Mitogenic activity of high molecular weight forms of insulin-like growth factor-II in amniotic fluid

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Abstract

Amniotic fluid (AF) collected from ewes and goats at mid gestation displayed mitogenic activity in mouse fibroblasts. Upon fractionation of this material by size exclusion chromatography, the mitogenic activity was resolved into two peaks, whose activity was inhibited by an anti-IGF type 1 receptor blocking antibody. One of the peaks contained IGF-I and IGF-II (mature form), whereas the other contained high M_r precursor forms of IGF-II. The presence in this latter fraction of IGF-binding proteins (IGFBP) suggests that the AF IGFBPs do not efficiently inhibit the mitogenic activity of the high M_r forms of

IGF-II. In agreement with this conclusion, exogenous IGFBP-1 failed to affect this activity. Analysis of IGF-II in sheep AF showed that the AF concentrations of both forms of IGF-II increased dramatically from mid pregnancy until 106–120 days of gestation, and fell thereafter. The amniotic IGFBPs followed a similar evolution. High M_r forms of IGF-II were also found in human AF, with a pattern of electrophoretic migration different from that of sheep. We suggest that the precursor forms of IGF-II may play an important role in foetal development.

Journal of Endocrinology (2001) **169**, 563–572

Introduction

The importance of amniotic fluid (AF) growth factors for normal foetal growth has been demonstrated in numerous studies. In the sheep, blocking AF ingestion by the foetus results in abnormal development of the gastrointestinal tract and the liver, and inhibits placental growth (Trahair *et al.* 1986, Avila & Harding 1991, Trahair & Harding 1992). By infusing insulin-like growth factor (IGF)-I to the foetus Kimble *et al.* (1999) were able to restore the disorders induced by ligation of the foetal oesophagus and therefore established the importance of the swallowing of AF growth factors, particularly of the IGFs, in normal foetal development. In the human, epidermal growth factor, transforming growth factor- α and hepatocyte growth factor are present in the AF and are thought to act in the development of the stomach and the respiratory tract (Kelly *et al.* 1997, Itakura *et al.* 1997). Growth factors or hormones present in the AF can be taken up by the foetus not only by AF deglutition, but can also penetrate the lung by inhalation (Haddad *et al.* 1995), can diffuse through the foetal skin and can be absorbed by the vascularized foetal surface of the placenta (Lind *et al.*

1972). The presence of IGF-I and IGF-II as well as of several IGF-binding proteins (IGFBPs) in human AF is well documented (Merimee *et al.* 1984, D'Ercole *et al.* 1985, Baxter *et al.* 1987, Busby *et al.* 1988, Rechler 1993, Chard *et al.* 1994, Nonoshita *et al.* 1994, Jones & Clemmons 1995). There are few studies concerning the IGF system in the AF of the ewe. Wang *et al.* (1990) found that ewe AF contains an IGFBP of 38 kDa with high affinity for IGF-II and lower concentrations of IGFs than foetal serum.

In this work, we have studied the properties of mitogenic components present in sheep and goat AF at 70 days of pregnancy, the time of the initiation of fast foetal growth in these animals (Ehrhardt & Bell 1995). Mouse BP-A31 fibroblasts, a cell line well characterized in terms of its response to mitogenic factors (Buchou *et al.* 1989, Buchou & Mester 1990, Fagot *et al.* 1993), were used as indicator cells. After chromatographic fractionation of AF, we have shown two peaks displaying mitogenic activity mediated by the type I IGF receptor. One of these peaks contained IGF-I and IGF-II (mature), whereas the other, eluting in the higher M_r region, contained precursor forms of IGF-II (17–30 kDa). In contrast with the mature form,

the high M_r forms of IGF-II displayed a mitogenic activity which was not inhibited by AF IGFBPs or by exogenous IGFBP-1.

