

## IGF binding protein-2 gene expression and the location of IGF-I and IGF-II in fetal rat lung

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### Summary

Binding proteins for the insulin-like growth factors (IGFBPs) are important modulators of the biological actions of IGF-I and IGF-II. The generation of IGFBPs within developing organs, and their spatial arrangement, may similarly determine IGF action at specific microanatomical sites. In situ hybridization studies with late gestation (days 16, 18 and 20) fetal rat lung using a cDNA probe for IGFBP-2 showed strong gene expression in the fetal lung epithelial structures (alveoli and airways). The sites of IGFBP-2 gene expression were associated with immunoreactive IGF-II at the apical surface of the epithelium. By day 20, there was also some IGFBP-2 gene expression and immunoreactive IGF-II at discrete sites in the mesenchyme. In contrast, immunoreactive IGF-I was

found predominantly distributed in a punctate pattern, consistent with its presence in the lumen or walls of small vessels or capillaries, and in a granular, intracellular form in both epithelial and mesenchymal cells. These studies suggest that endogenously generated IGFBP-2 may determine the distribution of IGF-II, principally at the apical surface of lung epithelia. IGF-I does not colocalise with IGF-II peptide or the sites of IGFBP-2 gene expression. We conclude that the spatial distributions of these two related growth factors are separately controlled, to some extent by endogenously generated binding proteins.

Key words: IGF, binding protein, lung, fetus, growth.

### Introduction

Growth factors acting at spatially defined microanatomical loci are likely major influences determining the pattern of cell division in the generation of organ structure during development. The requirement for a number of different growth factors in lung development has been suggested by in vitro studies (Stiles et al., 1986). While some endocrine growth factors may be important, endogenous factors have a major influence (Liggins, 1984). For example, fetal lung epithelia produce EGF (Raaberg et al., 1991), and fibroblasts have been shown to express the genes for IGF-I and IGF-II (Han et al., 1987), TGF- $\beta$  (Torday and Kourembanas, 1990) and transferrin (Skinner et al., 1991).

Although free diffusion of the endogenously produced growth-promoting agents is envisaged after their secretion by the cell, there is now good evidence that their freedom of movement is often constrained by specifically located binding proteins and by macromolecules in the extracellular matrix (Segarini et al., 1989, Ruoslahti and Yamaguchi, 1991). An example of this spatial constraint is TGF- $\beta$ , produced by fetal lung fibroblasts (Torday and Kourembanas, 1990), but found concentrated at the basal surface of fetal lung epithelia and throughout the basement membrane (Heine et al., 1990).

Insulin-like growth factors I and II are known to bind to a large family of binding proteins (IGFBPs; Ooi, 1990,

Lamson et al., 1991). Initially the IGFBPs were thought to provide a serum storage capacity for IGF-I and IGF-II, but recent reports suggest they may regulate IGF actions (Clemmons et al., 1986, Elgin et al., 1987). It has also been suggested that this regulatory function of IGFBPs may involve binding to the cell surface (Ooi, 1990, Wood et al., 1992). To date, six IGFBPs have been characterised (BP-1 to BP-6), of which IGFBP-2, a  $32 \times 10^3 M_r$  protein, is dominant in the fetus. The preferential binding of IGF-II to IGFBP-2 is described (Forbes et al., 1988), but IGFBP-2 gene splice variants, which may result in IGFBP-2 proteins of different sizes and binding characteristics, provides yet another element for consideration (Roghani et al., 1991).

In this report, we describe the gene expression of the dominant fetal IGF binding protein (IGFBP-2) in the alveolar and airway epithelium of the fetal rat in late gestation and its relationship to the location of IGF-I and IGF-II peptides, as determined by immunohistochemistry with specific antisera.

### Materials and methods

Ethical agreement was obtained for animal use from the Animal Ethical Committee, School of Medicine, University of Auckland. Fetal rats were obtained from CO<sub>2</sub> euthanated date-mated females at days 16-20 of gestation. They were excised from the uterus and were either frozen on dry ice for in situ hybridization or fixed in

Omnifix (Xenetics Biomedical Corp, San Diego, CA) on ice for 30 minutes and then at room temperature for 2 or more days for immunohistochemistry.

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company, MO, USA.

### Probes

The rat IGFBP-2 cDNA used in these studies was as described by Brown et al. (1989) and was kindly supplied by M. M. Rechler (NIH, Bethesda, MD, USA). The specific fragment corresponded to nucleotides 502-1087 of the coding region. The 720 base pair mouse IGF-II genomic probe was kindly supplied by P. S. Rotwein (Washington University, St. Louis, MO, U. S. A.) and covers the entire first exon of the gene (Daughaday and Rotwein, 1989). The probes were labelled with  $^{35}\text{S}$ -dCTP for in situ hybridization and with [ $^{32}\text{P}$ ]dCTP for Northern blot analysis using the random primers labelling system as described by the manufacturers (GIBCO-BRL, NY, USA).

