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# Human glomerular endothelial cells IGFBPs are regulated by IGF-I and TGF- $\beta_1$

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## Abstract

The release of insulin-like growth factor binding proteins (IGFBPs) and their regulation in human glomerular endothelial cells (GENC) was characterised. GENC produce IGFBP-4, IGFBP-2 and IGFBP-3 and express mRNA for IGFBP-2 to IGFBP-5. Due to the fact that IGF-I and TGF- $\beta_1$  modulate glomerular hypertrophy, their action on IGFBP release and GENC growth was studied. IGF-I increased IGFBP-3, IGFBP-2 and decreased IGFBP-4, while TGF- $\beta_1$  decreased IGFBP-3 and apparently increased IGFBP-4. All of the IGFBPs, except the TGF- $\beta_1$ -regulated IGFBP-4, were modulated at mRNA level. IGF-I stimulated GENC proliferation, while TGF- $\beta_1$  inhibited their growth. It was demonstrated that an IGFBP-3 antibody reduced GENC proliferation. However, rhIGFBP-3 alone had no effect on GENC, but after 48 h pre-incubation the IGF-I stimulated GENC growth was increased, suggesting that IGFBP-3 could modulate the IGF-I induced GENC proliferation. It was concluded that the stimulatory IGFBP-3 and the inhibitory IGFBP-4 could regulate GENC growth, although the IGFBP-3 seems to have a predominant effect in this control. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** IGF-I; TGF- $\beta$ ; IGFBP-3; IGFBP-4; Endothelial cells

## 1. Introduction

IGF-I and IGF-II have been demonstrated to be implicated in renal embryogenetic growth and function and in kidney diseases (Feld and Hirschberg, 1992; Zumkeller and Schofield, 1992; Flyvbjerg et al., 1995). IGFs play this role either as circulating or as autocrine or paracrine growth factors binding to specific membrane receptors. Studies showing the spatial distribution of the IGF system in the nephron have been predominantly performed in rats (Feld and Hirschberg, 1992), while less information is available on the human kidney. In the adult human kidney, IGF-II conversely

to IGF-I, has been found in the glomerular and peritubular vasculature as well as in the interstitium (Chin and Bondy, 1992; Chin et al., 1994). Moreover, it has been observed that IGF-I and IGF-II binding sites in human kidney sections appear partitioned to their respective specific receptors in glomeruli, tubular interstitial cortex and in the medulla and to specific carrier proteins (the insulin-like growth factor binding proteins, IGFBPs) largely in glomeruli (Chin et al., 1994). So far, six different IGFBPs (IGFBP-1 to IGFBP-6) (Shimasaki and Ling, 1991) with high affinity for IGFs (Baxter and Martin, 1989) have been identified and cloned and a recent report also characterised other IGFBP-related proteins with a lower affinity for IGFs (Kim et al., 1997). The IGFBPs can enhance or inhibit the IGF biological actions modulating the IGF access to their specific receptors. This transport of

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IGFs depends on the type of IGFBP, the experimental conditions (Clemmons, 1991) and the presence/absence of variants occurring for each IGFBP (proteolysation, glycosylation and phosphorylation) (Drop et al., 1992). In addition, there is evidence that IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-5 can be associated with components on the cell surface or within the extracellular matrix (Booth et al., 1995). Furthermore, IGFBP-3 has been demonstrated to have intrinsic biological activity, independent from IGFs and specific cell membrane binding sites (Oh et al., 1993a,b). Both rat and human kidney respond to IGF-I treatment with an increase of glomerular filtration rate and volume (Hirschberg and Kopple, 1989). Moreover, the renal hypertrophy observed in acromegalic patients, after monolateral nephrectomy and at the beginning of diabetic nephropathy, has been associated with an increase of systemic or local IGF-I (Flyvbjerg et al., 1988, 1992; O'Shea and Layish, 1992). The IGF system (Flyvbjerg et al., 1992; Pricci et al., 1996; Pugliese et al., 1996), together with TGF- $\beta$  (Flyvbjerg et al., 1988; Yamamoto et al., 1993; Shankland and Scholey, 1994), plays an important role in controlling glomerulosclerosis which is characterised by an enhancement of extracellular matrix production and altered cell growth. One of the primary effects of TGF- $\beta_1$  addition to cells in culture is the modulation of their growth, while the inhibition of cellular proliferation is the most frequent action played by TGF- $\beta_1$  on endothelial cells (Muller et al., 1987). Conversely, the IGFs have mainly been demonstrated to stimulate endothelial cell growth (Jialal et al., 1985; King et al., 1985; Grant et al., 1993), although, recent papers showed the lack of the mitogenic effect of IGFs on different types of endothelial cells (Dosso et al., 1993; Galli et al., 1994; Giannini et al., 1997). Moreover, both IGF-I and TGF- $\beta_1$  have also been demonstrated to regulate the IGFBP production in different cell types, including fibroblasts, mesangial and endothelial cells (Bar et al., 1987, 1989; Martin et al., 1992; Moser et al., 1992; Cohllick and Clemmons, 1993; Yang et al., 1993; Yateman et al., 1993; Erondur et al., 1996; Grellier et al., 1996; Giannini et al., 1997).

Since endothelial cells are anatomically positioned to determine a monolayer barrier between plasma and interstitial fluids, these cells can control the accessibility of IGFs to the target cells and the formation of IGF:IGFBP complexes. In this way, these cells provide an excellent experimental model to investigate factors that, regulating the endothelial IGFBP secretory pattern, could affect the autocrine or paracrine bioavailability of the IGFs. This study has identified and characterised the IGFBPs released by human glomerular endothelial cells and investigated the effect of IGF-I and TGF- $\beta_1$  on the modulation of the IGFBP release and consequently on cell growth.

## 2. Materials and methods

### 2.1. Cell isolation, culture and characterisation

Human glomerular endothelial cells (GENC) isolation was performed following a modification of the method previously described by Green et al. (1992). Homogeneous endothelial cell cultures were obtained from healthy slices of human kidneys, removed from patients less than 60 years old who underwent monolateral nephrectomy for cancer, and perfused with isotonic saline. Briefly, the capsule of human kidneys was removed and the cortex was aseptically dissected from the medulla and cut into small pieces. The pieces were cut and centrifuged three times at  $1000 \times g$  for 2 min to render them blood free (4235 centrifuge, PBI International, Milan, Italy). They were then incubated at 37°C for 3 h in 50 ml sterile solution containing 1 mg/ml collagenase type II (Worthington Biochemical, NJ). The solution was washed and centrifuged at  $1000 \times g$  for 5 min. The obtained pellet was vigorously resuspended in Coon by siliconated pipette and the solution was aspirated leaving out the biological material at the bottom of the tube. Then, the solution was left to sediment in order to obtain two phases. The lower of phase was harvested. This procedure was repeated five times to isolate the population of glomeruli. Finally, the solution was resuspended in a regular culture medium consisting of Coon's modified Ham F12 medium containing 10% Nu Serum IV (Collaborative Research, Bedford, MA), 1% Ultrosor G (IBF Biotechnics, Savage, MD), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin and plated onto gelatine-coated plates (Corning, NY). When confluent, GENC were dispersed with trypsin and transferred onto 100 cm<sup>2</sup> culture plates (Corning, NY).

Cells were identified as endothelial using morphological and biochemical markers as previously reported (Manuelli et al., 1995). In particular, GENC was characterised as endothelial cells by immunostaining for Factor VIII antigen correlated and for CD31, a platelet endocytosed adhesion molecule (Hewett and Murray, 1993) by using a Universal immunoperoxidase staining kit obtained from Vectro Laboratories (Burlingame, CA). The development reaction was performed using 3-amino-9-ethyl-carbazole (Sigma, St Louis, MO). GENC maintained endothelial markers from passage 1–10 with a positive pattern of 95%.