Materials and Methods

Reagents

Insulin was from Novo (Copenhagen, Denmark). Recombinant human IGF-I and IGF-II were a gift from Ciba-Geigy/Novartis (Basel, Switzerland). IGFBP-1 purified from human AF was kindly provided by Dr J W van Neck (Professor Drop's laboratory, Erasmus University, Rotterdam, The Netherlands). Recombinant human IGFBP-3 was a gift from Celtrix Pharmaceuticals (Santa Clara, CA, USA). The anti-IGF-I and anti-IGF-II antibodies were from Upstate Biotechnology (Euromedex, Souffelweyersheim, France). The monoclonal anti-IGF type 1 receptor blocking antibody (clone α IR3) was from Oncogene (France Biochem, Meudon, France). Methyl- 3 H-thymidine was from Amersham (Les Ulis, France). All other compounds (reagent grade) were from regular commercial sources.

Amniotic fluid

AF was collected by puncture of the amniotic sac of ewes or goats which were previously killed or anaesthetized in accordance with the rules of the European Convention (86/609/EEC), Slovak legislation and French recommendations for the use of experimental animals (guideline 04/19/1988). A single AF sample was collected per animal at different times of the pregnancy (from 41 days to term). Throughout this work, AF from sheep and goats were used and gave similar results. Human AF samples were collected for prenatal diagnosis purposes at the Service of Obstetrics and Gynaecology of Saint-Antoine University Hospital, according to French legislation and ethical rules. Aliquots of AF from 15 women (16–18 weeks of gestation) were supplied for this study by the cytogenetics laboratory of the hospital.

Aliquots of AF were maintained at -20°C . AF samples from individual subjects were analysed, except for peptide fractionation. In this case, sheep or goat AF samples collected at 70 days of pregnancy were pooled (250 ml) and delipidated by chloroform (Blahovec *et al.* 1997).

Peptide fractionation

Delipidated AF was concentrated at 37°C by rotary evaporator and submitted to gel filtration on a Sephadex G-10 column (Amersham Pharmacia, Saclay, France) equilibrated in $0.04\text{ M NH}_4\text{HCO}_3$. Void volume fraction (fraction A; $M_r \geq 700$) and total volume of the column

(fraction B) were collected and lyophilized. Peptide content of the fractions was measured by the ninhydrin reaction.

Peptides (100–150 mg) eluted in fraction A were dissolved in 10% acetic acid (15–20 ml) and loaded onto a Sephadex G-50 column ($30 \times 900\text{ mm}$; Amersham Pharmacia). Elution was carried out in the same medium at a flow rate of 9.5 ml/h , and monitored by optical density at 280 nm. The position of elution of ^{125}I -IGF-I as well as the void volume (elution of Blue dextran) and the total volume (elution of potassium chromate) were established under the same conditions. The fractions obtained were lyophilized and reconstituted with $0.5\text{ ml H}_2\text{O}$ for further analysis.

For further purification, G-50 column fractions of interest were pooled and loaded on a Sepharose 4B lentil lectin column (Amersham Pharmacia) to eliminate N-glycosylated components. The flow-through fraction was then loaded on an anion exchange column (Mono Q mini column, 0.1 ml ; Amersham Pharmacia) equilibrated with 30 mM Tris-HCl , pH 8.0. Elution was performed with a linear NaCl gradient ($0\text{--}0.2\text{ M}$).

Proliferation tests

Mouse BP-A31 fibroblasts were maintained in minimal essential medium modification α (MEM α) supplemented with 6% foetal bovine serum (FBS) in a humidified air/ CO_2 (5%) atmosphere. For the detection of mitogenic activity, 20×10^3 cells were seeded per well in 24-well boxes. After 24 h, the medium was replaced with $1\text{ ml MEM}\alpha$ plus $2.5\text{ }\mu\text{M FeSO}_4$ and the cells were allowed to enter quiescence for an additional 24 h. ^3H -Thymidine ($2\text{ }\mu\text{Ci}$) was added to the wells, together with substances and/or column fractions to be tested. After 24 h, the incorporation was terminated by acidification with 1 M ascorbic acid (3 drops/ml). The cells were fixed and washed with 5% trichloroacetic acid and solubilized in 0.1 M NaOH for counting in a liquid scintillation spectrometer.