### In situ hybridization

In situ hybridization was performed as described elsewhere (McCabe et al., 1986). Briefly, fresh frozen tissue was sectioned (10-20  $\mu\text{m}$ ) and the sections mounted on aminoacetylsilicon-treated slides. The frozen sections were fixed with paraformaldehyde (4% in PBS) while thawing (5 minutes), washed twice in PBS and dried in ethanol. Prehybridization was performed in a humidity box at 50°C for 2 hours with 1 ml prehybridization buffer [50% deionized formamide, 5  $\times$  hybridization salts, 5  $\times$  Denhardt's solution, 0.2% SDS, 10 mM DTT, 500  $\mu\text{g}/\text{ml}$  denatured herring sperm DNA and 50  $\mu\text{g}/\text{ml}$  poly(A) $^+$ ]. For hybridization, the prehybridization buffer was removed and 70  $\mu\text{l}$  of hybridization buffer (prehybridization buffer plus 10% dextran sulfate) with 3-4 ng denatured [ $^{35}\text{S}$ ]IGFBP-2 cDNA ( $10^5$ - $10^6$  cts/minute) was applied. Hybridization was performed overnight at 50°C. The sections were washed 5 times with 2  $\times$  SSC containing 20 mM  $\beta$ -mercaptoethanol for 10 minutes at room temperature (RT), 4 times with 2  $\times$  SSC for 10 minutes at RT, twice with 2  $\times$  SSC at 50°C for 15 minutes and twice with 0.2  $\times$  SSC at RT for 5 minutes. After drying, the sections were exposed to film emulsion (LM1, Amersham, UK) for approximately 14 days. The sections were lightly counterstained with haematoxylin and photographed with T-Max 100 (Eastman-Kodak) using bright-field illumination.

### Northern blotting

Livers of normal adult female rats, lactating rats and rats starved for 48 hours were extracted by a modification of the method of Chomzynski and Sacchi (1987) using guanidinium thiocyanate-phenol-chloroform extraction to provide total RNA samples. Aliquots of the RNA (10  $\mu\text{g}$ ) were separated on a denaturing 1% agarose gel and blotted onto nylon membrane (Gene-Screen Plus, DuPont-NEN, MA, USA) and probed with [ $^{32}\text{P}$ ]IGFBP-2 cDNA according to the manufacturers instructions. The membrane was washed twice for 30 minutes with 0.1  $\times$  SSC/0.1% SDS at 60°C and exposed to X-ray film for 5 days.

### Immunohistochemistry

Fixed tissue was processed for embedding in paraffin wax (Paraplast, Oxford Labware (Sherwood Medical), MO, USA). The tissues were sectioned at 5-6  $\mu\text{m}$ , positioned on poly-L-lysine-coated glass slides, deparaffinized in xylene and rehydrated in a descending ethanol series into Tris-buffered saline, pH 7.4 (TBS). The tissue sections were washed 3 times in TBS containing 0.1% (w/v) bovine serum albumin (Immunochemical Products, Auckland, NZ)

and incubated in 2% (v/v) hydrogen peroxide in TBS for 10 minutes to eliminate endogenous peroxidase activity. Polyclonal antisera to human IGF-I (#878/4) and human IGF-II (#C65) were raised in rabbits (gift from Dr B. H. Breier, Developmental Endocrinology Laboratory, Department of Paediatrics, School of Medicine, University of Auckland, Auckland, NZ) and used at a dilution of 1/250. The homology between human and rat IGF-I and IGF-II are 96% and 94% respectively (Daughaday and Rotwein, 1989). Control sections were incubated without the primary antiserum. Tissue sections were treated with the primary antiserum diluted in TBS containing 1% BSA for 48 hours at 4°C, washed 3 times with TBS-0.1% BSA and then incubated with biotinylated donkey anti-rabbit serum (Amersham, UK, diluted 1:300 in TBS-1%BSA) for 24 hours at 4°C. After three washes, sections were treated with streptavidin-peroxidase complex (Amersham, UK) for 3 hours at room temperature and then visualized by reacting with diaminobenzidine (0.05%) containing 0.01% hydrogen peroxide and nickel-cobalt reagents for intensification (Adams, 1981). The sections were dehydrated in alcohol to xylene, mounted in DPX mountant (BDH, Poole, UK) and coverslipped.

Preabsorption of the diluted (1/250) antisera by overnight incubation with the homologous peptide (10  $\mu\text{M}$ ) resulted in 93% (IGF-I) and 43% (IGF-II) loss of staining, as determined by morphometric analysis (JAVA Scientific, CA, USA). Crossreactivity between these antisera and the heterologous peptides was less than 0.5% at dilutions of 1/100,000 under radioimmunoassay conditions. Preabsorption of the antisera with the heterologous peptide (10  $\mu\text{M}$ ) at the higher concentrations of antisera used in these immunochemical studies (1/250) resulted in some blocking of the IGF-I staining by IGF-II (decreased by 27%) but IGF-II staining was relatively unaffected by IGF-I (decreased by 5.3%).

## Results

Hybridization with the  $^{32}\text{P}$ -labelled IGFBP-2 cDNA probe showed a single band at approximately 1.7 kb with total RNA extracts from rat liver by northern analysis. As described previously (Ooi et al., 1990), IGFBP-2 mRNA was significantly increased in the livers of fasted adult female rats (Fig. 1), suggesting that IGFBP-2 gene expression is sensitive to hormonal and nutritional influences. In addition, we have shown that lactation is a major stimulus for IGFBP-2 gene expression, supporting a role for hormonal factors in the control of IGFBP-2 production (Fig. 1).

