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Original Article

In vivo localization of the insulin-like growth factors I and II (IGF I and IGF II) gene expression during human lung development

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ABSTRACT The insulin-like growth factors I and II (IGF I and IGF II) are synthesized in many organs during human development and are involved in the growth and differentiation of tissues. Correlations between lung growth and maturation and the local production of IGFs have been poorly explored in humans. Using *in situ* hybridization we localized the synthesis of IGFs in the human fetal respiratory tract over an extended period of the gestation and we demonstrated time dependent changes. IGF mRNAs were expressed throughout gestation with a clear predominance of IGF II and a decreasing expression of both IGFs after the 20th week of gestation. They were mainly detected in the mesodermal-derived components of the respiratory tract, especially in the undifferentiated mesenchyme of the lung buds up to 20 weeks of gestation. At this time the local production of collagen and the proliferation of adjacent epithelial cells were predominant features. Later, mesenchymal hybridization decreased. Weak epithelial hybridization was observed during the first stages of growth and progressively decreased when the epithelium underwent maturation: early in the trachea, later in the distal lung buds. A consistent expression of IGF II, but not IGF I, in the endothelium, throughout gestation, was also observed. The IGFs may act on the near epithelial cell proliferation in both autocrine and paracrine ways. They may also stimulate the maturation of the connective tissue. This endogenous production of growth factors may play a role in the somatic growth during prenatal life.

KEY WORDS: *insulin-like growth factors, human fetus, lung, in situ hybridization*

Introduction

The insulin-like growth factors (IGF I and IGF II) are polypeptides with a structural homology to pro-insulin (Zapf *et al.*, 1984). They were first characterized by Salmon and Daughaday by their actions in promoting somatic growth (Salmon and Daughaday, 1957). The mitogenic actions of the IGFs are now well documented from *in vitro* experiments (Van Wyk, 1984; D'Ercole, 1989). The IGFs are also able to influence the differentiation of a variety of cell types, such as myoblasts (Florini *et al.*, 1986), and induce a differentiated cell function, like stimulation of glycogen synthesis in fetal rat hepatocytes (Freemark *et al.*, 1985) or type II collagen formation in cartilage (Demarquay *et al.*, 1990). IGFs are present in blood bound to carrier proteins called IGF binding proteins (IGF-BPs) (Zapf *et al.*, 1984). These binding proteins are synthesized in the human fetus in a tissue-specific fashion. Pannier *et al.* (1994) showed that IGF-BP 4 mRNA is abundantly expressed in fetal lung. IGFs are thought to act on their target cells by binding to specific cell surface receptors (Morgan *et al.*, 1987; Lammers *et al.*, 1989). Great emphasis has been placed on the paracrine action of IGFs (D'Ercole, 1987). However they are not devoid of autocrine action, as demonstrated during muscle development by Tollefsen *et al.* (1989) or in the kidney (Kobayashi *et al.*, 1991). The theory of an endocrine action may be supported by their role in fetal growth (Lassare *et al.*, 1991).

The role of IGFs in normal mammalian development is a subject of increasing interest to developmental biologists. These growth factors are active during prenatal development (Froesch *et al.*, 1985; Liu *et al.*, 1989) and play an important role in cellular proliferation and differentiation (Jetten, 1991). Their action on fetal growth has been demonstrated: newborn mice bearing a targeted disruption of the IGF I and IGF II gene exhibit severe growth deficiency (Baker *et al.*, 1993; Liu *et al.*, 1993). The widespread production of IGFs has been demonstrated in fetal tissues using immunohistochemistry (Han *et al.*, 1987b) or mRNAs detection (Scott *et al.*, 1985; Han *et al.*, 1988). Transcripts for IGF II have

Abbrevations used in this paper: IGF I, insulin-like growth factor I; IGF II, insulin-like growth factor II.

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TABLE 1

IGF II GENE EXPRESSION IN HUMAN FETAL LUNG

Age(weeks of gestation)	10	11	12	12	15	17	19	20	23	25	28	30	35
Localization													
*Undiff. mesenchyme	++	++	++	++	++	++	++	++	+	no	+	+	+
*Dense conn. tissue	no	+	+	+	+	+	+	+	+	+	+	+	+
*Perichondrium	no	++	++	++	++	no	+	no	+	+	+	no	+
*Cartilage	no	+	+	+	+	no	+	no	-	-	-	no	-
*Smooth muscle	no	+	++	++	++	+	+	no	+	++	+	no	+
*Endothelium	++	++	++	++	++	++	++	++	++	++	++	++	++
*Epithelium in the airways	no	+	-	+	+	-	-	no	-	-	-	no	-
*Epithelium in the lung buds	+	+	+	+	+	+	+	+	+	no	+	+	+

++, strong hybridization (strong signal after a short exposure); +, weak hybridization (faint signal but higher than background levels); -, negative hybridization (autoradiographic grains density not higher than background levels); no, not observed component (absent in the sample)

been detected in human from as early as day 18 of gestation (Brice *et al.*, 1989) and for IGF I from at least mid-gestation (Han *et al.*, 1987a). Some other studies have suggested that the synthesis of IGFs may begin earlier (Smith *et al.*, 1987). IGFs receptors have also been identified from the 9th week of human gestation (Grizzard *et al.*, 1984). All these results support the hypothesis that IGFs are active throughout human development.