Forskolin (10^{-5} M) and phorbol myristate acetate (PMA; 100 ng/ml) were used to investigate the role of protein kinase A and protein kinase C (PKC) signalling pathways.

The presence and mitogenic activity of components of the IGF system in chromatographic fractions of AF were investigated by using IGF-I (1 nM), IGF-II (10 nM), anti-IGF-I ($10\text{ }\mu\text{g/ml}$) or anti-IGF-II ($10\text{ }\mu\text{g/ml}$) antibodies, neutralizing anti-IGF type 1 receptor antibody (α IR3; $2\text{ }\mu\text{g/ml}$), IGFBP-1 (70 nM) or IGFBP-3 (10 nM).

Western immunoblot and ligand blot

Analysis of IGF-II by Western immunoblot was performed with a specific anti-IGF-II antibody ($1\text{ }\mu\text{g/ml}$) directed against rat IGF-II, after electrophoresis under reducing

conditions in 15% polyacrylamide gels (Laemmli 1970) followed by transfer onto nitrocellulose filters (Interchim, Montluçon, France).

IGFBPs were analysed by Western ligand blot as described by Hossenlopp *et al.* (1990). Aliquots of crude AF or column fractions were adjusted to 1% sodium dodecyl sulfate (SDS) in non-reducing Laemmli sample buffer and boiled for 5 min. They were fractionated by electrophoresis in 12% polyacrylamide gel and electro-blotted onto nitrocellulose filters. The membrane was then incubated with a mixture of 125 I-labelled IGF-I and IGF-II (1/1; 500 000 c.p.m. per filter). After extensive washing, the filters were exposed with an X-ray sensitive film (Kodak).

Assay of IGFs

The IGF concentration in Sephadex G-50 column fractions was determined by radioimmunoassay for IGF-I (Bessone *et al.* 1999) and protein binding assay for IGF-II (Binoux *et al.* 1986). Before the assays, the column fractions were adjusted to 1 M acetic acid, 0.15 M NaCl and 0.1% bovine serum albumin. In order to eliminate IGFBPs which would interfere with the assays, the samples were submitted to a filtration on Centricon C 30 (Amicon; Merck Eurolab, Strasbourg, France). Samples were tested in duplicate at two different concentrations. Intra- and interassay coefficients of variation were 4.8 and 10% respectively for IGF-I and 4.8 and 9.5% for IGF-II. The sensitivities of the assays were 0.85 ng for IGF-I and 0.03 ng for IGF-II.

Results

Detection and characterization of mitogenic activity

When the delipidated AF was added to serum-deprived BP-A31 cells, there was no effect on 3 H-thymidine incorporation (data not shown). However, after gel filtration on Sephadex G-10, the fraction eluted in the void volume (fraction A) was able to initiate the cell cycle in serum-deprived BP-A31 cells; in contrast, fraction B, eluted in the total volume of the column, contained small molecules that inhibited the cell cycle (Fig. 1) and were toxic at high concentration.

In order to obtain information concerning the functional characteristics of the growth factor(s) responsible for the observed mitogenic effect of fraction A, we tested the activity of these fractions in the presence of forskolin (activator of adenyl cyclase) or PMA (activator of PKC) (Fig. 1). Forskolin did not alter the mitogenic activity of fraction A, whereas it abolished the effect of PMA. The activities of fraction A and PMA were approximately additive. The same results were obtained with two different pools of AF.