### 2.2. Collection of conditioned medium, identification and immuno-characterisation of IGFBPs

Cells were seeded in 25 cm<sup>2</sup> tissue culture flasks at a density of  $35 \times 10^3$  cells/cm<sup>2</sup> in regular medium. After a period of 24 h, cells were washed with phosphate-buffered saline (PBS) and cultured in 1.5 ml serum-free

culture medium for 48 h (basal conditions) or in serum-free culture medium for 24 h and then in serum-free culture medium containing IGF-I or TGF- $\beta_1$  (Sigma) at different concentrations for 24 h. The conditioned medium (CM) was then collected and stored at  $-80^{\circ}\text{C}$  in polypropylene tubes (Nalgene, Nalge/Sybron, NY), already treated as previously described (Giannini et al., 1994) to reduce non specific binding of proteins to tubes. After the CM collection, the cell number was determined and the volume of CM analysed and adjusted accordingly.

IGFBPs in the CM were examined by Western blotting, carried out according to the method of Hossenlopp et al. (1986). Briefly, 100  $\mu\text{l}$  of CM along with prestained molecular weight marker proteins (Bio-Rad, Richmond, CA) underwent sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel;  $18 \times 12$  cm) in nonreducing conditions. Proteins separated on the gel were then transferred onto a nitrocellulose membrane in a buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol) at 10 V for 16 h using a transblotting cell apparatus (Bio-Rad). The nitrocellulose membrane (Sartorius AG, Gottingen, Germany) was dried at  $37^{\circ}\text{C}$  for 5 min and then prewashed with 1% BSA buffers containing detergents and incubated for 2 h at room temperature with  $1 \times 10^6$  cpm [ $^{125}\text{I}$ ]IGF-II (Amersham, Milan, Italy). The membrane was then washed, dried and autoradiographed after 3-D exposure of the film (Eastman Kodak, Rochester, NY) at  $-80^{\circ}\text{C}$ .

Protein band intensity in Western blot was analysed by scanning densitometry of the original autoradiographic film with a Flowvision densitometer (Lynx, San Mateo, CA). To quantify the intensity of radioactivity of various IGFBPs, several autoradiographs developed after different times of exposure of the same blot were measured and data within a linear range were used.

For immunoblotting, CM was subjected to SDS-PAGE under nonreducing conditions and then electrophoretically transferred onto a nitrocellulose membrane as described above for Western ligand blotting. Polyclonal antibodies against IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 were purchased from UBI (UBI, Lake Placid, NY) and goat antibody immunoglobulin G conjugate with alkaline phosphatase was obtained from Sigma Immuno Chemicals. The nitrocellulose membrane was incubated at  $4^{\circ}\text{C}$  with 1:200 dilution AbBP-2 and AbBP-5, 1:500 dilution AbBP-3 and AbBP-4, in PBS containing 3% non-fat dry milk. The nitrocellulose membrane was rinsed three times with PBS-3% milk and incubated at  $25^{\circ}\text{C}$  for 3 h with an antirabbit immunoglobulin G-alkaline phosphatase conjugate in PBS-3% milk. The membrane was then washed once with 0.1 mol/l Tris-Cl buffer (pH 9.5) containing 0.1 mol/l NaCl and 5 mmol/l  $\text{MgCl}_2$  and then incubated with bromochloroindole phosphate-ni-

tro blue tetrazolium substrate solution. The reaction was stopped with PBS containing 20 mmol/l EDTA.

For glycosylation studies a 10-fold concentrated lyophilised GENC serum free CM was analysed as follows: 2 mg/ml solution of GENC CM was prepared and 5  $\mu\text{l}$  7.5% NP-40 and then 8  $\mu\text{l}$  *N*-Glycanase enzyme (Genzyme Corporation, Cambridge, MA) were added to a 10  $\mu\text{l}$  aliquot of the above obtained solution. After the addition of 7  $\mu\text{l}$  of distilled water, the solution was incubated for 24 h at  $37^{\circ}\text{C}$ . Instead for O-glycosylation studies, GENC CM was incubated at  $37^{\circ}\text{C}$  for 2 h with 15  $\mu\text{l}$  of Neuraminidase (Boehringer Mannheim Biochemicals, IN) and then for 12 h with 8  $\mu\text{l}$  of O-glycanase (Genzyme, Boston, MA). The reactions were finally stopped on the addition of a proper amount of PAGE loading buffer and the samples were analysed by ligand blot after SDS-PAGE. For protease analysis, CM was prepared by equilibration with sodium acetate buffer (0.1 M, pH 5.5). In order to detect protease activity, 100  $\mu\text{l}$  GENC CM were incubated with protease inhibitors ethylene-diamino-tetraacetic acid (EDTA), phenyl-methyl-sulphonyl-flouride (PMSF), *N*-ethyl-maleinide (NEM) (Sigma) at a final concentration of 10 nM at  $37^{\circ}\text{C}$  for 24 h. Proteolyzed samples were analysed by SDS-PAGE as previously indicated.

### 2.3. RNA preparation and Northern blot analysis

Total RNA was prepared from GENC using the guanidium thiocyanate-phenol-chloroform extraction method (Giannini et al., 1997). A 20  $\mu\text{g}$  lane of total RNA was loaded onto 1% agarose formaldehyde gel and run at 80 V for 3.5 h in 3-morpholinopropanesulfonic acid (MOPS). The gel was then stained with ethidium bromide to check that the ribosomal RNAs were intact and equal amounts of RNA had been loaded. The gel was incubated with 50mM NaOH, 10mM NaCl for 10 min and neutralised with 1M ammonium acetate and blotted onto GeneScreen (Du Pont-NEN, Boston, MA). The gels were then hybridised with complementary DNA (cDNA) probes for IGFBP-1 to IGFBP-6, obtained from plasmid human IGFBPs (a kind gift from Dr Shunichi Shimazaki, JCR Biopharmaceuticals, San Diego, CA) and human  $\beta$ -actin cDNA (a kind gift from Dr C. Mavilia). All of the probes were labelled using the Prime-H random primer kit (Stratagene, La Jolla, CA). Each blot was hybridised with  $2 \times 10^6$  cpm/ml probe in 50% formamide,  $3.6 \times \text{SSPE}$  (0.5 M NaCl, 5 mM EDTA and 50 mM sodium phosphate, pH 6.8), 1% SDS, 10% dextran sulphate,  $5 \times \text{Denhart's}$  solution) solution at  $42^{\circ}\text{C}$  overnight. The blot was finally washed in  $2 \times \text{SSPE}$ , 0.2% SDS at room temperature followed by  $0.1 \times \text{SSPE}$  0.2% SDS at  $60^{\circ}\text{C}$  and autoradiographed ( $\alpha\text{IR3}$  was a kind gift from Dr M. Maggi, University of Florence, Italy).

## 2.4. Measurement of cell growth

In order to evaluate cell proliferation, GENC were plated in multiwell plates (Corning, NY) in regular medium (RM) and in serum-free medium (SFM) and were counted after trypsin dispersion by using a Burkert chamber at the times 0, 24, 48, 72 and 96 h. For stimulation experiments cells were spread in multiwell plates in RM for 24 h and at semi-confluence were again incubated for 24 h: (A) in RM with TGF- $\beta_1$ , TGF- $\beta_1$  + IGF-I (Sigma) and a Polyclonal antibody against IGFBP-3 (UBI); and (B) in SFM with IGF-I alone or in combination with TGF- $\beta_1$ . In some experiments human recombinant IGFBP-3 (UBI) was incubated in absence or presence of IGF-I and Des(1–3)IGF-I (Peninsula, Belmont, CA). Cells were counted in triplicate.

## 2.5. Data analysis

Results are expressed as mean values  $\pm$  SD. Statistical significance was calculated by an ANOVA and Dunnett's multiple comparison test. Experiments were repeated at least three times, each time being consistent.