However, there is little information about the synthesis of IGFs in human developing tissues and the studies focus only on part of the gestational development (Han *et al.*, 1987a; Brice *et al.*, 1989). We are not aware of recent studies specifically concerned with the synthesis of IGFs in the human fetal lung *in vivo* during an extended period of gestation. Most studies have been carried out *in vitro* on fibroblasts (Stiles and Moats-Staats, 1989), on mouse fetal lung explants (D'Ercole *et al.*, 1980), or on tracheal epithelial cells (Retsch-Bogart *et al.*, 1990).

Precise information about local synthesis of the IGFs may be important for the understanding of their role during normal development of the organ. Moreover, a better knowledge of the expression of IGFs during periods of active cell proliferation is probably a first step towards understanding lung hypoplasia and other growth defects, repair following injury or neoplastic cell proliferation. Consequently, we undertook a study using *in situ* hybridization applied to a wide range of developmental stages, to localize the IGF I and IGF II messenger RNA and to see if there might be time-dependent changes occurring in the course of human lung growth and maturation.

Results

We analyzed the gene expression of both IGF II and IGF I in the human fetal respiratory tract between 10 and 35 weeks of gestation. For each case the transcripts for the IGFs were examined first in the mesodermal structures (lamina propria in the airways, cartilage, smooth muscle, connective tissue of the interlobular septa or pleura, undifferentiated mesenchyme of the distal areas, vessels) and then in the endodermal derived epithelium at different levels of the respiratory tract (trachea and airways, pulmonary buds).

IGF II gene expression in the human fetal respiratory tract

Table 1 shows the levels of labeling obtained in each structure of the respiratory tract between 10 and 35 weeks of gestation. The intensity of the signal is based on a comparison of 8 sections of each sample exposed for 2 exposure times to photoemulsion (4 and 6 weeks).

At every stage of development the mesodermal components hybridized with the probe for IGF II mRNA. Much of the hybridization appeared to be localized in the undifferentiated mesenchyme of the distal lung buds. High levels of autoradiographic grains were detected in the vicinity of the growing epithelial tubes and canalicules up to the 20th week of gestation (Figs. 1A and 2A). We observed a slight loss of hybridization after this time (Figs. 4A and 5A), however the signal remained clearly detectable and was always higher than in the negative control slides.

The connective tissue of the main airways, the interlobular septa (Fig. 3A) and the pleura expressed IGF II transcripts. However the labeling was much lower than in the undifferentiated mesenchyme except in the perichondrium (Fig. 2C) and in areas of developing smooth muscle, which were sites of high hybridization throughout gestation.

Hybridization also occurred in developing cartilage but the chondrocytes appeared to lose most of their transcripts as they assumed a differentiated appearance.

We have consistently demonstrated an important hybridization in the vessels throughout gestation. In particular high levels of IGF II mRNA were found in the endothelium of the major vessels (Figs.