Figure 1 Effect of Sephadex G-10 column fractions on 3 H-thymidine incorporation by BP-A31 cells. The void volume (fraction A) or total volume (fraction B) of the Sephadex G-10 column were lyophilized and taken up in 10% acetic acid to a final concentration of 7 mg dry material/ml. Portions of the fractions were then added to quiescent BP-A31 cells in wells containing 1 ml serum-free MEM α medium; the final concentrations were 25 μ g/ml. At the same time, the effect of PMA was determined, alone and in combination with fraction A. The inhibition of the mitogenic effects of PMA (100 ng/ml) and of fraction A by forskolin (10^{-5} M) were also assayed. FBS (6%) was used as positive control, fresh serum-free medium as negative control. 3 H-Thymidine incorporation was evaluated as described in Materials and Methods. Results are expressed as means $\pm$ S.E.M. of triplicate cultures. The experiment was repeated twice with similar results. C, control; S, FBS; A, fraction A; B, fraction B; Fk, forskolin; P, PMA.

When fraction A was submitted to size-exclusion chromatography on Sephadex G-50, two peaks of similar mitogenic activity were resolved (Fig. 2). The first peak of activity (peak I; maximum in fractions 20 and 21) eluted immediately after the void volume of the column, while the second one coincided with the position of elution of 125 I-IGF-I (peak II; maximum in fractions 27 and 28). This pattern was reproducible in four experiments using different AF pools from sheep or goats.

IGF-I and IGF-II were assayed respectively by immuno- and binding assay in mitogenically active Sephadex G-50 fractions, after a filtration which eliminated proteins of $M_r > 10$ kDa, as described in Materials and Methods. IGF-I as well as IGF-II were detected in peak II, with a maximum in fractions 27 and 28, at the position of elution of I^{125} -IGF-I (Fig. 3b). The concentrations of IGF-II were much higher than those of IGF-I (maximal concentrations of 123.2 and 9.6 ng/ml respectively). Small amounts of IGFs were also present in peak I fractions. Western immunoblot analysis of the Sephadex

Figure 2 Mitogenic activity present in fraction A was separated into two components by Sephadex G-50 gel filtration. Fraction A of the Sephadex G-10 column was further fractionated on Sephadex G-50. The fractions were lyophilized and reconstituted with 0.5 ml H₂O. Twenty microlitres of the solubilized fractions were added to quiescent BP-A31 cells in serum-free MEM α medium and ³H-thymidine incorporation was measured over 24 h. The position of ¹²⁵I-IGF-I elution is indicated. Data are means $\pm$ S.E.M. of triplicate cultures. The same profile of elution was obtained with four different AF preparations.

G-50 fractions with a specific anti-IGF-II antibody (Duguay *et al.* 1998) showed, however, that IGF-II immunoreactive material was present in large amounts both in peak I and II (Fig. 3a). The IGF-II immunoreactive peptides observed in peak I differed from those in peak II by their M_r values. Fractions 26–29 of peak II contained an IGF-II signal which migrated at the same position as recombinant IGF-II (6 kDa), the intensity of the signal varying in parallel to the concentrations of IGF-II determined by binding assay (Fig. 3b). Fractions 18–27 of peak I contained IGF-II immunoreactive peptides which migrated with a higher apparent M_r (17–30 kDa), with a maximum in fractions 20–24 (Fig. 3a). IGF-II found by binding assay in fractions 18–24 corresponds to the mature (low M_r) form present at amounts too low for detection by Western blotting (note that large M_r precursor forms are eliminated together with IGFBPs by Centricon filtration). The concentrations of IGF-I estimated by immunoassay in Sephadex G-50 fractions were also too low to be detected by immunoblotting (data not shown).

Two IGFBP bands were detected by Western ligand blotting with ¹²⁵I-labelled IGFs in Sephadex G-50 fractions 18–22 (Fig. 3c). These IGFBPs migrated respectively as human IGFBP-3 (39–45 kDa) and IGFBP-1 (29 kDa). Attempts to reveal goat and sheep IGFBPs by immunoblotting using polyclonal antibodies raised against human IGFBP-1 and IGFBP-3 were unsuccessful, probably due to the absence of cross-reactivity with ruminant proteins.