## 3. Results

### 3.1. IGFBPs identification and characterisation on GENC conditioned medium

CM from GENC (identified as endothelial cells by the positive reaction to Factor VIII, see Fig. 1A and to CD31, data not shown) was analysed using the Western ligand blotting procedure (Fig. 1B) with [ $^{125}$ I]IGF-II as a labelled ligand, chosen because this peptide showed

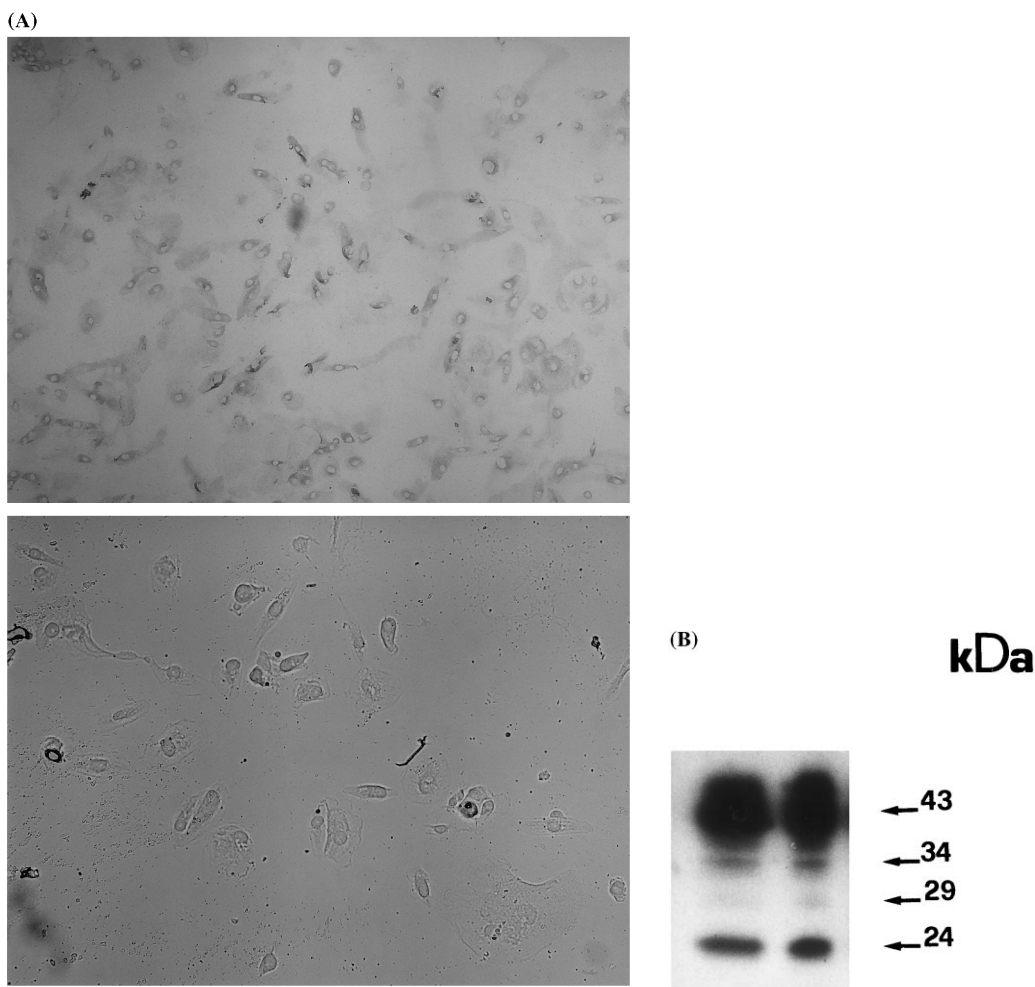


Fig. 1. (A) Upper: immunostaining for specific endothelial cell marker (Factor VIII) of GENC culture (original magnification  $\times 125$ ). The figure was obtained by using a Universal immunoperoxidase staining-kit and the development of the reaction was carried out with 3-amino-9-ethyl-carbazole. Lower: negative control (original magnification  $\times 250$ ). (B) Identification of the forms of IGFBPs secreted by GENC in vitro. CM (48 h serum free culture) was collected from two different GENC cultures under basal conditions and subjected to ligand blotting analysis. The ligand blots were prepared by incubating Western blots with [ $^{125}$ I]IGF-II. The molecular weights are represented on the right part of the gel. The autoradiogram is obtained after 1 week film exposure at a low temperature. A single representative experiment is shown.

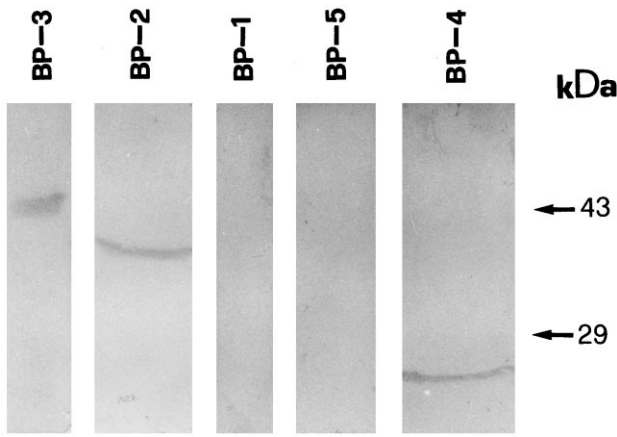


Fig. 2. Identification of the forms of IGFBP secreted by GENC in unstimulated conditions. CM was collected from GENC cultures and subjected to immunoblotting. Immunoblots were prepared by incubating Western blots with different antisera against IGFBPs as indicated in the figure, at the dilution reported in Section 2. On the right side of the figure the molecular weight of proteins expressed in kDa is marked.

high affinity for most of the IGFBPs compared to IGF-I. Under basal conditions these cells predominantly secreted two different IGFBPs that migrated on gel electrophoresis; one as a strong doublet band with a molecular weight (MW) ranging between 43 and 39 kDa and the other as a single weaker band of 24 kDa. The autoradiogram of Fig. 1B also showed the appearance of two faint bands of about 34 and 29–30 kDa. This pattern of GENC IGFBPs production was not modified after the collection of CM until the 10th cell culture passages (data not presented). When CM obtained from GENC was immunoblotted with five different polyclonal IGFBP antisera (from IGFBP-1 to IGFBP-5 antibodies), only the bands of 43–39, 34 and 24 kDa observed in ligand blot analysis were reactive (Fig. 2). The proteins recognised were IGFBP-3, IGFBP-2 and IGFBP-4, respectively. IGFBP-1 and IGFBP-5 antibodies, previously tested in human serum and in CM of human prostatic cancer cells as positive controls for IGFBP-1 and IGFBP-5 respectively, even at very high concentrations, and after deglycosylation studies with *N*-glycanase, neuraminidase and O-glycanase (data not reported) did not show positive bands of reaction suggesting the lack of presence of these proteins. It was not possible to characterise the eventual presence of IGFBP-6 because its antiserum is not commercially available for immunoblot purposes. Moreover, in order to determine whether the 29–30 kDa protein in GENC CM could correspond to the glycosylated form of IGFBP-4, concentrated CM of GENC was examined by ligand blot procedure before and after deglycosylation using *N*-glycanase (Fig. 3).

As shown in this figure, the 29–30 kDa band was completely abolished by the treatment with *N*-glycanase but on the contrary, the 24 kDa (IGFBP-4) band was unmodified, or apparently increased, by the enzymatic treatment suggesting that the 29–30 kDa band could represent the *N*-glycosylated form of the 24 kDa IGFBP-, as confirmed by the immunoblot study with the IGFBP-4 antiserum after the deglycosylation treatment (Fig. 3, inset). In addition, a reduction of IGFBP-3 intensity was also observed. Thus, the two bands of 24 and 29–30 kDa were identified to be IGFBP-4. These results indicate that, together with IGFBP-3, IGFBP-4 is the predominant IGFBP secreted by GENC. To further characterise the IGFBPs identified into GENC CM by ligand and immunoblot procedures, Northern blot analysis has been carried out using <sup>32</sup>P labelled cDNA probes from human IGFBPs. Fig. 4 indicates the Northern blot studies of total RNA obtained from GENC cultured in standard growth conditions. As reported in the figure, in basal conditions GENC show the presence of mRNA for IGFBP-2, IGFBP-3, IGFBP-4 and a fainter signal for IGFBP-5. A single band of hybridisation of about 2.5, 2.6, 2.2 and 6 kb in length was observed in each gel, respectively. On the other hand, lack of positive signal was found with IGFBP-1 and IGFBP-6 cDNA human probes even at a longer exposure of the autoradiograms.