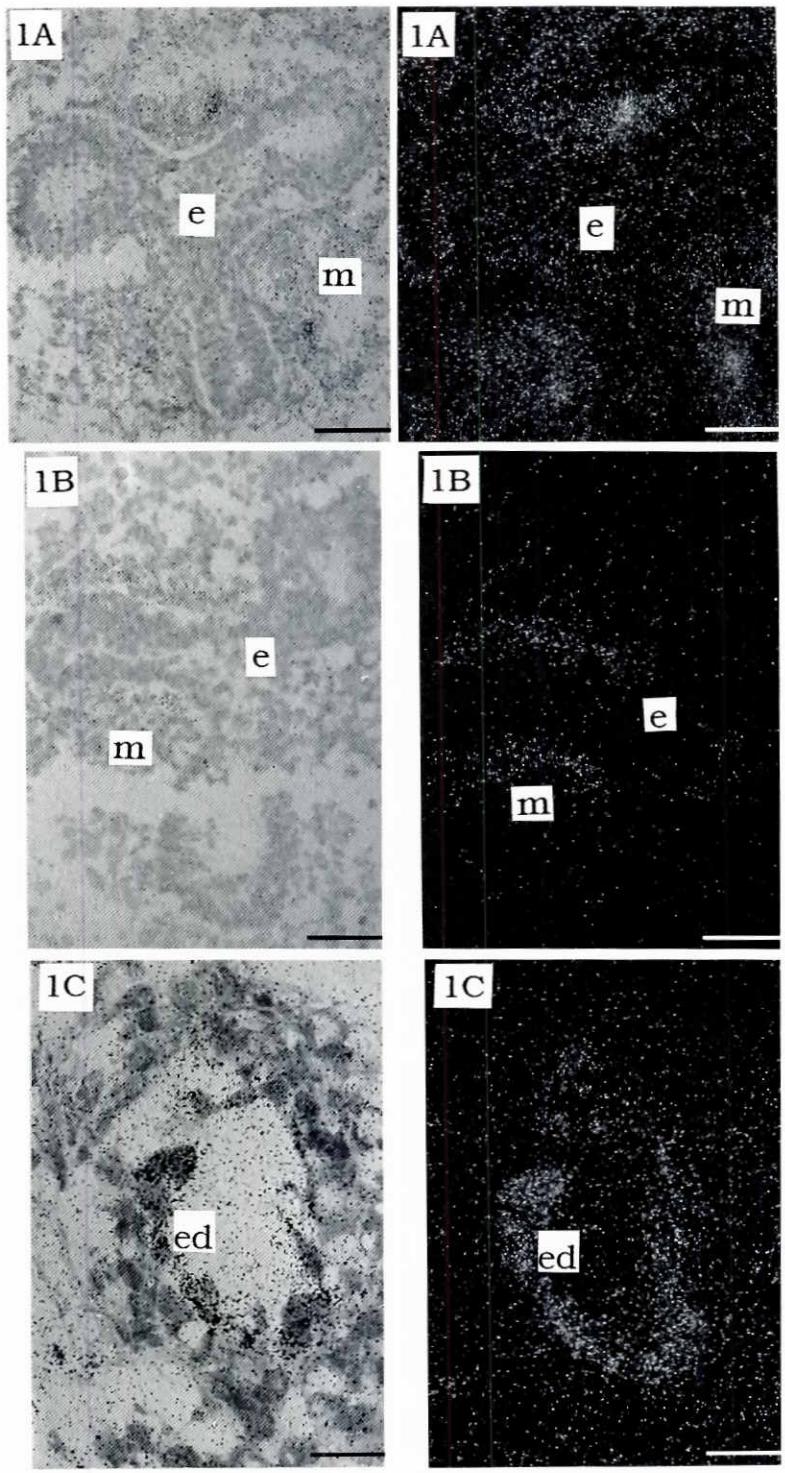

Fig. 1. Pattern of IGFII and I gene expression in human fetal respiratory tract at 12 weeks of gestation. (A) *IGF II mRNA expression in a dividing epithelial tube surrounded by undifferentiated mesenchyme (bright and dark field). The transcripts are mainly found in the mesenchyme immediately surrounding the epithelial tubes. The undifferentiated epithelial cells also express a few IGF II mRNA.* **(B)** *IGF I mRNA expression in the same sample on serial sections. A faint hybridization is observed, mainly in the mesenchyme. No epithelial expression is detectable.* **(C)** *IGF II mRNA in a vessel showing high hybridization especially in the endothelium. e, epithelium. ed, endothelium. Scale bar, 20 μm.*

1C and 3A). The capillaries appeared as nests of autoradiographic grains. There was no alteration in the distribution or density of reaction as pregnancy progressed.

The endodermally derived epithelium weakly hybridized with the probe for IGF II mRNA. The few autoradiographic grains were mainly observed in the undifferentiated epithelium of the distal lung buds (Fig. 1A). Labeling decreased and became difficult to detect when the lung underwent alveolization because of the thinness of the alveolar walls. The epithelial cells of the main airways lost their activity earlier, as they assumed their differentiated appearance.

IGF I gene expression in the human fetal respiratory tract

Table 2 shows the level of labeling observed in each case when probing for IGF I mRNA. The hybridization signal was always weaker than with the probe for IGF II mRNA.