The activities of the pooled fractions of peak I or II of Sephadex G-50 column were both strongly inhibited by the α IR3 antibody (Fig. 4a) which blocks the IGF type 1 receptor (Kull *et al.* 1983, Rohlik *et al.* 1987). The anti-IGF-I antibody, in conditions in which it inhibited 1 nM IGF-I by approximately 80%, strongly inhibited peak II in which both IGF-I and IGF-II were detected by radioimmunoassay (74% inhibition), but had no effect on peak I activity. IGF-I is a more potent mitogen than IGF-II in BP-A31 cells; 10 nM IGF-II was required to produce an effect similar to 1 nM IGF-I (Fig. 4b).

As peak I contained IGFBPs (Fig. 3c) together with large amounts of high M_r forms of IGF-II, we investigated the effects of IGFBP-1 and IGFBP-3 in the BP-A31 cells and their inhibition of the mitogenic effect of recombinant IGF-II. No IGFBPs were detected by Western ligand blotting in the supernatant of quiescent BP-A31 cells (Fig. 3c). Recombinant IGF-II (10 nM) produced a large increase in ³H-thymidine incorporation (500%) compared with control. IGFBP-3 alone at 10 nM was not mitogenic in these cells (Fig. 5). IGFBP-3 present during the 24 h before IGF-II had a very low inhibitory effect on IGF-II mitogenic activity, and displayed a weak inhibitory effect when added together with IGF-II (Fig. 5). Purified human AF IGFBP-1 at 70 nM efficiently inhibited the mitogenic activity of 1 nM IGF-I, and reduced the effect of 10 nM IGF-II by 45% (Fig. 4b); when added together with fractions of peak I or II, IGFBP-1 produced only a weak inhibition of the ³H-thymidine incorporation induced by peak I, while the activity of peak II was reduced by approximately 60% (Fig. 4b).

In order to further characterize the substances contained in peak I of the Sephadex G-50 column, pooled fractions of peak I were loaded on a lentil lectin column. After passage through the column, the mitogenic activity was found in the flow-through, indicating that it did not carry N-linked sugar residues. When this material was further fractionated on a Mono Q anion exchange column (Fig. 6a), four peaks absorbing at 280 nm and 256 nm were detected. Two of these peaks (fractions 10–12 and 13–15) were endowed with mitogenic activity (Fig. 6b). Analysis of these fractions by Western immunoblot with an anti-IGF-II antibody revealed a band at approximately 19 kDa in fraction 11, while fraction 14 contained IGF-II-immunoreactive proteins with M_r ranging from 17 to 30 kDa, similar to those found in the initial material (Fig. 6c). There was no IGF-II signal in fractions devoid of mitogenic activity (Fig. 6b and c).

Analysis of IGF-II and IGFBPs in sheep AF in the course of gestation

When crude sheep AF was submitted to Western immunoblot analysis with an anti-IGF-II antibody (Fig. 7a), a band migrating as recombinant IGF-II (6 kDa) was found at all stages of gestation investigated

Figure 3 Presence of IGFs and IGFBPs in Sephadex G-50 fractions. (a) Western immunoblotting. One hundred microlitres of the indicated fractions were brought to dryness under vacuum, reconstituted with 50 µl of reducing sample buffer and fractionated by SDS-PAGE (15% acrylamide), prior to electrotransfer and revelation with anti-IGF-II antibody. Recombinant IGF-II (rIGFII; 5 ng) was loaded in parallel as a control. The position of molecular weight standards is indicated. (b) Radioimmuno- and binding assays. The levels of IGF-I and IGF-II were determined as described in Materials and Methods in the fractions as shown. Minus sign indicates non-detectable level of IGF-I (less than 0.85 ng/ml). (c) Western ligand blotting. Ten microlitres of the fractions indicated were loaded in non-reducing sample buffer on 12% SDS-PAGE and analysed by ligand blotting with ¹²⁵I-IGF-I and ¹²⁵I-IGF-II. Human serum (2 µl) and human AF (2 µl) (AF+S), and recombinant human IGFBP-3 (rhBP3; 5 ng) were used as positive controls. CM, conditioned medium of BP-A31 cells (50 µl) after 24 h of serum depletion. The positions of human IGFBP-1 and -3 are indicated. The same result was obtained with two different pools of AF.