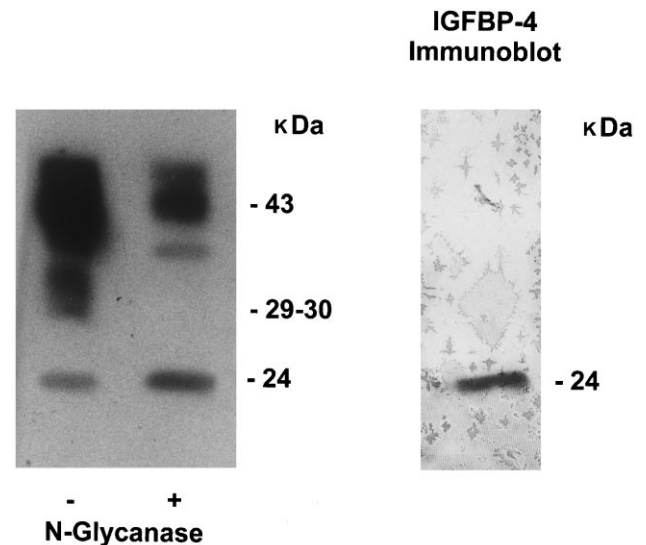


Fig. 3. [<sup>125</sup>I]IGF-II ligand blot of GENC CM with or without *N*-glycanase treatment. CM from GENC was incubated in the absence (–) or presence (+) of *N*-glycanase as reported in Section 2. The IGFBP molecular weights are reported in kDa. The arrow marks the positions of glycosylated form IGFBP-4 of 29–30 kDa. The autoradiogram is the result of 1 day exposure of the film at a low temperature. On the right the IGFBP-4 immunoprobings of 24 kDa band after *N*-glycanase treatment is represented.

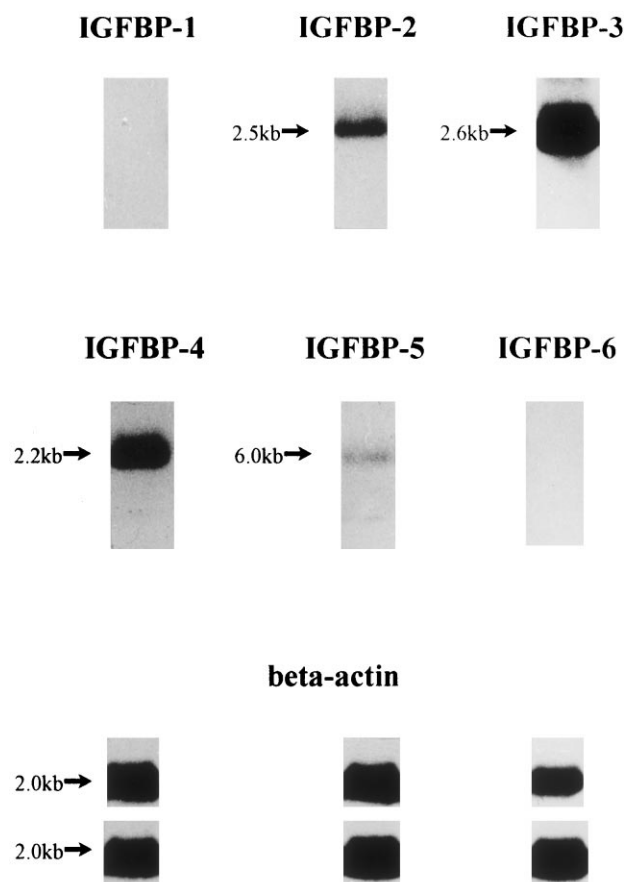


Fig. 4. Total cellular RNA was prepared, as described in Section 2, from unstimulated GENC cultures. Aliquots of 20  $\mu$ g total RNA were electrophoresed and transferred to nylon membrane and then hybridised with  $^{32}$ P-labeled human IGFBPs (from IGFBP-1 to IGFBP-6) cDNA probes. The arrows denote the IGFBP kb messages that are present in these endothelial cells (upper). The housekeeping gene  $\beta$ -actin total RNA for equal loading meaning (lower).

### 3.2. Regulation of glomerular endothelial cell IGFBPs

Treatment of GENC with IGF-I resulted in a differential regulation of the IGFBP population found in glomerular endothelial cells. The results of this study indicate that this effect was observed at concentrations ranging between 0.1 and 1 nM of IGF-I. As shown in Fig. 5, IGF-I, in a dose–response manner increases significantly the levels of IGFBP-3 (1.7-fold stimulation compared to the control) at 0.1 nM and reaches a plateau of stimulation at the high dose of 1  $\mu$ M (2.3-fold stimulation). The IGFBP-2 secretion was significantly stimulated starting at 1 nM (4.17-fold stimulation) reaching the maximum effect at higher IGF-I concentrations. The densitometric analysis of the ligand blot study is represented in Table 1. Conversely, an opposite effect of IGF-I was observed concerning the regulation of IGFBP-4. As reported in Fig. 5 and in Table 1, the addition of 0.1 nM IGF-I determined a dramatic and constant reduction (10-folds less) of

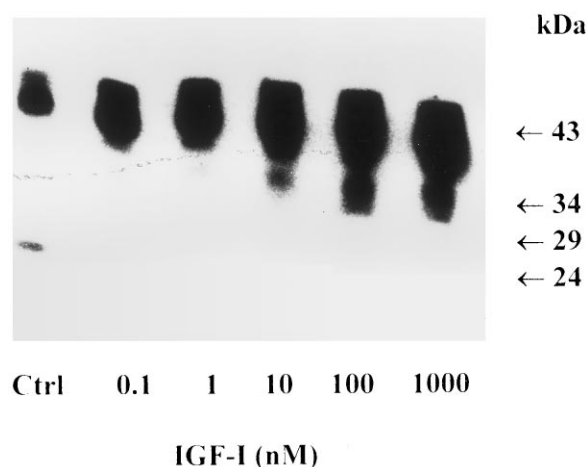


Fig. 5. [ $^{125}$ I]IGF-II Western ligand blot of GENC CM harvested after 48 h serum free culture in presence of increasing concentrations of IGF-I (0.1–1000 nM) (24 h stimulation). The autoradiogram is the result of a 1 day film exposure. Indicative molecular weights expressed in kDa are shown on the right. A single representative experiment is shown.

IGFBP-4 in all the experimental points of the dose–response curve. Moreover, the IGF-I was not apparently able to modify the level of the faint band of 29–30 kDa, even at longer exposure of the gel.

Next examined was the effect of TGF- $\beta_1$  on IGFBP production into GENC CM. In Fig. 6 the addition of TGF- $\beta_1$  determined a dose–response regulation of IGFBPs. In particular, the 24 h treatment with 1 nM TGF- $\beta_1$  significantly decreased the bands representative of IGFBP-3 and the glycosylated form of IGFBP-4 (1.6- and 2.4-fold, respectively). On the contrary, 1 nM TGF- $\beta_1$  was apparently able to increase the 24 kDa IGFBP-4 band production (about 2.4-fold). The regulatory action of TGF- $\beta_1$  on these IGFBPs secretion reached a plateau-effect at a concentration of 10 nM. IGFBP-2, compared to the other IGFBPs, was unaf-

Table 1  
Densitometric quantification of IGF-I effect on GENC IGFBP-modulation<sup>a</sup>

	BP-3	BP-2	29–30 kDa	BP-4
Control	35.8 $\pm$ 6	7.5 $\pm$ 1.5	1.8 $\pm$ 1.5	34.3 $\pm$ 0.7
IGF-I	61.2 $\pm$ 7.5*	10.4 $\pm$ 1.5	nd	3.7 $\pm$ 0.7**
0.1 nM				
IGF-I	65.7 $\pm$ 7.5**	31.3 $\pm$ 3**	nd	3.7 $\pm$ 0.9**
1 nM				
IGF-I	79.1 $\pm$ 10.4**	22.4 $\pm$ 6*	nd	5.2 $\pm$ 1.5**
10 nM				
IGF-I	82.1 $\pm$ 6**	48.6 $\pm$ 2.1**	nd	3.1 $\pm$ 1.5**
100 nM				
IGF-I	82.1 $\pm$ 6**	45.3 $\pm$ 4.5**	nd	3 $\pm$ 1.8**
1000 nM				

<sup>a</sup> Data are expressed as mean values  $\pm$  SD.