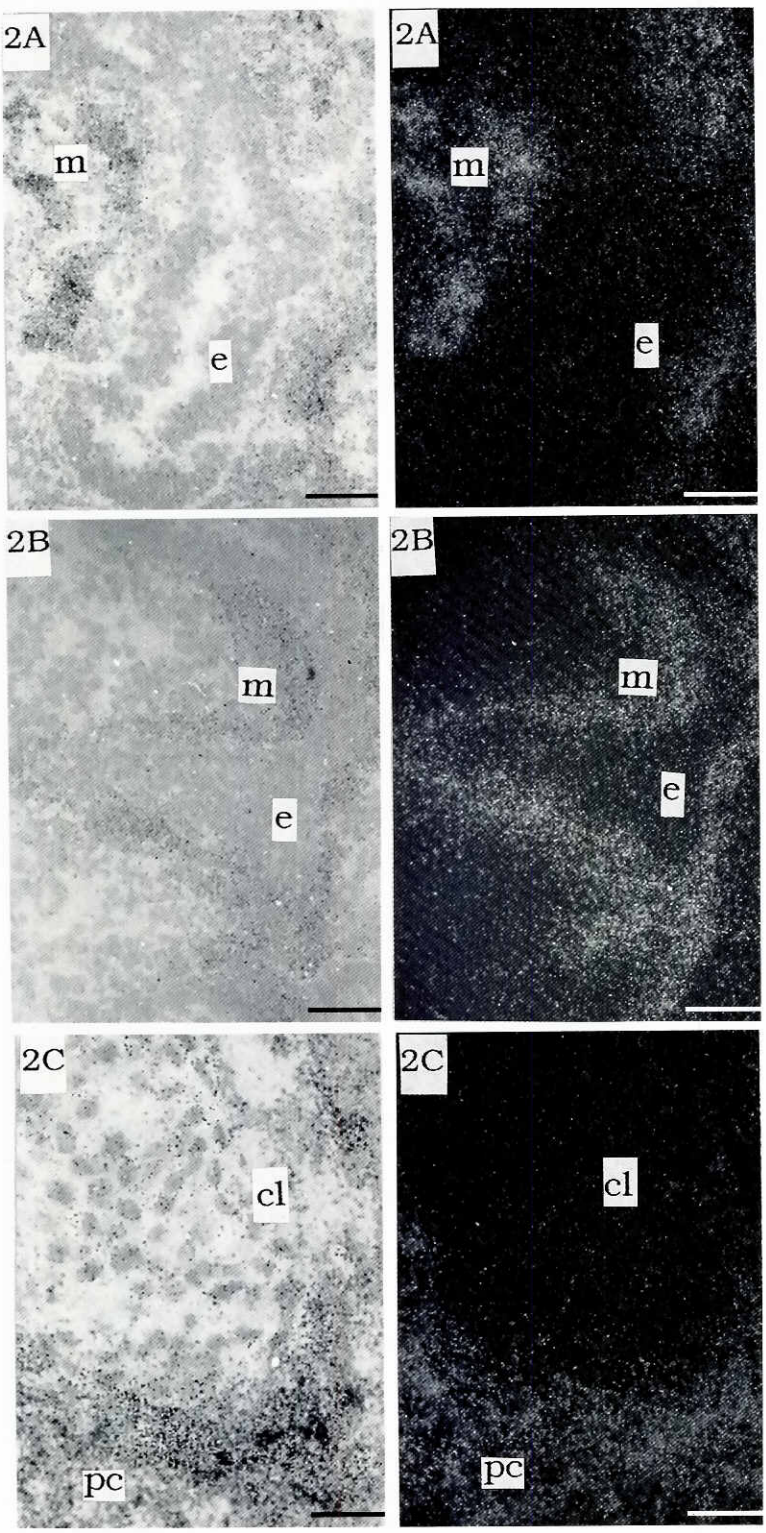

Fig. 2. Pattern of IGF II and IGF I gene expression in human fetal respiratory tract at 15 weeks of gestation. **(A)** *IGF II mRNA in the developing lung. The transcripts are mainly localized in the mesenchyme closely related with the dividing epithelial tubes.* **(B)** *IGF I mRNA in the same area on serial section. The signal is lower than for IGF II, always localized in the mesenchyme.* **(C)** *IGF II mRNA in the tracheal wall. The perichondrium is strongly labeled and the chondrocytes express a few transcripts. e, epithelium; m, mesenchyme; pc, perichondrium; cl, cartilage. Scale bar, 20 µm.*

Among the mesodermal components, the undifferentiated mesenchyme surrounding the epithelium of the lung buds appeared to be the most important site of hybridization. The labeled mesenchymal cells were closely adjacent to the growing epithelial tubes and canalicules (Figs. 1B and 2B). The signal progressively decreased after the 20th week of gestation (Figs. 4B and 5B). The dense connective tissue of the airways, interlobular septa and pleura inconstantly expressed very scarce transcripts, except in the perichondrium which appeared slightly labeled. We always failed to reveal evidence for IFG I mRNA in the vessels,