(41–140 days). The intensity of this band was low until 75 days, rose thereafter to a maximum between 106 and 120 days, and then decreased again. A band migrating at 17 kDa was also recognized by the anti-IGF-II antibody. This signal was variable during early gestation, became strong between 80 and 90 days and reached a maximum between 106 and 120 days. Similarly, human AF (18 weeks of gestation) contained the 6 kDa form of

IGF-II, as well as higher apparent *M_r* forms of 12 and 15 kDa; additional bands of 32 and 37 kDa were also present (Fig. 7a). Fourteen other human AF samples obtained at 16–18 weeks of pregnancy exhibited a similar pattern of IGF-II forms (data not shown). Sheep AF samples analysed by Western ligand blot (Fig. 7b) contained IGFBPs whose pattern varied with the time of gestation. At the earlier stages, two IGFBPs bands

Figure 4 Effects of anti-type 1 IGF receptor, anti-IGF-I and IGFBP-1 on the mitogenic activities of Sephadex G-50 fractions. Incorporation of ^3H -thymidine in BP-A31 cells over 24 h was measured after stimulation by 20 μl of pooled fractions of peak I or II, as described in Materials and Methods, in the presence or absence of (a) antibodies directed against type 1 IGF receptor (anti-R-IGF I, αIR3 , 2 $\mu\text{g}/\text{ml}$) or IGF-I (anti-IGF-I, 10 $\mu\text{g}/\text{ml}$) and (b) IGFBP-1 (70 nM). The inhibitory effect of IGFBP-1 on recombinant IGF-I (1 nM) or IGF-II (10 nM) is also shown. Results are expressed as means $\pm$ S.E.M. of triplicate cultures. The experiment was repeated twice with similar results.

migrating at approximately 40 and 45 kDa were shown and two other IGFBPs bands, migrating respectively at approximately 30 and 25 kDa, were detectable after day 90. From day 90 onwards, the overall levels of IGFBPs increased dramatically to reach a maximum by day 120 and decreased thereafter.

Figure 5 Effects of IGFBP-3 on the mitogenic activity of IGF-II. Quiescent BP-A31 cells were incubated for 24 h with IGF-II (10 nM), IGFBP-3 (10 nM) or IGF-II+IGFBP-3, in the presence of ^3H -thymidine. Where indicated, IGFBP-3 was present during the 24 h prior to the addition of ^3H -thymidine (pre-incub). The ^3H -thymidine incorporation was determined as described in Materials and Methods. The results shown (means $\pm$ S.E.M. of triplicate cultures) are representative of two experiments.

Discussion

The AF collected from sheep and goats at 10 weeks of gestation contained mitogenic activity detectable after the removal of inhibitory, low M_r components. This mitogenic activity displayed the characteristics of IGF. In particular, it was not inhibited by forskolin, inducer of cAMP accumulation which acts as an inhibitor of several other mitogenic agents in the BP-A31 fibroblasts used as indicator cells, such as platelet-derived growth factor and PMA (Buchou *et al.* 1989), bovine fibroblast growth factor (Buchou & Mester 1990) and prostaglandin $F_{2\alpha}$ (Fagot *et al.* 1993). Moreover, the effects of the mitogenic component(s) of AF were approximately additive with those of a saturating concentration of PMA (100 ng/ml), indicating different signal transduction pathways. The mitogenic activity of AF was separated by size exclusion chromatography into two peaks, both inhibited by an anti-type 1 IGF receptor blocking antibody. In agreement with these results, assays of IGF and Western immunoblot confirmed that both IGF-I and IGF-II were present in the fractions with mitogenic activity. Assay of IGFs showed that IGF-I and the 6 kDa mature form of IGF-II were eluted in peak II. On the other hand, peak I contained essentially IGF-II immunoreactive material of high M_r (17–30 kDa) as revealed by Western immunoblotting. The presence of the low M_r mature form of IGF-II in peak II, at the position of elution of ^{125}I -IGF-I, was confirmed by Western immunoblotting. The levels of IGF-II and IGF-I indicate that their ratio (12:1) is elevated in sheep AF at mid gestation, similar to that found at the end of

gestation by Wang *et al.* (1990) and as reported by Merimee *et al.* (1984) and Nonoshita *et al.* (1994) in human AF throughout pregnancy.