In situ hybridization of  $^{35}\text{S}$ -labelled IGFBP-2 probe with frozen sections of day 18 gestation fetal rat lung showed a distribution that was strictly limited to the epithelial structures (Fig. 2A). At this stage of gestation the mesenchymal cells of the fetal rat lung appear to have no significant IGFBP-2 gene expression. The different intensities of labelling evident in these pulmonary epithelia suggest that the message levels are not uniform and that in some structures, such as the large airways, there is patchy distribution indicative of different levels of gene expression in adjacent cells. Over all, the larger airways and some of the small terminal sacs appear less intensely labelled than adjacent alveolar structures and their associated ducts. The pleural mesothelium by comparison, contains only moderate levels of message. In comparison, the localisation of IGF-II gene expression, as demonstrated by in situ hybridization with a  $^{35}\text{S}$ -labelled IGF-II probe, was widespread in the day 18 fetal rat lung (Fig. 2B). At this stage in gestation there was less IGF-II gene expression in the lung than in the adjacent liver,

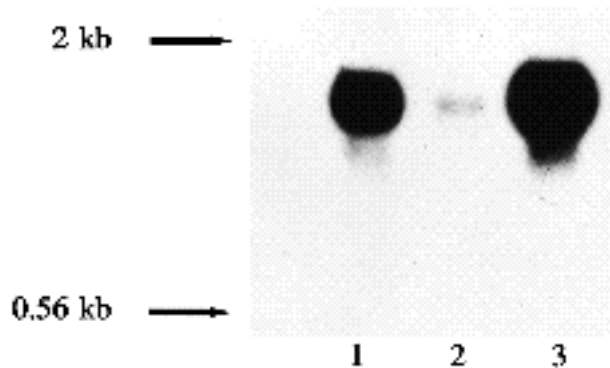


Fig. 1. Demonstration of IGFBP-2 mRNA in extracts of rat liver by northern analysis. RNA extracts from the livers of female adult rats which were (1) lactating, (2) normal and (3) starved were separated by denaturing agarose gel electrophoresis and blotted onto nylon membrane. The membrane was probed with  $^{32}\text{P}$ -labelled IGFBP-2 cDNA as described in the Methods. The IGFBP-2 mRNA (1.7 kb) was increased by lactation (lane 1) and starvation (lane 3) in comparison to normal controls (lane 2).

while the rib has an extremely dense concentration of message (Fig. 2B). These two *in situ* studies demonstrate that IGFBP-2 and IGF-II gene expression are not significantly co-localised. In comparing the images produced with IGFBP-2 and IGF-II probes, it is clear that the high density of IGFBP-2 mRNA in the epithelial structures cannot be attributed to non-specific binding or trapping (Fig. 2A). Similarly, the tissue specificity of the IGF-II probe, with dense localisation in rib and the relatively diffuse labelling in the lung, demonstrates the specificity of the *in situ* procedures.

Over the latter part of gestation in the rat, on days 16, 18 and 20, IGFBP-2 mRNA is present in most of the fetal lung epithelium (Fig. 3A-C). The intensity of labelling appears to increase from day 16 to day 18, and by day 20 is no longer confined to the epithelium, seeming to be present also in the mesenchyme (Fig. 3C). Although epithelial expression is almost exclusively seen in the day 16 and 18 sections, the pattern at day 20 suggests that mesenchymal expression cannot be excluded (Figs 2A, 3A-C).

Since it was expected that IGFBP-2 would bind IGF-II and, to a lesser extent, IGF-I (Ooi, 1990), it was useful to compare the location of these growth factors in fetal lung as possible indicators of functional activity of IGFBP-2. These peptides were located by immunohistochemistry using relatively specific polyclonal rabbit antisera (Breier et al., 1991). Localisation was determined with biotinylated second antibody and streptavidin-peroxidase, reacted with diaminobenzidine and enhanced with a nickel/cobalt preparation which results in a black stain at positive sites (Adams, 1981).

IGF-II was found mainly at the apical surface of the lung epithelium at days 16 and 18 (Figs 3D-E, 4B). In day 16 lung sections, there was also faint staining at the basal surface of the epithelium (Fig. 3D). The intensity of IGF-II staining was greatest on the apical surface of the stratified epithelium of the larger airways (Fig. 4A). In the day 20 sections, the apical staining of lung epithelium for IGF-II was decreased,

while some mesenchymal cells were stained in an irregular pattern, principally at the margins and extremities of some lobes (Fig. 3F). The pattern of staining for IGF-II on the apical surface of much of the epithelium (Fig. 3D-F) was generally consistent with the appearance of the IGFBP-2 mRNA within these epithelial cells (Fig. 3A-C).