\*\*  $P < 0.01$ .

\*  $P < 0.05$ . These data were obtained from triplicates.

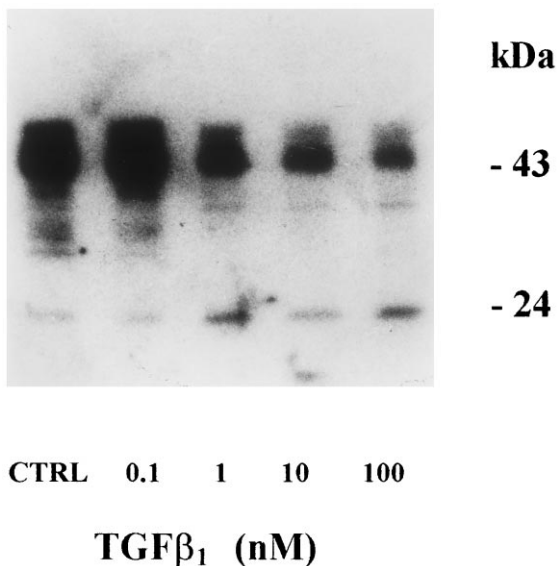


Fig. 6. Effect of TGF- $\beta_1$  on IGFBP secretion. CM was collected from cultures incubated in absence (control) or presence of increasing concentrations of TGF- $\beta_1$  (0.1–100 nM). Samples were analysed using ligand blot analysis. The molecular weights of the IGFBPs are indicated on the right expressed in kDa. The autoradiogram is the result of 3 days film exposure.

ected by the TGF- $\beta_1$  addition in each experimental dose used. Table 2 represents the densitometric determination of TGF- $\beta_1$  on IGFBPs modulation.

To further characterise the IGFBP-3 regulation observed in our experimental conditions, we determined whether the effect of TGF- $\beta_1$  was related to IGFBP-3 proteolytic activation or to the controlled protein transcription. Fig. 7 represents the effects of various protease inhibitors on the IGFBP-3 levels in GENC CM. As clearly shown, the presence of 10 nM of EDTA, PMSF and NEM did not modify the downregulating action observed after the addition of TGF- $\beta_1$  100 nM, moreover, protease inhibitors alone did not

Table 2  
Densitometric quantification of TGF- $\beta_1$  effect on GENC IGFBP-modulation<sup>a</sup>

	BP-3	BP-2	29–30 kDa	BP-4
Control	207 ± 4.5	194.1 ± 2.6	166.9 ± 5.6	67.1 ± 3.9
TGF- $\beta_1$ 0.1 nM	217.1 ± 2.6	188.5 ± 3	123.7 ± 3.4	65.4 ± 3.5
TGF- $\beta_1$ 1 nM	128.6 ± 7.8**	186.3 ± 3.5	68.4 ± 2.9**	171.3 ± 5.8**
TGF- $\beta_1$ 10 nM	76.9 ± 7.3**	189 ± 3	26.7 ± 3.8**	162.4 ± 4.2**
TGF- $\beta_1$ 100 nM	68.4 ± 9.4**	184.9 ± 4.9	2.3 ± 3.7**	173.6 ± 5.2**

<sup>a</sup> Data are expressed as mean values ± SD.

\*\*  $P < 0.01$ . These data were obtained from triplicates.

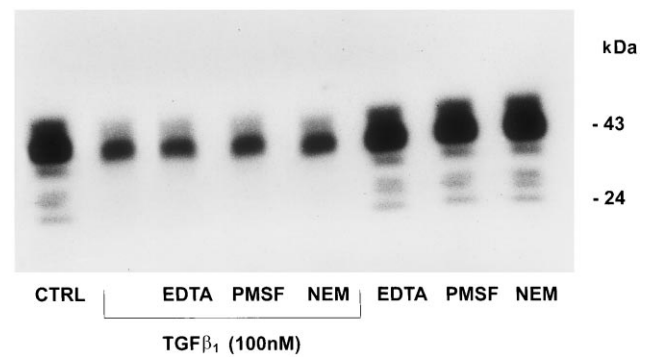


Fig. 7. Effect of protease inhibitors on IGFBP-3 production. GENC CM in basal conditions (control lane) and after treatment with 100 nM TGF- $\beta_1$  alone and 100 nM TGF- $\beta_1$  with or without the presence of 10 nM protease inhibitors (EDTA, PMSF, NEM), were obtained. On the right of the gel the protein molecular weights are expressed in kDa. The autoradiogram obtained by [<sup>125</sup>I]IGF-II ligand blot analysis is the result of 1 day film exposure.

show any kind of activity on IGFBP-3 release. These results, together with the lack of proteolytic fragment detection at the bottom of the gel, suggest that TGF- $\beta_1$  works at mRNA level.

### 3.3. Regulation of IGFBP-3 and IGFBP-4 mRNAs by IGF-I and TGF- $\beta_1$

Northern blot analysis of total RNA from GENC (Fig. 8) revealed a single 2.5 kb mRNA species that hybridised to a human IGFBP-3 cDNA probe. The

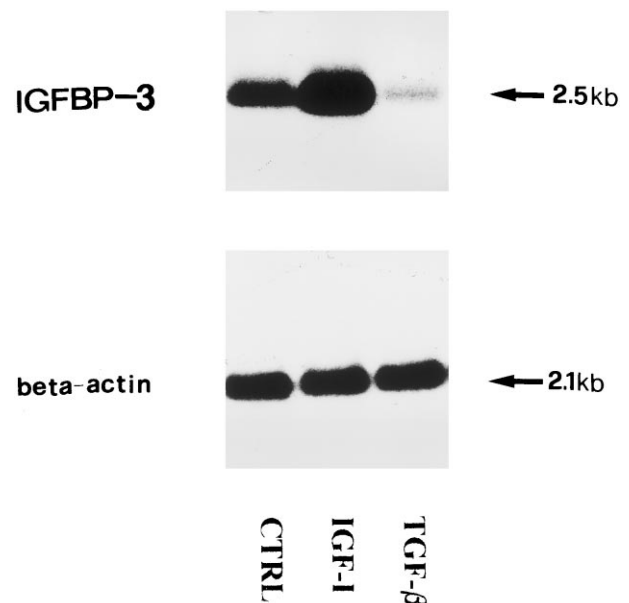


Fig. 8. Regulation of IGF-I and TGF- $\beta_1$  on IGFBP-3 mRNA levels. Cells were grown until 90% confluent and incubated with SFM containing 1 nM IGF-I and 10 nM TGF- $\beta_1$  for 24 h. Total RNA was extracted and analysed using Northern blot analysis. A single representative blot from two different experiments is shown.

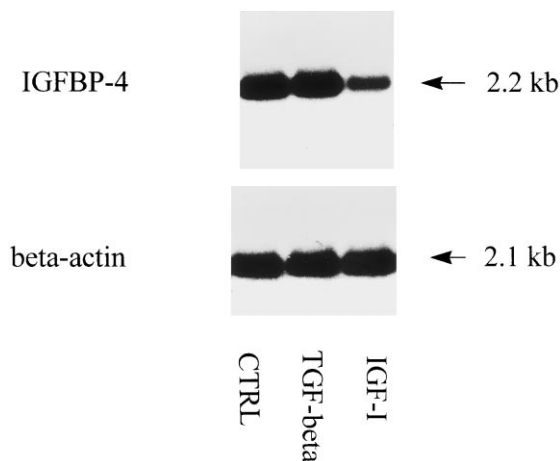


Fig. 9. Effect of TGF- $\beta_1$  and IGF-I on IGFBP-4 mRNA. GENC were grown until subconfluence and incubated for 24 h in SFM containing 1 nM IGF-I or 10 nM TGF- $\beta_1$ . Total RNA was extracted and analysed using Northern blot analysis. A single representative blot from two different experiments is shown.