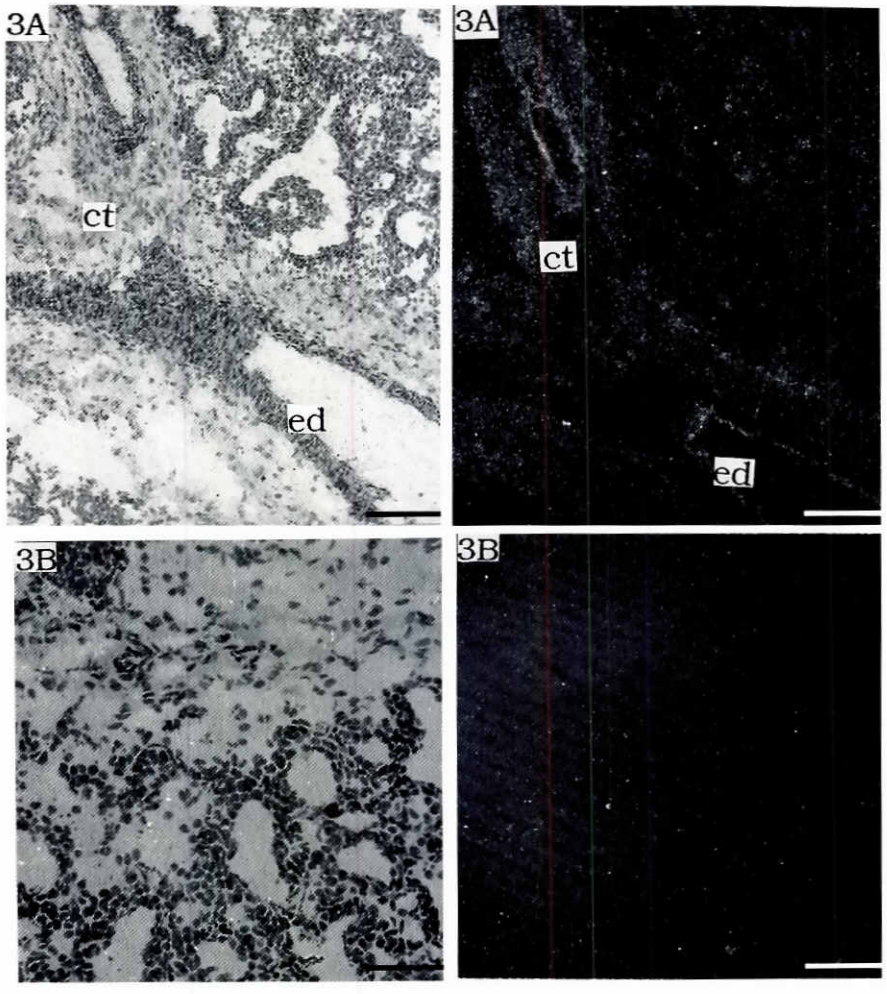

Fig. 3. Detection of IGF II mRNAs in the lung at 19 weeks. (A) *A low power view of an interlobular septum shows the labeling of the dense connective tissue and the high level of transcripts in the large vessels.* **(B)** *The negative control slide treated with RNAse before hybridization with the IGF II cDNA probe shows a very low background. ct, connective tissue; ed, endothelium. Scale bar, 20 μm.*

either in the endothelium or in the smooth muscle. Labeling was not higher than in the negative control slides. No hybridization was detectable in the epithelium during the whole development.

Discussion

The aim of this work was to provide precise information concerning the synthesis of both IGF I and IGF II over an extended period of human lung development and to correlate these factors to early cell proliferation and to later cell maturation. The results may help to support the hypothesis of the role of IGFs during lung development. The implication of IGFs in the development of the respiratory tract is probably predominant: the null mutant newborn mice for Igf1r gene die of respiratory failure (Liu *et al.*, 1993).

We have demonstrated a widespread synthesis of IGFs in the fetal lung, especially in the components of mesodermal origin, and a low or inexistent hybridization in the endodermally derived epithelium.

The IGF II transcripts were predominant whatever the stage of development and we observed a progressive decrease with gestational age. The predominance of IGF II during prenatal development has already been demonstrated in midtrimester human fetal tissues (Han *et al.*, 1988) and in fetal blood (Lassare *et al.*, 1991; Bang *et al.*, 1994). However the major role of IGF I during fetal growth has been demonstrated by Muaku *et al.* (1995) with dietary protein restriction experiments in pregnant rats. IGF I may also be involved in embryonic processes of cell differentiation (Liu *et al.*, 1993).

The IGFs genes are expressed primarily in the mesoderm-derived cells. Previous *in vitro* results highlighted the major role of fibroblasts in IGFs production (Atkison *et al.*, 1980; Snyder and D'Ercole, 1985). However, the few morphological studies available in humans (Han *et al.*, 1987a; Brice *et al.*, 1989) or in animals (Beck *et al.*, 1987; Stylianopoulou *et al.*, 1988) gave inconsistent results concerning the localization of the IGFs mRNA, even among same species.