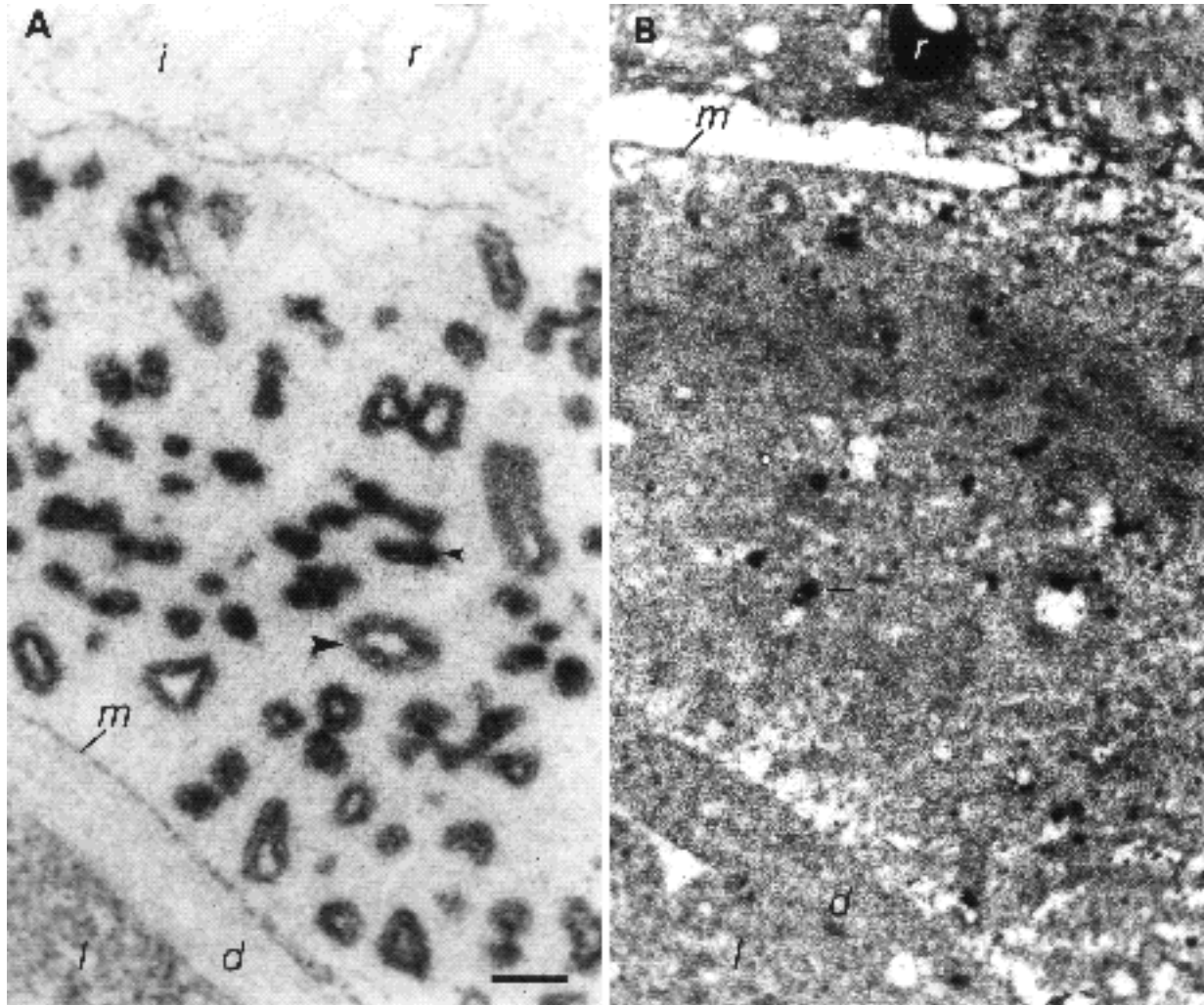
The staining pattern for IGF-I was qualitatively different from IGF-II. Although there was significant staining at the luminal surface of the epithelium at day 16 (Fig. 3G), the majority of the IGF-I staining was punctate throughout the mesenchyme on days 16, 18 and 20 (Fig. 3G-I). The identity of these punctate structures is not clear. While many are consistent with the position and shape of small blood vessels or capillaries (Fig. 4C), they often appear within or underlying the epithelium (Fig. 4D). The identity of the punctate bodies in the mesenchyme as small pulmonary vessels enriched for IGF-I is supported by the observation that immunohistochemical staining for von Willebrand Factor (Factor VIII-related antigen), which is specific for endothelium, produces a similar staining pattern. Whether this reflects IGF-I in blood, blood cells, or in vessel endothelium is difficult to determine with the techniques employed here.

The experiments to determine specificity of the antisera in immunohistochemistry are shown in Fig. 5A-C. In procedures where the primary antiserum was omitted the image was extremely faint with no distinguishing features (Fig. 5A). Preabsorption of the IGF-II antiserum with IGF-II peptide (10  $\mu\text{M}$ ) for 24 hours resulted in a darker image than seen in controls (Fig. 5A) but again with only marginal features and no staining of the apical surface of the epithelium (Fig. 5B). Preabsorption of the IGF-I antiserum with the IGF-I peptide (10  $\mu\text{M}$ ) using the same protocol provided an image with marginal features occasionally recognisable as punctate or darkening on the basal surface of the epithelium (Fig. 5C).

## Discussion

In this study, we have shown that IGFBP-2 gene expression occurs in the late gestation fetal rat lung, principally in the epithelium, and may be a major factor in the localisation of IGF-II within the fetal lung. In contrast, the distribution of IGF-I appears to be distinct from IGF-II and does not colocalize with IGFBP-2. Whereas IGF-II is found principally at the apical surface of lung epithelia in contact with the fluid-filled lumen of the alveoli and developing airways, the IGF-I immunoreactivity is most often found distributed in a punctate pattern at some distance from the luminal surface of the epithelia.