treatment with 10 nM TGF- $\beta_1$  for 24 h resulted in a significant decrease in IGFBP-3 mRNA, after correction for  $\beta$ -actin mRNA (qualitative data not shown). However, a regulatory effect on mRNA level of transcription, as reported for TGF- $\beta_1$ , was also observed for IGF-I. Fig. 8 also shows that a 24 h treatment with 1 nM IGF-I determined a significant increase of 2.5 kb mRNA, thus demonstrating that both growth factors could regulate the IGFBP-3 production at protein expression level in opposite ways. Since IGF-I and TGF- $\beta_1$  also showed a different action in controlling IGFBP-4 mRNA levels, Northern blot analysis of total RNA from GENC revealed that the single 2.2 kb mRNA hybridised to a human IGFBP-4 cDNA probe was decreased 3.2-fold (qualitative data not reported), after treatment with 1 nM IGF-I, compared to TGF- $\beta_1$  which, at 10 nM, had no effect on IGFBP-4 mRNA (Fig. 9). In order to establish whether the observed change of IGFBP-4 and IGFBP-3 mRNAs and respective protein production in GENC CM was due to the ability of IGF-I to bind with its cell surface specific receptor (previously identified in GENC, but data not reported) or to control the mRNA stability, the monoclonal antibody  $\alpha$ IR3, specific antagonist for the IGF-I receptor was used. Fig. 10 shows that the addition of 1 nM IGF-I together with equimolar concentrations of  $\alpha$ IR3 did not determine significant modifications in the mRNA levels for IGFBP-4 and IGFBP-3, suggesting that the modifications of mRNA levels for these two IGFBPs are mediated through the activation of IGF-I receptors. Concerning the IGFBP-2 regulation, IGF-I, compared to TGF- $\beta_1$  which showed a lack of control on this IGFBP, determined an increase of IGFBP-2 mRNA (data not reported).

### 3.4. Effect of IGF-I and TGF- $\beta_1$ on GENC number

Fig. 11 in its upper panel represents GENC growth in RM and in SFM conditions. The graphic of GENC growth in SFM conditions did not show any significant variation in cell number in each of the investigated experimental points. On the contrary, when GENC were grown in RM, they significantly duplicated their number after 24 h and reached the maximum cell density after 48 h. During the following hours, there was no further increase in cell number. For this reason, the effects of IGF-I and TGF- $\beta_1$  on GENC growth after 48 h of culture were studied. The results are represented in the lower panel of Fig. 11. In SFM conditions, the growth of GENC was significantly stimulated by the addition of 1 nM IGF-I ( $P < 0.01$ ) (lane 1) and this effect was further increased after the addition of 100 nM IGF-I ( $P < 0.05$ ) (lane 2). The presence of a polyclonal anti-IGFBP-3 antiserum (1:100) alone (lane 3) dramatically blocked the cell growth. A strong inhibitory effect on GENC growth was observed in the presence of 10 nM TGF- $\beta_1$  (lane 4) and this inhibitory action was also observed when the cells were cultured in the co-presence of 10 nM TGF- $\beta_1$  and 1 nM IGF-I (lane 5).

Since the presence of IGFBP-3 per se could modulate the stimulatory action of IGF-I, the effect of recombinant human IGFBP-3 (rhIGFBP-3) on IGF-I induced proliferation in GENC was investigated. As reported in Fig. 12, the only presence of 50 nM rhIGFBP-3 (lane A) had no effect on GENC growth compared to the effect obtained by the presence of 1 nM IGF-I alone

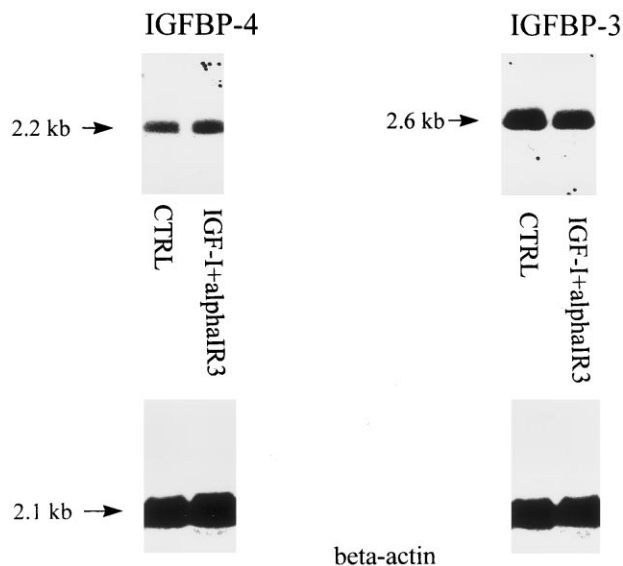


Fig. 10. Northern blots demonstrating the effect on IGFBP-4 and IGFBP-3 mRNA levels after the addition of 1 nM IGF-I and an equimolar concentration of  $\alpha$ IR3, the polyclonal antibody against the IGF-I receptor. Representative blots from two separate experiments are shown.

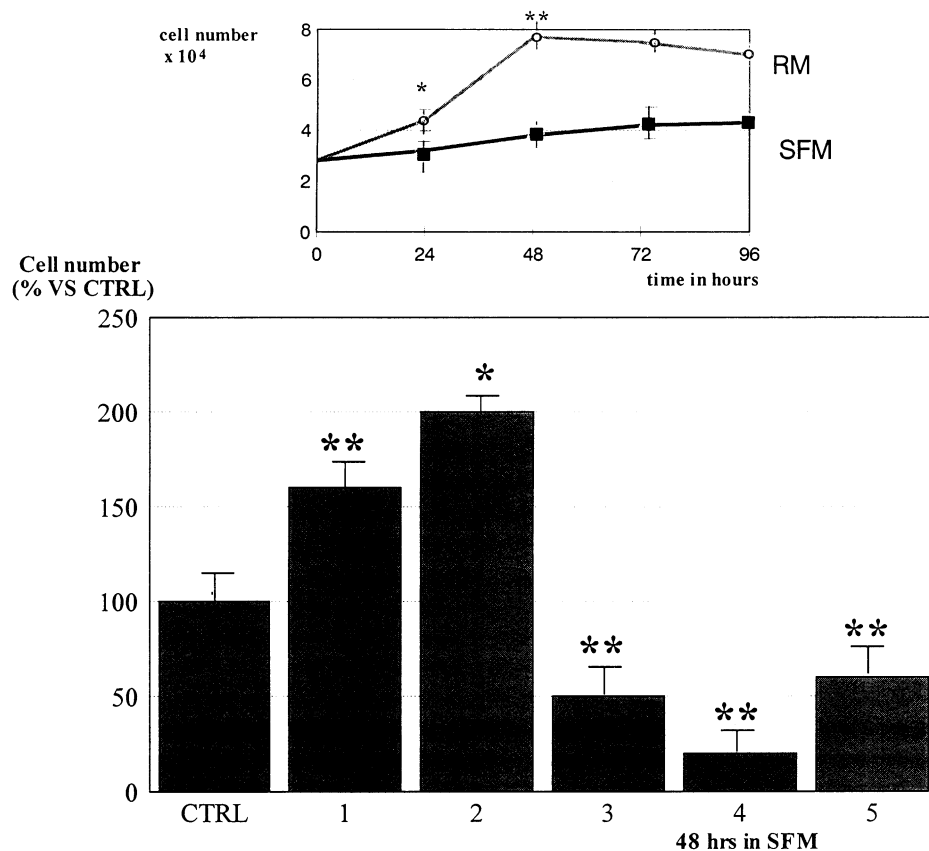


Fig. 11. Upper panel: GENC growth in RM and in SFM conditions. Lower panel: Effect of IGF-I and TGF- $\beta_1$  on GENC growth. GENC monolayers were grown in 12-well tissue culture plates to 75% confluence in SFM according to different experimental conditions: 1 nM IGF-I (lane 1), 100 nM IGF-I (lane 2), anti IGFBP-3 polyclonal antibody 1:100 final dilution (lane 3), 10 nM TGF- $\beta_1$  alone (lane 4) or with 1 nM IGF-I (lane 5). Each bar represents mean  $\pm$  SE from six wells. \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