In specifically examining the human fetal lung we demonstrated that the most important synthesis occurred in the undifferentiated mesenchyme surrounding the expanding epithelia and not in the dense connective tissue of interlobular septa or pleura, as expected from the data of Han *et al.* (1987a). Mesenchymal gene expression decreased with connective tissue maturation after the 20th week. Mesenchymal cells are known to synthesize the extracellular matrix proteins and there may be a correlation between mesenchymal collagen and IGFs production.

In vitro, IGF I has been shown to stimulate matrix synthesis (Abrahamson *et al.*, 1991; Gillery *et al.*, 1992). In our observation

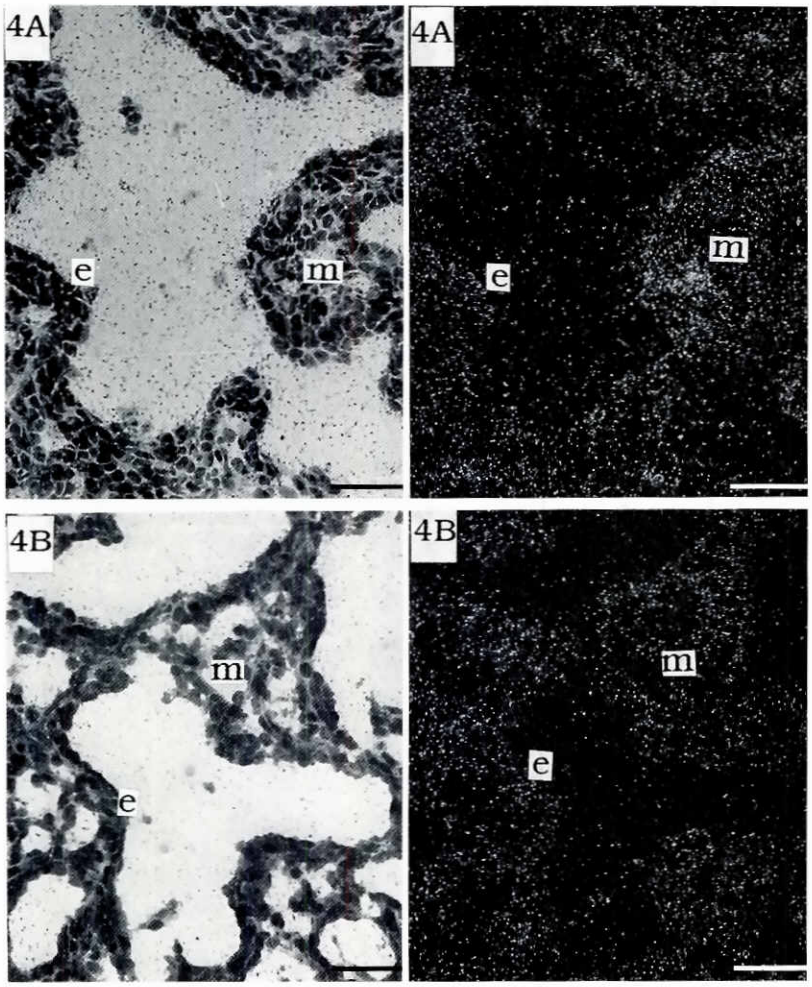

Fig. 4. Detection of IGF II and IGF I in the lung at 23 weeks of gestation. (A) *IGF II mRNA in the lung. The transcripts are still abundant with a slight decrease in the canalicule walls.* **(B)** *IGF I mRNA in the same sample on serial section. The hybridization signal is lower than for IGF II mRNA, localized in mesenchyme. e, epithelium; m, mesenchyme; scale bar, 20 μm.*

the most important production of IGFs occurred at about 20 weeks of gestation and Clark (1983) described a maximum in the synthesis of collagen at this very stage of development. The IGFs may be considered as an autocrine stimulus for collagen synthesis *in vivo* and a factor of maturation for the connective tissue.

We were also able to show a faint endogenous synthesis of IGF II by epithelial cells during the early developmental stages of human lung. In human fetus, Brice *et al.* (1989) demonstrated the existence of IGF II transcripts in the lung epithelium between 12 and 14 weeks of gestation. On the other hand, Han *et al.* (1987a) did not find any epithelial hybridization between 16 and 20 weeks. In our observation there was no striking change in the levels of labeling between 14 and 16 weeks of gestation. The dividing epithelial cells expressed a weak hybridization of IGFs mRNA, differentiating epithelial cells lost their ability to produce IGFs mRNA, early in the trachea, later in the distal epithelium of the alveoli.

The immunoreactivity of the epithelial cells for IGFs (Han *et al.*, 1987b) and the detection of specific receptors for IGFs in the rat gastro-intestinal mucosa (Heinz-Erian *et al.*, 1991) indicate a local action. The IGFs may stimulate the nearby cell proliferation in both autocrine and paracrine ways.