The importance of the IGF binding proteins (IGFBPs) is suggested by their ability to modify the biological activity of IGFs (Knauer and Smith, 1980, Clemmons et al., 1986, Elgin et al., 1987), but it is not clear how this occurs. The majority of circulating IGFBP is produced by the liver, largely determining the biological activity of IGFs in blood. Local production of IGFBPs may likewise affect the activity of IGFs produced locally, whether autocrine or paracrine, and also endocrine IGFs. In our studies, it is clear that the primary site for IGFBP-2 gene expression in the fetal rat lung from days 16-20 of gestation is the epithelium (Figs 2A, 3A-C). Speci-

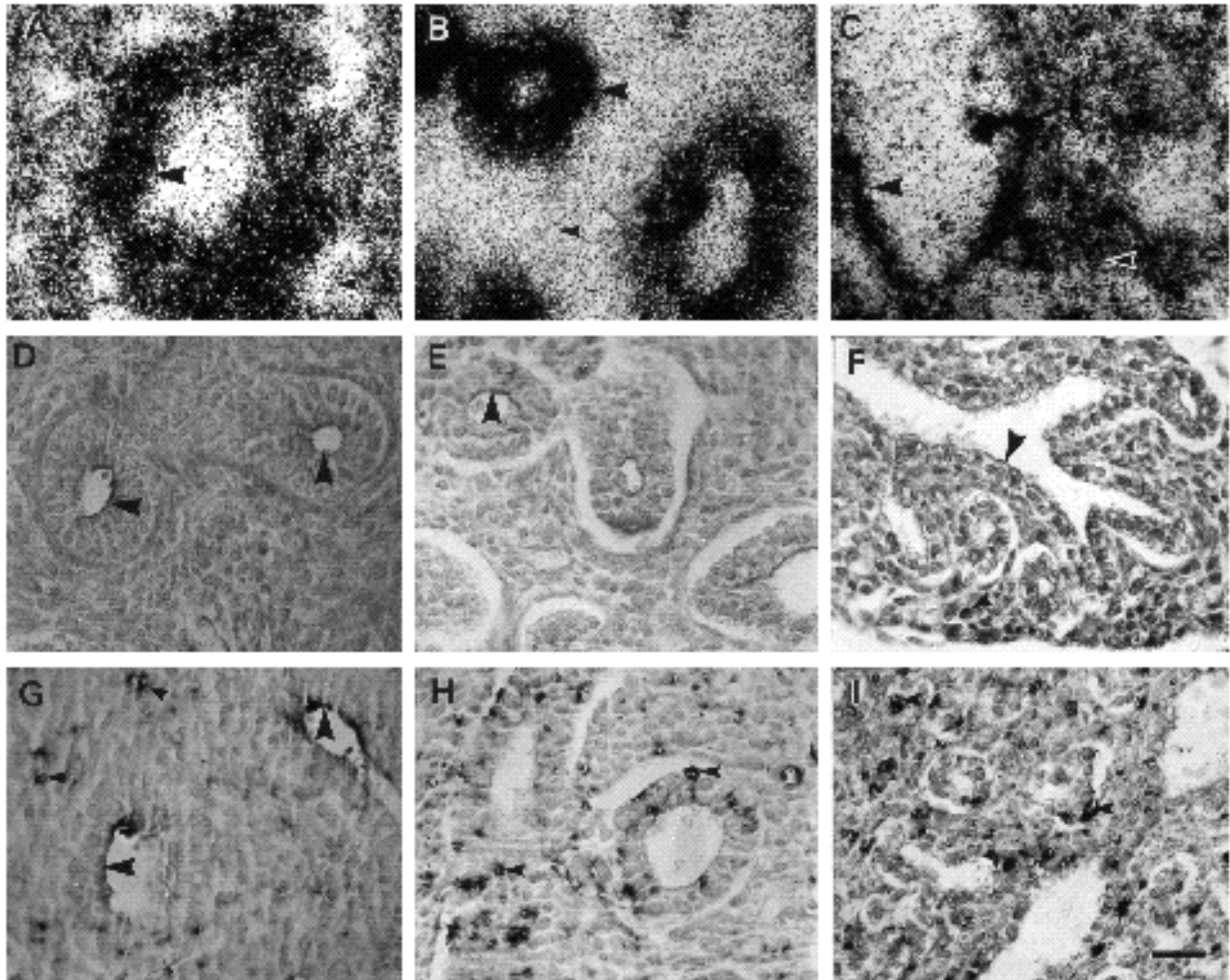


**Fig. 2.** The locations of IGFBP-2 and IGF-II gene expression in fetal rat lung by in situ hybridization. These two photomicrographs are derived from equivalent parasagittal sections through 18 day fetal rats with examples of lung, liver, striated muscle (diaphragm and chest wall) and rib. (A) The photomicrograph shows an in situ image of fetal rat lung and associated tissues after hybridization with  $^{35}\text{S}$ -labelled IGFBP-2 cDNA and exposure to photographic emulsion. In lung the signal is almost entirely restricted to the epithelium with minimal levels in the mesothelium (m) of the pleura. The mesenchymal tissues, including endothelia, appear completely devoid of IGFBP-2 message. The IGFBP-2 mRNA is not uniformly distributed in the epithelium. There appears to be less expression in epithelia of some larger airways (large arrow) and greater concentrations of message in adjacent alveolar and duct structures (small arrow). Diaphragm (d), intercostal muscle (i) and rib (r) have little IGFBP-2 while liver (l) contains moderate amounts. (B) The photomicrograph shows an in situ image of fetal rat lung and associated tissues after hybridization with  $^{35}\text{S}$ -labelled IGF-II DNA and exposure to photographic emulsion. The labelling in the lung is dispersed but with some regional differences, with an absence of signal in areas close to the mesothelium (m) which are likely terminal alveoli. Most of the labelling in the lung is consistent with mesenchymal gene expression, with occasional poorly defined circular formations which may correspond to large airways or vessels. There is strongest labelling in the rib (r), with lesser amounts in the liver (l) and diaphragm (d). Some black granular artefacts are also present (-). The scale bar in 2A equals 100  $\mu\text{m}$  and is the same for 2B.