(lane B). A stimulatory effect of rhIGFBP-3 was reached when 1 nM IGF-I (lane C) was added to a 48 h preincubation of increasing concentrations of rhIGFBP-3 (10–75 nM). The increased growth (2.5-fold) was statistically significant with 50 nM of rhIGFBP-3. In contrast, when 50 nM rhIGFBP-3 together with 1 nM IGF-I was added (lane D) a significant ( $P < 0.05$ ) reduction of the IGF-I effect was observed compared to the effect of IGF-I alone. In addition, the presence of 1 nM Des(1–3)IGF-I (lane E), an analogue of IGF-I with a low affinity for IGFBPs, showed a significant ( $P < 0.05$ ) increase of cell number compared to the control but its effect was not statistically different to the same concentration of IGF-I (lane B). A further experiment was carried out to allow a clearer understanding of the role of endogenous and exogenous IGFBP-3 in controlling GENC growth. In Fig. 13 quiescent cells (48 h serum free) were incubated in the presence or absence of 50 nM rhIGFBP-3 and then treated for 24 h with 1 nM IGF-I without changing the medium before the addition of this growth factor to avoid the loss of endogenous IGFBP-3 secreted by GENC. As shown in Fig. 13, IGF-I caused a statistically significant increase ( $P < 0.01$ ) of cell num-

ber even in the absence of preincubation with rhIGFBP-3, suggesting that the presence of endogenous IGFBP-3 could positively affect IGF-I action, moreover, the preincubation with rhIGFBP-3 significantly stimulated the IGF-I induced GENC growth if compared to the effect obtained in absence of rhIGFBP-3 ( $P < 0.05$ ). This result suggests that the co-presence of endogenous and exogenous IGFBP-3 have an additive effect on IGF-I biological action and confirms that the preincubation with IGFBP-3 plays an important role in controlling the bioavailability of IGF-I in GENC.

#### 4. Discussion

The IGF system is widely represented in the human kidney (Feld and Hirschberg, 1992) and involved in renal haemodynamics (Hirschberg et al., 1993) and tubular function (Caverzasio and Bonjour, 1992; Hirschberg et al., 1993). Other observations emphasise the importance that the IGF system, including the IGFBPs, has in modulating cell proliferation in kidney diseases as diabetic hypertrophy (Feld and Hirschberg, 1992; Flyvbjerg et al., 1992; Gelato et al., 1992; Pugliese

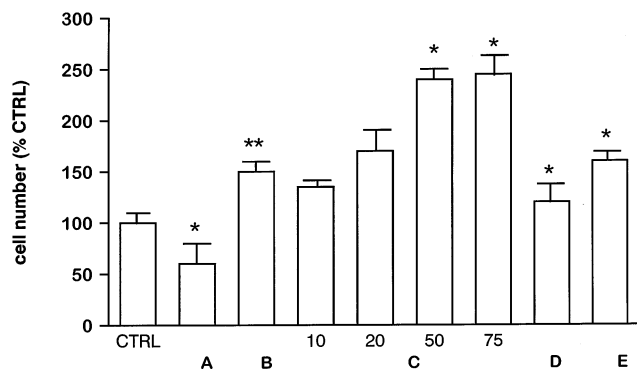


Fig. 12. Modulatory effect of hrIGFBP-3 on IGF-I-induced GENC proliferation. GENC were plated at subconfluence and maintained for 48 h in SFM and were then incubated for 24 h as follows: A, 50 nM rhIGFBP-3; B, 1 nM IGF-I; C, 50 nM rhIGFBP-3 and 1 nM IGF-I added together; D, 1 nM Des(1–3)IGF-I. For the experiment in lane C the cells were preincubated for 48 h in SFM in the presence of increasing concentrations of rhIGFBP-3 (10–75 nM) and then treated for 24 h with 1 nM IGF-I. This experiment was performed three times with similar results. Values shown are the mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$

et al., 1996). Together with the IGF system, various growth factors participate in renal disease; among these, TGF- $\beta_1$  is a multifunctional cytokine playing a crucial role on cell proliferation and in diseases characterised by excessive fibrosis (Border et al., 1995). At the kidney level, TGF- $\beta$  is capable of modulating cell growth (Pugliese et al., 1996), matrix turnover (Pricci et al., 1996) and regulation of the bioavailability of IGFs through local control of IGFBP release (Grellier et al., 1996). Since the concentration of IGFBPs depends on the regulation of local growth factors on each single IGFBP, the effect that IGF-I and TGF- $\beta_1$  could have on the control of GENC IGFBP was studied.

Previous reports have demonstrated a direct secretion of IGF-I (Grant et al., 1993; Ohashi et al., 1993) and

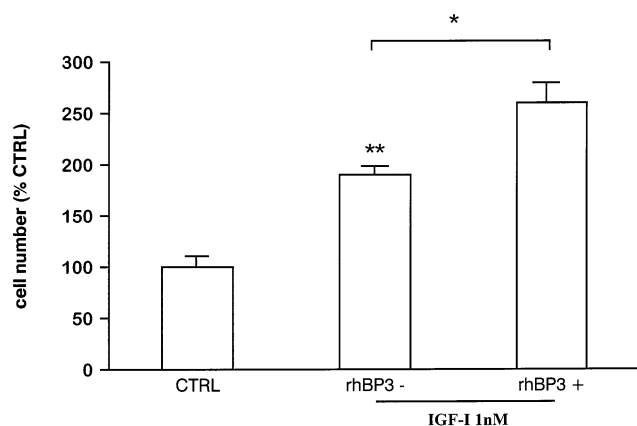


Fig. 13. GENC were incubated for 48 h in the absence or presence of 50 nM rhIGFBP-3. The cells were then stimulated for 24 h with 1 nM IGF-I without a medium change. Data represent the mean  $\pm$  SD of triplicate determinations. \* $P < 0.05$ ; \*\* $P < 0.01$

IGFBPs (Bar et al., 1987; Moser et al., 1992; Giannini et al., 1997) by endothelial cells, suggesting that a pivotal role played by these cells in controlling IGF-I autocrine or paracrine actions. The time of maximum IGFBP release is represented by 48 h serum free culture conditions, as observed in different cell types including endothelial cells (Giannini et al., 1994, 1996; Kimura et al., 1996; Giannini et al., 1997). Ligand blot analysis of 48 h GENC CM showed the presence of IGFBP-3, IGFBP-2 and IGFBP-4. Moreover, another 29–30 band which could represent the glycosylated form of IGFBP-4 (Boes et al., 1992; Moser et al., 1992; Yang et al., 1993; Giannini et al., 1997), the IGFBP-6 (Jones and Clemmons, 1995) or the other reported IGFBP-related proteins (Kim et al., 1997) was identified by the *N*-glycanase treatment as a glycosylated IGFBP-4. Although bovine cells from micro-vessels present mRNAs from IGFBP-2 to IGFBP-6 (Moser et al., 1992), only IGFBP-2 to IGFBP-5 mRNAs were detected in GENC. According to recent observations in bovine retinal endothelial cells (Giannini et al., 1997), it was not possible to detect any signal for IGFBP-6 mRNA in this study confirming the data obtained by Chin et al. (1994) in human kidney. Together with IGFBP-1, IGFBP-5 appears to be constantly not expressed in endothelial cells (Moser et al., 1992). It was recently discovered that bovine retinal endothelial cells in culture seem to produce a material that can cross-react with a specific IGFBP-5 radioimmunoassay (Giannini et al., 1997). Therefore, the lack of IGFBP-5 protein detection in endothelial cells and in GENC CM could be due to proteolysis of this IGFBP-5 (Nam et al., 1996). This different expression of IGFBP mRNAs suggests that the spread distribution of the endothelial wall could be accompanied by a multiple IGFBP release, which may reflect a specific control for each particular anatomical district interested. The ratio between one IGFBP and another may be a critical factor on controlling the autocrine or paracrine effect of IGF-I.