We observed that both epithelial and mesenchymal IGFs mRNA levels were predominant during the period of active epithelial growth corresponding to the first stages of lung development. Lambert *et al.* (1990) found an overexpression of IGF II gene in colorectal tumors. Neoplastic cells are known to divide actively. These findings may underline the mitogenic role of IGFs in normal developing tissue and help to understand the pathophysiology of neoplastic growth.

Beside this mitogenic action on epithelial cells the correlation between IGFs mRNA levels and the incidence of mitosis in the mesenchyme was not striking in our experiment. The mitogenic actions of the IGFs on fibroblasts remain unclear: Gillery *et al.* (1992) did not find any stimulating effect of IGFs, while Abrahamson *et al.* (1991) described a dose dependent stimulation of fibroblastic proliferation. However Liu *et al.* (1993) described a reduction in the number of myocytes in respiratory muscles of the mutant mice for Igf1r gene, indicating a role for IGFs in the proliferation of such mesenchymal cells.

The production of IGF II mRNA by the blood vessels was striking whereas IGF I synthesis remained undetectable throughout gestation. There is little information concerning the synthesis of

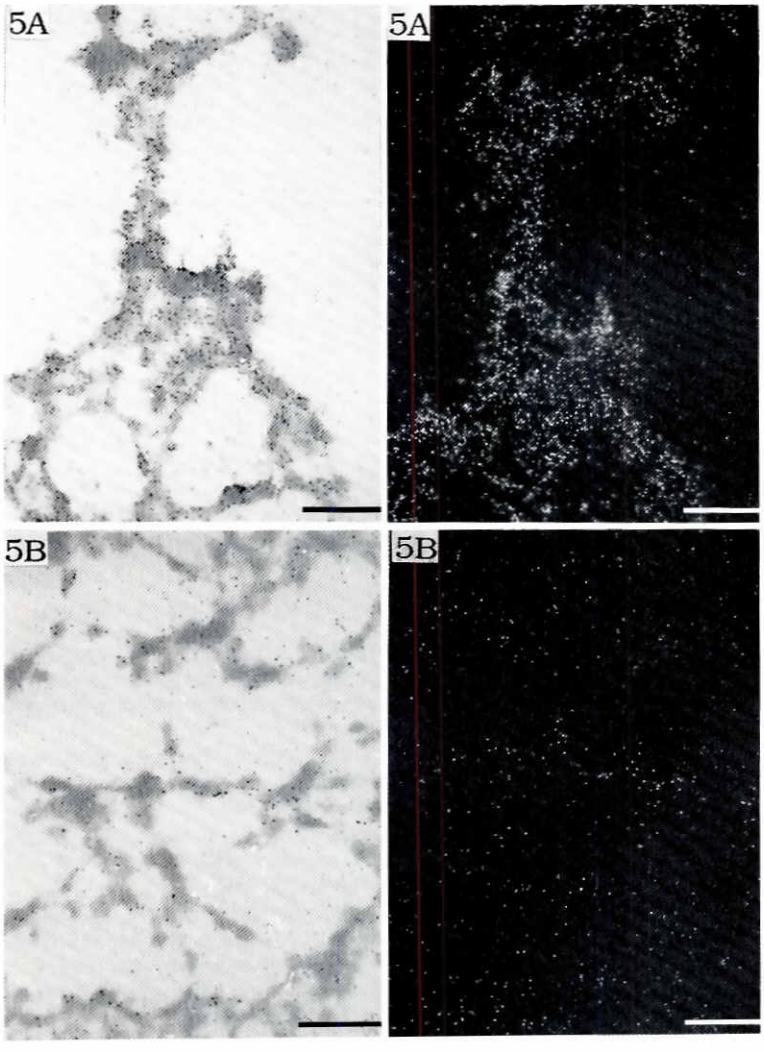

Fig. 5. Pattern of expression of the IGF II and IGF I genes in the lung at 35 weeks of gestation. (A) *Detection of IGF II mRNA in the developing alveoli. The labeling remains detectable in the thin alveolar walls, with a slight decrease.* **(B)** *Detection of IGF I mRNA in the same sample on serial section. The hybridization signal is very low in the alveolar walls. e, epithelium; m, mesenchyme. Scale bar, 20 μm.*

IGFs in the fetal cardio-vascular system in which Han *et al.* (1987a) and Brice *et al.* (1989) reported contradictory results. The data concerning adult murine material (Murphy *et al.*, 1990; Delafontaine *et al.*, 1991) can hardly be compared with results obtained from human fetal vessels. The high levels of IGF II mRNA we observed in the endothelial cells may partly explain the important labeling that Han *et al.* (1987a) reported in the pulmonary connective septa in which large vessels are found. The vascular source of IGF II during fetal life may be related to the high levels of IGF II detected in the fetal blood (Lassare *et al.*, 1991). The immunoreactivity of the endothelium (Hansson *et al.*, 1989), the detection of IGF II specific receptors in vessels (Young *et al.*, 1990) and the release of IGFBP 3 by cultured endothelial cells (Bar *et al.*, 1987) indicate a local role for IGFs. However, the actions of IGF II of endothelial origin and their target cells have to be further explored in normal fetal tissues.