ficity of the IGFBP-2 probe was confirmed by the abolition of the signal by RNAase pretreatment, by the finding of a single band on northern analysis (Fig. 1), and by the generation of different images with other probes during parallel experiments. Of particular relevance was the observation that IGF-II mRNA does not co-localise with IGFBP-2 mRNA (Fig. 2B). The IGF-II probe labelled throughout the lung, with no discernible concentration in any structure. In contrast, there was dense labelling of the rib and comparatively strong labelling of the liver (Fig. 2B). These two

examples of in situ hybridization also demonstrate that the procedure used has sufficient resolution to exclude non-specific binding or trapping of the labelled probes.

In our studies, the IGFBP-2 mRNA was not uniformly distributed throughout the epithelium. The images suggest that some areas have greater gene expression (most alveoli and associated ducts) than others, such as large airways and some terminal alveoli (Fig. 2A). There were common examples of high and low levels of expression in adjacent cells of large airways and, where a duct system could be traced, a gradual

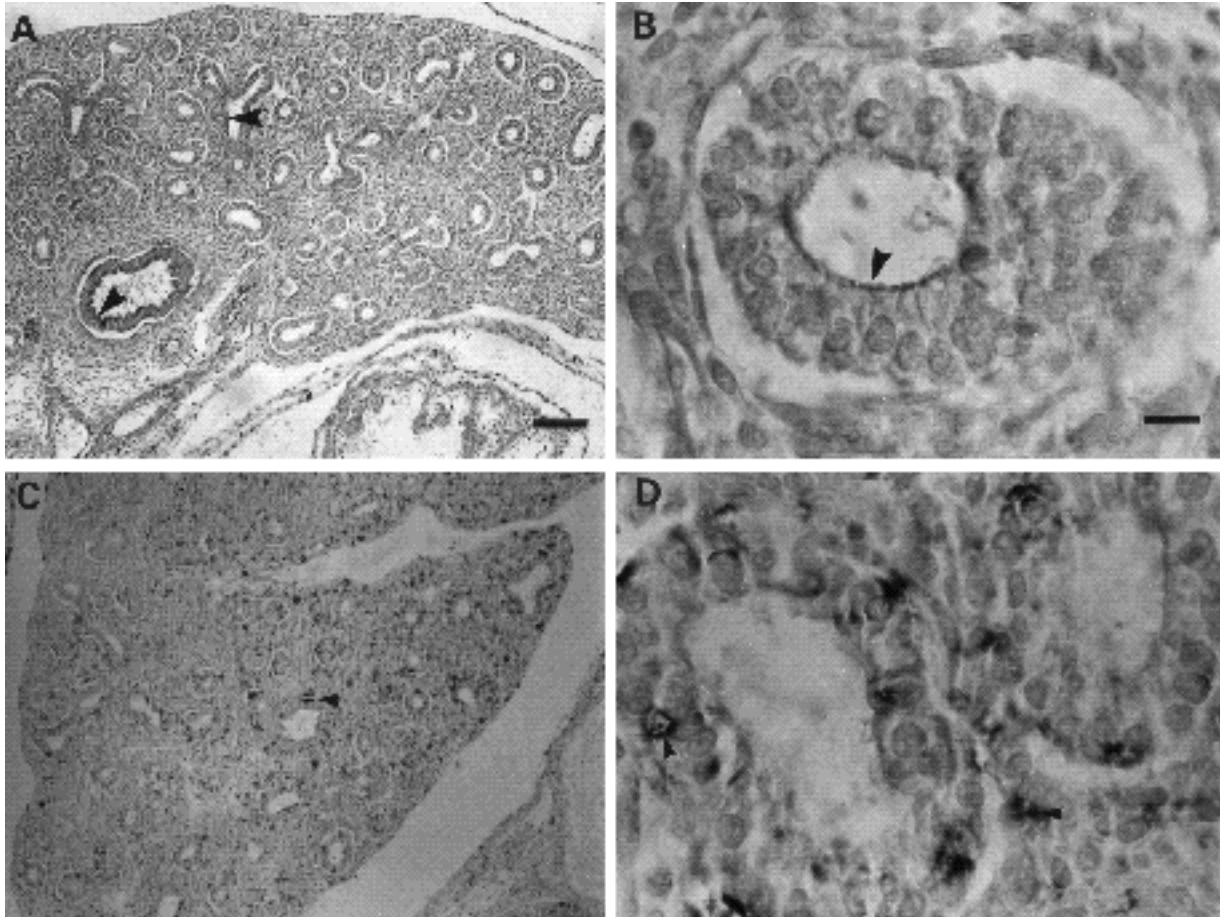


**Fig. 3.** A comparison of the location of IGFBP-2 gene expression with the location of immunoreactive IGF-I and IGF-II in fetal rat lung at days 16-20 of gestation. The scale bar in I represents 25  $\mu$ m and is the same for all the photomicrographs in. A-C show in situ images of fetal rat lung at days 16, 18 and 20 of gestation. The message is almost entirely restricted to the lung epithelium (large arrows). The mesenchymal tissues (small arrows) have little IGFBP-2 gene expression, except in the day 20 specimen. The close association between IGFBP-2 and IGF-II in the fetal rat is indicated in the immunohistochemistry images obtained with an antiserum for IGF-II. At days 16, 18 and 20 of gestation there is very distinct staining of the apical surface of lung epithelia (large arrows) with IGF-II antiserum (D-F). At gestation day 20 the IGF-II stain in the lung became more diffuse (F) with some labelling of the mesenchyme (small arrow), similar to the pattern seen with IGFBP-2 mRNA (C). The localization pattern for IGF-I was entirely different, with a mixed distribution which was predominantly punctate throughout the mesenchyme (small arrows) of the lung from days 16-20 (G, H, I) with only partial staining of the apical surface of the epithelium (large arrows) on day 16 (G). The apical surface stain disappeared at days 18 and 20 but the punctate staining remained undiminished (H, I).