The Sephadex G-50 peak I of mitogenic activity contained little of the mature IGF-II but instead important amounts of IGF-II immunoreactive peptides of 17–30 kDa. After further fractionation by anion exchange chromatography, these high M_r IGF-II immunoreactive proteins were again recovered in the mitogenically active fractions. We concluded that the mitogenic peptides eluted in peak I after chromatography on Sepharose G-50 are high M_r precursor forms of IGF-II. Big IGF-II isoforms were first described by Haselbacher & Humbel (1982), Daughaday *et al.* (1988), Shapiro *et al.* (1990) and Zapf *et al.* (1992). Jansen *et al.* (1985) have predicted, according to the cDNA sequence, that IGF-II is synthesized as a precursor, and Hudgins *et al.* (1992) identified O-glycosylated precursors of IGF-II. High M_r forms of IGF-II are found in several body fluids of healthy individuals. When produced at high levels by certain types of tumours, they induce severe hypoglycaemia (reviewed in Koch *et al.* 1999). In agreement with our observations, a proIGF-II-immunoreactivity has been detected in human AF (Liu *et al.* 1993). At least 12 different forms of high M_r IGF-II molecules, with a similar protein backbone of 87 amino acid and different amounts of sialylated, O-linked sugars linked to the E-peptide extension, were identified by Valenzano *et al.* (1995) and Valenzano *et al.* (1997) in foetal bovine serum. Other IGF-II isoforms with high M_r are produced by alternative splicing (Jansen *et al.* 1985) and by processing at Arg 104 (Duguay *et al.* 1998). In our study, we have shown that ewe AF contains at least three high M_r forms of IGF-II and that human AF also contains high M_r IGF-II forms, with a different pattern of migration in SDS-PAGE, suggesting that the process of maturation of IGF-II differs between ruminants and humans. These results indicate that, in both species, the high M_r precursor forms of IGF-II are secreted into the AF. It is necessary to take this result into account to measure IGF-II concentrations in AF correctly, as the classical assays evaluate only mature IGF-II. By Western immunoblot, we found that

Figure 6 Ion-exchange chromatography of the large M_r IGF-II containing Sephadex G-50 fractions. Pooled fractions of peak I from the Sephadex G-50 column were loaded onto Sepharose 4B lentil lectin column. The flow-through (FT; 1.5 ml) was loaded onto a Mono Q column and eluted with an NaCl gradient (0–0.2M). Fractions of 75 μ l were collected. (a) Optical density (at 280 and 256 nm) of the fractions eluted from the MonoQ column. (b) Mitogenic activity present in fractions separated on Mono Q column. Five microlitres of each fraction were added to quiescent BP-A31 cells and ³H-thymidine incorporation was measured over 24 h. The same profile of elution was observed with two different AF preparations. (c) Western blot analysis. Forty microlitres of lectin column FT and 25 μ l monoQ fractions were submitted to SDS-PAGE (15%) under reducing conditions. rIGF II, recombinant IGF-II. The immunoblot was revealed with anti-IGF-II antibody.

Figure 7 Analysis of IGF-II and IGFBPs in sheep amniotic fluid in the course of gestation. The AF was obtained from different animals by puncture on the days of gestation indicated. (a) Western immunoblot with anti-IGF-II. Samples (50 μ l) were run on SDS-PAGE (15%) in reducing conditions. A human AF sample (18 weeks of pregnancy) was run in parallel (hAF); the same pattern was obtained with 15 different human AF samples at 16–18 weeks of pregnancy (data not shown). Positions of pre-stained molecular weight standards are indicated. (b) Western ligand blot. Samples (30 μ l) were run on 12% SDS-PAGE in non-reducing conditions. Ligand blot was performed with 125 I-IGF-I and -II as described in Materials and Methods.

