of 5-μm thickness were mounted on glass slides treated with 3-aminopropyltriethoxysilane (Sigma St. Louis, MO). Each slide contained sections of the four treatments. Before hybridization, slides were dewaxed in toluene, rehydrated through an ethanol series, and washed in 2X SSC (1X: 150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0). Control slides only were subjected to prehybridization RNase treatment (RNase I "A", Pharmacia, Baie d'Urfe, PQ) 50 μg/mL in RNase buffer (500 m*M* NaCl, 10 m*M* Tris-HCl, 10 m*M* EDTA, pH 8.0) for 30 min at 37°C, and 2 × 15 min washes at 37°C in 2X SSC. All slides were postfixed for 20 min in 4% paraformaldehyde, permeabilized with proteinase K (20 μg/mL in 2X SSC) for 30 min at room temperature, refixed for 20 min in 4% paraformaldehyde, washed for 2 × 5 min in 4X SSC, acetylated in 6% (v/v) acetic anhydride in 100 m*M* triethanolamine, pH 8.0, for 10 min and washed for 2 × 5 min in 4X SSC. Prehybridization and hybridization were carried out at 50°C and sections were washed to 0.5X SSC at 50°C following the procedure described by Watson et al. (1992). Immunological detection of the DIG-labeled probes followed the protocol recommended by Boehringer Mannheim. Specific conditions were as follows: the alkaline phosphatase-anti-DIG Fab fragment was used at a concentration of 1:500 and incubated for 2 h; color development using X-phosphate/NBT substrate was terminated after 30 min for U2 snRNA antisense treatment, and after 4 h for all other slides. Sections were left unstained, dehydrated, cleared in xylene, and mounted with Cytoseal 60 (Stephen Scientific, Riverdale, NJ).

Controls included U2 snRNA sense probes to assess background levels of signal generated by nonspecific hybridization of RNA probes. RNase treatment of control slides before hybridization was used to demonstrate that the hybridization signal was generated by binding of probes to RNA. Control slides without probes showed levels of background signal generated by interactions not involving probes. In immunohistochemical procedures, control slides were included to assess background levels of signal generated by the anti-DIG Fab fragment and by residual endogenous alkaline phosphatase activity.

Immunohistochemistry

Two IGF-I antisera raised against recombinant human IGF-I were kindly provided by P. D. Gluckman (Auckland, New Zealand). Rabbit antirat IGF-I 878/4 is a high-affinity polyclonal antibody with <0.05% crossreactivity with IGF-II in radioimmunoassay (Breier et al., 1991). Rabbit antirat IGF-I 861/5 has lower affinityand <0.5% cross-reactivitywith IGF-II (Breier et al., 1991). Two IGFBP-2 antisera were used. Rabbit antirat IGFBP-2 antiserum Rb20 was kindly provided by N. Ling (La Jolla, CA) and has been characterized by Liu et al. (1993) and rabbit antibovine IGFBP-2 antiserum was purchased from UBI (cat. no. 06-107, Lake Placid, NY) and has been characterized by Cohick and Clemmons (1991). Rabbit antihuman IGFBP-3 was provided by C. Maack (CELTRIX Pharmaceutical Inc.), and guinea pig antihuman IGFBP-5 was purchased from UBI (cat. no. 06-110, Lake Placid, NY). Both were characterized by Camacho-Hubner et al. (1992).

Tissues were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in 17 m*M* phosphate buffer, pH 7.0, for 12 h at 4°C. Tissues were washed for 3 d at 4°C with 2 changes of PBS/d, and then embedded and sectioned as described in the previous section.

The avidin–biotinylated peroxidase complex method was used with reagents from Vectastain (kit # PK-4001, Burlingame, CA). Sections were deparaffinized in xylene, rehydrated through an ethanol series, and washed in PBS. Endogenous peroxidase was inhibited with 1% H_2O_2 in PBS for 10 min. Sections for the IGF-I immunohistochemistry were permeabilized with 0.0625% trypsin for 5 min, washed in PBS, and incubated for 10 min in 0.1% normal goat serum in PBS. Sections were then incubated overnight at 4°C with either:

1. Primary antisera 1:500–1:2500 in PBS with 1% BSA;
2. Nonimmune rabbit serum 1:500; or,
3. IGF-I antiserum 878/4 1:2500 preabsorbed with 2.5×10^{-1} μg/mL recombinant human IGF-I peptide.

Sections were washed for 10 min in PBS and further incubated for 2 h at room temperature with either goat antirabbit IgG-biotin (Vectastain PK-4001) or goat anti-guinea pig IgG-biotin (Vectastain BA-7000) 1:500 in PBS with 1% BSA. Sections were washed for 10 min in PBS, further incubated for 2 h with the avidin–biotinylated peroxidase complex per kit instructions, and washed for 10 min in PBS. The color reaction was allowed to proceed for 10 min using DAB substrate. Sections were washed in PBS, lightly counterstained with Carazzi's hematoxylin, dehydrated, cleared in xylene, and mounted with Permount (Sigma).

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