change in the intensity of the image. This suggests that gene expression may depend on the differentiation state of the epithelial cells in different zones of the connecting alveolar and duct system. There was also a distinct difference observed between the in situ images of gestation day 18 and day 20 lungs (Fig. 3B-C). The IGFBP-2 gene expression appeared to be present in the lung mesenchyme of the day 20 fetuses, suggesting that IGFBP-2 production by mesenchyme cannot be excluded.

Immunohistochemical studies of IGFBP-2 localisation by Hill et al. (1989) showed that IGFBP-2 was present in lung epithelia and at their luminal surface. This work was carried out on human fetuses aborted early in gestation (14-16

weeks). Immunohistochemical staining for IGFs, using an antiserum that did not differentiate between IGF-I and IGF-II, suggested a close association between IGFs and IGFBP-2 (Hill et al., 1989). In our studies, we have used antisera developed for high specificity radioimmunoassays of serum IGF-I and IGF-II (Breier et al., 1991), which we have shown are also relatively specific for these two peptides in fixed tissue sections with only minor crossreactivity. We have also used date-mated fetal rats (term is day 22) at days 16-20 of gestation, a model that is quite different to the human abortus, but intensively studied in terms of lung development (Post et al., 1984; Williams and Dobbs, 1990). In the rat fetus there is strong staining for both IGF-I and IGF-II at the lume-



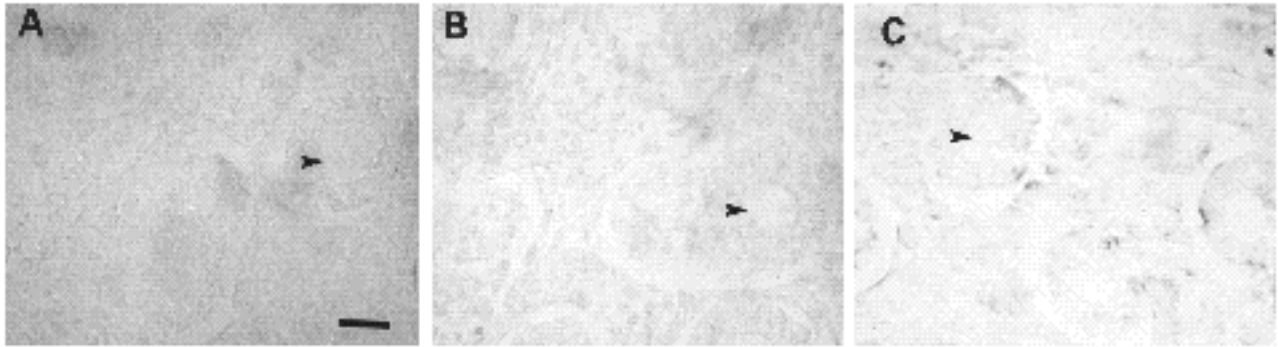
**Fig. 4.** Details of IGF-I and IGF-II staining in fetal rat lung at day 18 of gestation. The greatest concentrations of immunoreactive IGF-II were seen at the surface of large airways (large arrows, A) but at higher magnification, IGF-II can be seen within or upon the apical epithelial surface in small saccular alveoli (large arrow, B). The low magnification view of IGF-I staining (C) shows the wide distribution of the punctate pattern with a tendency for more frequent staining sites at the extremity of the lobe. Some staining (small arrow) extended over the basal surface of several cells. At high magnification it appeared that the small punctate stained areas could surround a lumen (small arrows), indicating small vessels or capillaries (D). The scale bar in A equals 100  $\mu$ m and is the same scale in C. The scale bar in B equals 10  $\mu$ m and is the same scale in D.

nal surface of lung epithelia at the earlier time of day 16 (Fig. 3D,G), consistent with the observations on the early human fetus by Hill et al. (1989), but the pattern changes markedly by days 18 and 20 when virtually all IGF-I staining is punctate in the mesenchyme or in a granular pattern in the epithelium (Figs 3H,I, 4C,D). Many of the punctate immunoreactive IGF-I sites have an inner lumen and are possibly small vessels or capillaries. This conclusion was supported when a similar pattern was seen with sections stained for von Willebrand Factor (Factor VIII-related antigen). However, the von Willebrand factor stain did not co-localise with intra-epithelial IGF-I stain as seen in Fig. 4D (data not shown). It is possible that these granular structures within epithelia containing immunoreactive IGF-I are endocytotic material. The IGF-II immunoreactive material was seen most frequently at the apical surface of epithelia (Figs 3D-F, 4A,B) but spreading to certain mesenchymal cells by day 20. It is interesting to note that the co-localisation of IGF-II and IGFBP-2 message is consistent at all three gestation times and is not likely to involve the IGF-II (mannose-6-phosphate) receptor since the expression of this gene appears to be restricted to the

smooth muscle of larger vessels in the lung at this stage in gestation (Senior et al., 1990).