Materials and Methods

Collection of the specimens

Thirteen normal non-infected human embryos and fetuses between 10 and 35 weeks of gestation were studied. They were the product of spontaneous abortions, medical induction or voluntarily interrupted pregnancies, collected between 15 min and 5 h after the abortion. A ß actin cDNA probe (1200 bp long, cloned in the Pst1 site of pGen, provided by Dr. Mayeux, INSERM U152, Hôpital Cochin, Paris, France) was used as a positive control in order to ensure proper protection from RNAs activity.

Fetal age was assessed using the menstrual age and usual morphological criteria: weight, crown-rump length, foot length and organ weight (Trolle, 1948; Tanimura *et al.*, 1971). The tracheo-bronchial tree of the youngest embryos was dissected under a dissecting microscope. The tracheal samples corresponded to the 2nd cartilage ring under the thyroid. The pulmonary sections were taken from the proximal part of the middle lobe of the right lung. The samples were immediately frozen and stored in liquid nitrogen.

In situ hybridization

5 μm thick serial frozen sections of the pulmonary samples were collected on chrome-alum (0.4%) gelatin (0.5%) coated microscope slides, immediately fixed in 4% paraformaldehyde (pH 7.4) for 10 min, washed in PBS (20 mM sodium phosphate -0.7% NaCl pH 7.4), dehydrated in ethanol and stored in 70% ethanol at 4°C before use. Molecular probes were IGF I cDNA, 600 pb long, cloned in the Pst1 site of PSPT18 and IGF2 cDNA, 800-bp PstI fragment of the IGF II clone phigf2 (Bell *et al.*, 1984) kindly provided by Dr. Bell (Howard Hugues Inst., Chicago, USA). They were labeled by random priming, using the DNA labeling system

TABLE 2

IGF I GENE EXPRESSION IN HUMAN FETAL LUNG

Age (weeks of gestation)	10	11	12	12	15	17	19	20	23	25	28	30	35
Localization													
*Undiff. mesenchyme	+	+	+	+	+	+	-	-	-	no	+	+	+
*Dense conn. tissue	no	-	-	-	+	+	-	-	-	-	-	-	-
*Perchondrium	no	+	+	+	+	no	-	-	-	+	+	no	-
*Cartilage	no	-	-	-	-	no	-	-	-	-	-	no	-
*Smooth muscle	no	-	+	+	-	-	-	-	-	-	-	no	-
*Endothelium	-	-	-	-	-	-	-	-	-	-	-	-	-
*Epithelium in the airways	no	+	+	-	+	-	-	-	-	-	-	no	-
*Epithelium in the lung buds	+	-	-	-	-	-	+	+	+	no	-	-	-

++, strong hybridization (strong signal after a short exposure); +, weak hybridization (faint signal but higher than background levels); -, negative hybridization (autoradiographic grains density not higher than background levels); no, not observed component (absent in the sample)

(Amersham, Little Chalfont, UK) and a ^{35}S dCTP (specific activity: 650 Ci/mM) (Amersham) and purified through a Sephadex G50 column followed by ethanol precipitation. Specific activity of the resulting ^{35}S labeled DNA was $3x10^8$ cpm/mg. The slides were pre-treated by heating to 70°C in 2XSSC (1XSSC= 0.15 M sodium chloride and 0.015 M sodium citrate) for 10 min to facilitate probe penetration and then dipped in a solution containing triethanolamine (0.1 M pH 8) and acetic anhydride (0.25%) at room temperature for 10 min. They were carefully rinsed. The denatured labeled DNA was mixed with 50% formamide, 0.6 M NaCl, 10 mM Tris, 1 mM EDTA, 1X Denhardt, 250 mg/ml denatured salmon sperm DNA, 500 mg/ml tRNA, 10 mM DTT, 10% dextran sulphate. 10 µl of the hybridization solution was applied to each slide corresponding to 150000 cpm. Hybridization was carried out for 18 h at 42°C in a humidified chamber. Then the slides were washed in SSC of degrading concentration, dehydrated, and then air dried. Finally sections were dipped in K5 emulsion (Ilford Ltd., Mobberley, Cheshire, UK) for autoradiography, exposed at 4°C for 4 to 6 weeks, developed, counterstained and photographed.

As the probes were cDNA, RNase A was used for negative controls. In each case and for each probe, the control slides were incubated with 10 mg/ml of RNase A for 1 h at 37°C before pre-treatment and hybridization.

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