AF concentrations of the different forms of IGF-II and of the IGFBPs follow the same pattern of evolution in the course of gestation.

The importance of IGF-II in foetal growth has been confirmed by the inactivation of the IGF-II gene in the mouse (DeChiara *et al.* 1990). In the sheep, IGF-II mRNA is produced at a high level in the maternal and foetal placenta (Wathes *et al.* 1998) and foetal plasma is rich in IGF-II, with a concentration at 130 days correlated with foetal weight (Owens *et al.* 1994). Almost all foetal tissues, particularly the liver, produce IGF-II at higher concentrations than IGF-I (McLellan *et al.* 1992, Kind *et al.* 1995). The origin of the IGF-II in sheep AF has not been determined. The increase of IGF-II (mature and precursors forms) in AF occurs after the placental maximal weight has been reached and coincides with the exponential growth of the foetus after 80 days of pregnancy (Ehrhardt & Bell 1995). It is also to be noted that there is a drop in the IGF-II in AF at the end of gestation as well

as a fall in IGF-II mRNAs in foetal tissues and of IGF-II in foetal plasma at term (Mesiano *et al.* 1989, O'Mahoney *et al.* 1991, Delhanty & Han 1993, Forhead *et al.* 1998, Wathes *et al.* 1998). The AF IGF-II could originate from the foetus through excretion in the urine. However, after infusion of labelled IGF-II into the femoral vein of the ovine foetus, no movement of IGF-II out of the foetal circulation into foetal fluids was observed (Bassett *et al.* 1990). The foetal membranes may constitute another potential source of IGF-II, as they are for other components of AF (Wintour *et al.* 1986).

The peak II, in which mature IGF-II is eluted together with IGF-I, and the peak I, in which high M_r forms of IGF-II are eluted, displayed similar mitogenic activities. As they were both inhibited by the anti-IGF type 1 receptor antibody, we conclude that they do not contain mitogens other than the IGFs active in BP-A31 cells. IGFBP-1 purified from human AF acted as a potent inhibitor of peak II but did not inhibit the activity of peak

I (note also that IGFBPs co-eluted with the high M_r forms of IGF-II in peak I). From these data, we can assume that the IGFBPs are not efficient inhibitors of the high M_r forms of IGF-II present in the AF. This is probably not due to a lack of binding of high M_r forms of IGF-II to IGFBPs. Valenzano *et al.* (1997) found no difference in the binding to IGFBP-1 and IGFBP-3 of the foetal bovine high M_r weight forms of IGF-II. Binding characteristics of pro-IGF-II, extracted from tumours or serum from patients with cancer, to IGFBP-1 to -6 were established: precursor forms of IGF-II did not show any difference from mature IGF-II in their binding to IGFBPs (Bond *et al.* 2000), except for the formation of a ternary complex with the acid labile subunit (ALS) and IGFBP-3. However, ALS is absent from AF. These results suggest that the precursor forms of IGF-II are particularly efficient activators of the type 1 IGF receptor. In agreement with this interpretation, Valenzano *et al.* (1997) have shown that the high M_r weight forms of IGF-II bind to the purified IGF type 1 receptor with a higher affinity than mature IGF-II, and that they have a greater biological activity.

Taken together our data indicate that the precursor forms of IGF-II in AF constitute an important pool of biologically active growth factors.

Acknowledgements

J B was supported by a grant from INSERM and Z K by a European Union grant (Tempus/PHARE). We thank Dr N Joyé for the supply of human AF, L Perrin for the radioimmunoassay of IGFs, M Garbi for help with certain experiments and Professor Y Le Bouc and Professor D Cabrol for helpful discussions.

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Received 12 February 2001

Accepted 22 February 2001