The possibility of histological artefacts giving rise to the images produced by IGF-I and IGF-II immunohistochemistry was minimised by analyzing sections from additional samples of tissue fixed with Bouins solution and fluorescence immunohistochemistry (fluorescent second antibody), which does not rely on biotin-streptavidin coupling or peroxidase activity. The images produced by these alternate techniques were virtually identical and confirmed the observations presented here. The control and antiserum preabsorption studies (Fig. 5A-C) provide good evidence of immunohistochemical specificity, excluding the possibility of significant image artefacts.

Previous work on the activity of IGF-I and IGF-II gene expression in the human fetus has demonstrated that mesenchymal cells are the major site of production in 16-20 week gestation human embryonic tissues (Han et al., 1987). IGF-II and, to a lesser extent, IGF-I gene expression were reported in the mesenchymal tissues, especially in the connective tissues of the interlobular septa and some vessel



**Fig. 5.** Control studies for IGF-I and IGF-II immunohistochemistry. In general, there is no evidence of physical trapping of primary or secondary antisera which could give rise to artefactual images. The photomicrographs were deliberately overexposed to produce a visible image. (A) Control study with biotinylated second antibody and with no primary antiserum. The arrow marks an alveolar lumen. There is no evidence of specific or non-specific staining in this experiment. The scale bar is equal to 10  $\mu\text{m}$  and is the same for (B) and (C). (B) Preabsorption of the IGF-II antiserum with IGF-II (10  $\mu\text{M}$ ) for 24 hours before immunohistochemistry resulted in 43% decrease in staining. The arrow marks an alveolar lumen. There is no significant staining of the epithelial surface. (C) Preabsorption of the IGF-I antiserum with IGF-I (10  $\mu\text{M}$ ) for 24 hours before immunohistochemistry resulted in 93% decrease in staining. The arrow marks the alveolar lumen. The faint staining of punctate structures and some basal epithelial structures is just visible.

walls. In marked contrast to the human studies, IGF-II mRNA was reported present only in epithelium of major bronchi, and IGF-I mRNA was present in insignificant amounts during late gestation (day 17.5) in fetal rat lung (Beck et al., 1987). In earlier rat embryos (day 13.5), Wood et al. (1990) demonstrated IGFBP-2 and IGF-II co-expression in some endodermal structures, notably the liver and bronchial epithelium, but the two genes were expressed separately in the majority of tissues. In our experiments with an IGF-II probe, we found little evidence of specific expression in lung, while the developing skeletal structures gave a very strong signal and the liver, an expected site for IGF-II gene expression, was greater than lung but less than the rib (Fig. 2B). The dispersed labelling of the lung with the IGF-II probe is more consistent with mesenchymal gene expression and contrasts with the sharply defined epithelial labelling evident in most of the IGFBP-2 *in situ* images (Fig. 2A).

Comparing these previous observations on IGF gene expression with the location of immunoreactive IGF-I and IGF-II in fetal lung reported here, and by Hill et al. (1989) on the human fetus, it appears that IGF-I and IGF-II peptides are often found, presumably sequestered by binding proteins, at locations distant from site(s) of gene expression and synthesis in the fetal lung. For example, IGF-II generated in the mesenchyme must diffuse or be transported to the luminal surface of the epithelium before binding at the apical surface. Similarly, if IGF-II is generated in the epithelium of the large airways, it must be translocated to smaller airways and alveoli.

The presence of IGFBPs and IGF-II in amniotic fluid (D'Ercole et al., 1985, Wang et al., 1990) raises the possibility that these may be available to the fetus transdermally and by swallowing. Since there is reported to be little mixing of amniotic fluid with lung (tracheal) fluid (Harding et al., 1986), the access of the fetal lung to this source of IGFBPs and IGF-II would seem to be only indirect. Even so, high concentrations of IGFBP-2 at the apical surface of lung epithelium would be ideally placed to glean IGF-II intro-

duced into the luminal fluid of the fetal lung, whatever its origin.

Our studies show that IGF-I immunoreactivity does not co-localize with IGF-II, especially at days 18 and 20 of gestation in the fetal rat lung. It is possible that the partial colocalization seen on day 16 (Fig. 3D, G) may reflect a sufficient excess of IGFBP-2 capacity to bind some IGF-I. Alternatively, there may be sufficient crossreactivity of the IGF-I antiserum with IGF-II to give a reaction with the relatively high levels present at the apical surface of the alveolar epithelium (Fig. 3D, G). It is clear, however, that IGF-I at days 18 and 20, and even at day 16, is present in a punctate pattern, which is not consistent with IGFBP-2 gene expression, IGFBP-2 immunoreactivity (Hill et al., 1989) or IGF-II immunoreactivity. It is likely that IGF-I is localized by binding to proteins other than IGFBP-2 or possibly by some form of intracellular sequestration.

Spatial control of growth, for example by different patterns of expression of related proto-oncogenes, is an established phenomenon in lower life forms such as *Drosophila* (McGinnis et al., 1984). The microanatomical separation of related peptides such as IGF-I and IGF-II in the lung and other fetal tissues by gene expression products such as specific binding proteins may be considered a similar, if more complicated process.

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