IGF-I stimulated the release of IGFBP-3 and IGFBP-2, while at the lowest dose used IGF-I determined a decreased IGFBP-4. In bovine retinal endothelial cells it was recently observed that IGFBPs have an inhibitory effect on IGF actions and that the addition of IGFs enhanced the level of IGFBP-2 (Giannini et al., 1997). On the contrary, purified IGFBP-2 in the CM of microvascular endothelial cells has been reported to weakly enhance the positive action of IGF-I on glucose transport and amino acid uptake (Bar et al., 1989). Instead, IGFBP-4 is constantly observed in different endothelial cell lines (Boes et al., 1992; Yang et al., 1993; Giannini et al., 1997) and have an inhibitory modulation on IGF bioavailability. The Northern blot analysis that was carried out in the absence and presence of  $\alpha$ IR3, suggests that IGF-I decreases the band of

IGFBP-4 in GENC CM modifying the transcriptional levels of its mRNA, since, no proteolytic extra-bands on Western ligand- or Immunoblot studies lower than 24 kDa have been detected (Cheung et al., 1994). IGF-I at lower experimental doses was also a potent inducer of IGFBP-3 release in GENC. Studies in several cell types suggest that this IGFBP-3 regulation may depend on different mechanisms, including a protecting action against IGFBP-3 proteolysis (Camacho-Hubner et al., 1992; Salahifar et al., 1997), a direct stimulation of IGFBP-3 mRNA levels (Ramagnolo et al., 1994) or, as already observed in retinal endothelial cells (Giannini et al., 1997), an IGF-receptor independent mechanism. However, in this study, the Northern blot analysis performed in the presence or absence of  $\alpha$ IR3 demonstrated that the IGF-I induced IGFBP-3 increase is regulated by a rise in IGFBP-3 mRNA levels, confirming other recent observations in endothelial cells by Erondur et al. (1996).

It is believed that this is the first report that demonstrates the *in vitro* mitogenic action of IGF-I on human glomerular endothelial cells, confirming the large number of reports about the stimulatory effect of this growth factor on different types of endothelial cells (King et al., 1985; Grant et al., 1987, 1993; Canalis et al., 1989; Ohashi et al., 1993). A minority of papers showed a uniform lack of mitogenic effect of IGF-I on bovine endothelial cells (Dosso et al., 1993; Galli et al., 1994; Giannini et al., 1997). This difference in terms of cell growth compared to our results on GENC could derive from the different cell types used as experimental model. However, another explanation could arise. As mentioned previously, the inhibitory role played by IGFBP-4 *in vitro* systems, concerning IGFBP-3, stimulatory (De Mellow and Baxter, 1988; Blum et al., 1989; Oh et al., 1993b; Ramagnolo et al., 1994; Slootweg et al., 1995; Conover et al., 1996) and inhibitory (De Mellow and Baxter, 1988; Liu et al., 1992; Oh et al., 1993a; Cohen et al., 1994; Pratt and Pollak, 1994; Cohen et al., 1996) effects, have been demonstrated in several cellular types. Even if there is not enough available data, so far one could hypothesize that in endothelial cells IGFBP-3 and IGFBP-4 are necessary for the IGF-I mediated mitogenesis. In fact, in bovine endothelial cells where there is a large amount of IGFBP-4 but not IGFBP-3, the addition of IGF-I did not affect the growth of these cells or the IGFBP-4 and IGFBP-3 bands (Giannini et al., 1997). On the contrary, the heavy presence of both IGFBP-3 and IGFBP-4 into GENC was modified after the addition of IGF-I which stimulated the IGFBP-3 band and decreased IGFBP-4, dramatically modifying these two IGFBPs and the cell number. Moreover, the presence of an IGFBP-3 polyclonal antibody during GENC regular growth blocked the mitogenesis of these cells, independently from the levels of IGFBP-4 and of the other

IGFBPs. This could suggest that, together the inhibitory levels of IGFBP-4, the control of IGFBP-3 concentration or bioavailability, counteracting the inhibitory effects of other IGFBPs (probably including IGFBP-2), is the crucial step regulating *in vitro* the IGF-I induced growth of human GENC. The mechanism underlying this effect of IGFBP-3 on glomerular human endothelial cell growth needs further studies elucidated. However, it was observed that rhIGFBP-3 alone, *per se*, had no effect on GENC proliferation and instead a 48 h rhIGFBP-3 incubation seemed to potentiate the IGF-I induced GENC number. Moreover, the lack of response in terms of GENC growth after the simultaneous addition of IGF-I and rhIGFBP-3 suggests that the positive effect of IGFBP-3 on IGF-I action is present only when this binding protein is cell-surface associated (Conover et al., 1990). The different behaviour observed after the addition of IGF-I, between the endogenous GENC IGFBP-3 and the preincubation with rhIGFBP-3, could be due to the different degree of glycosylation of this IGFBP, being the rhIGFBP-3 probably less bound by oligosaccharides chains. Since the affinity of IGFBP-3 for IGF-I in the non-glycosylated form is reported to be higher with respect to the glycosylated form (Bach and Rechler, 1995), the rhIGFBP-3 could better control the bioavailability of IGF-I to its specific membrane receptors avoiding the IGF-I-induced down regulation of IGF-I receptors, as already described (Conover et al., 1990). The use of the IGF-I analogue, Des(1–3)IGF-I with reduced affinity for IGFBPs, significantly increased the GENC cell number when compared with the control, but its action was not significantly different when compared with the cell preincubated with rhIGFBP-3. Taken together, these data suggest that a potentiation of the IGF-I action in GENC occurs when IGFBP-3 is cell-surface associated.

A partial confirmation of the main role played by IGFBP-3 in modulating GENC growth is the observation that the strong TGF- $\beta_1$  inhibitory effect on GENC proliferation was accompanied by a decrease in IGFBP-3 release. There are several reports concerning the uniform inhibitory effect on cell endothelial growth by TGF- $\beta$  (Muller et al., 1987; Morello et al., 1995). To date, the precise mechanism underlying this inhibitory action of TGF- $\beta_1$  is unknown (Lawrence, 1995), however, in some cell types this control of mitogenesis seems to be mediated by the IGF system, as observed in human fibroblasts (Martin and Baxter, 1991; Yateman et al., 1993), in breast cancer cells (Oh et al., 1995) and in mesangial cells (Grellier et al., 1996). This study hypothesizes that, at least in GENC, this inhibiting action of TGF- $\beta_1$  on cell growth, regardless of an apparent increase of inhibitory IGFBP-4, was mediated by a marked reduction of IGFBP-3 levels. Northern blot analysis and protease inhibitor experiments confi-

irmed previous reports on endothelial cells (Erondu et al., 1996) concerning the TGF- $\beta_1$  reduction of IGFBP-3 levels decreasing the expression of IGFBP-3 mRNA, without activating specific IGFBP-3 proteolytic enzyme (Jones and Clemmons, 1995). Moreover, the reduction of the 29–30 kDa glycosylated form of IGFBP-4 with the apparent contemporary increase of the 24 kDa band, was not supported by an altered IGFBP-4 mRNA transcription (Chen et al., 1993). Although this mechanism still remains to be clarified, it could reflect a direct action of TGF- $\beta_1$  on potential sites of glycosylation as already reported for extracellular matrix components (Sharma and Ziyadeh, 1994). However, these results are in contrast with other data obtained with different cell types, where the inhibitory action of TGF- $\beta_1$  on cell mitogenesis was mediated through an increase of IGFBP-3 (Yateman et al., 1993; Oh et al., 1995; Grellier et al., 1996). Although the different cells used as an experimental model could explain the divergent results reached, we cannot completely exclude the presence of other factors involved in the modulation of TGF- $\beta_1$  action on GENC, such as the modified IGFBP-4 profile or an IGF-I post-receptor pathway control, as described in other experimental models (Besset et al., 1994).

In summary, the present study demonstrates that human GENC release three different IGFBPs. It also reports that IGF-I and TGF- $\beta_1$  modulate, in different ways, the release of these IGFBPs and, respectively stimulate or inhibit, cell growth. The data suggests that IGFBP-3 and IGFBP-4 could be the two critical IGF-BPs regulating the cell growth and that the factor playing the most important role in GENC proliferation could be the control of IGFBP-3, which is up- and down-regulated at the transcriptional level, respectively by IGF-I and TGF- $\beta_1$ . The role of IGFBPs in renal diseases requires further study to understand the exact physiological meaning, however, we can hypothesize that the distrectual regulation of these IGFBPs could address the circulating or locally produced IGFs to the target cells. Together with a recent report (Grellier et al., 1996) on human mesangial cells, the data suggest a new emerging role for IGFBP-3 in GENC.

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