Modulation of insulin-like growth factor (IGF) and IGF binding protein biosynthesis by hypoxia in cultured vascular endothelial cells

M Tucci, K Nygard, B V Tanswell, H W Farber¹, D J Hill and V K M Han

MRC Group in Fetal and Neonatal Health and Development, Department of Paediatrics, Obstetrics and Gynaecology, Biochemistry and Anatomy, Lawson Research Institute, University of Western Ontario, London, Ontario, Canada N6A 4V2 and ¹The Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts 02118, USA

(Requests for offprints should be addressed to V K M Han, Lawson Research Institute, St Joseph's Health Centre, 268 Grosvenor Street, London, Ontario, Canada N6A 4V2)

Abstract

Endothelial cells (EC) are hypoxia-tolerant and their capacity to proliferate in low oxygen tension is essential to maintain vascular endothelium integrity. The present study addresses whether hypoxia alters the expression of insulin-like growth factor (IGF) and IGF binding protein (IGFBP) genes in bovine aortic EC (BAEC) and bovine pulmonary artery EC (BPAEC). EC were cultured in normoxic (21%) conditions and exposed to 0% oxygen for 24, 48, or 72 h; some cells were reoxygenated by exposure to 21% oxygen for 24 or 48 h following hypoxia. IGF-I peptide and mRNA levels were very low in both cell types, and decreased further with exposure to hypoxia. Ligand blotting showed that both cell types synthesized 24 kDa (IGFBP-4), 30 kDa (IGFBP-5 and/or IGFBP-6), 43 kDa and 48 kDa IGFBPs (IGFBP-3 glycosylation

variants). IGFBP-4 was the predominant IGFBP expressed by both cell types and did not change with exposure to hypoxia. Hypoxia caused a significant increase in IGFBP-3 secretion in BPAEC but not in BAEC. IGFBP-3 stable mRNA levels in BPAEC were increased correspondingly. IGFBP-5 was expressed only in BAEC and decreased with exposure to hypoxia. IGFBP-6 mRNA expression was low and increased in both cell types with exposure to hypoxia. These results demonstrate that EC IGFBP baseline expression as well as its expression in hypoxia vary in different vascular beds and suggest that the IGFBPs may be the dominant paracrine regulators of proliferation of EC as well as maintenance of endothelium integrity during hypoxia.

Journal of Endocrinology (1998) **157**, 13–24

Introduction

Endothelial cells (EC) occupy a strategic location in the vasculature where they play an important role in the maintenance of vascular integrity and the passage of growth mediators and cytokines into the extravascular space. Hypoxemia is one of the most frequent and common insults to the vascular endothelium. Several previous studies have demonstrated that EC possess remarkable tolerance and adaptation to hypoxic conditions (Farber & Rounds 1990, Ogawa *et al.* 1990, Shreenivas *et al.* 1991, Zimmerman *et al.* 1991, Graven *et al.* 1993, Tucci *et al.* 1996). These studies have shown that cultured vascular EC tolerate acute hypoxia (0% oxygen) for periods up to five days and chronic hypoxia (3% oxygen) for periods up to several months, continue to divide at rates dependent on the origin of the EC as well as the ambient oxygen concentration to which they are exposed, and demonstrate no evidence of significant cellular damage despite exposure to these seemingly adverse hypoxic

conditions (Farber & Rounds 1990, Zimmerman *et al.* 1991, Graven *et al.* 1993, Tucci *et al.* 1996, 1997). The mechanisms underlying this hypoxia tolerance as well as the factors affecting EC proliferation during hypoxia are not fully understood.

The insulin-like growth factors (IGF-I and IGF-II) and the six insulin-like growth factor binding proteins (IGFBP-1 to -6) are important modulators of cellular proliferation and differentiation (Rechler & Nissley 1990, Clemmons 1991, Drop *et al.* 1991, Shimasaki & Ling 1991, Cohick & Clemmons 1993, Jones & Clemmons 1995) that are produced in multiple cell types including vascular EC (Bar *et al.* 1987b, 1989, Moser *et al.* 1992, Gajdusek *et al.* 1993, Taylor *et al.* 1993). IGFBPs serve as carrier proteins for the IGFs in both vascular and extra-vascular tissues, can inhibit or potentiate IGF biologic activity in a tissue-specific manner and participate in the local regulation of growth and repair processes (Rechler & Nissley 1990, Clemmons 1991, Drop *et al.* 1991, Shimasaki & Ling 1991). In EC, IGFs and IGFBPs

are known to affect several metabolic processes (King *et al.* 1985, Bar *et al.* 1987a, 1988a,b), are involved in potential interplay between vascular endothelium and subendothelial components (Hansson *et al.* 1987, 1989, Delafontaine *et al.* 1991, Khorsandi *et al.* 1992, Nakao *et al.* 1992, Taylor *et al.* 1993) and may play a role in the intrinsic growth of normal and diseased blood vessels (King *et al.* 1985, Nicosia *et al.* 1994).

In other cell types and biological systems, exposure to decreased oxygen alters the expression of the genes encoding IGF system peptides. Numerous studies have described modifications of various IGF system peptides following hypoxic-ischemic brain injury (Klempt *et al.* 1992, 1993, Lee *et al.* 1992, Beilharz *et al.* 1993, Lee & Bondy 1993, Stephenson *et al.* 1995, Johnston *et al.* 1996, Sandberg *et al.* 1996), hypoxia induced by reduced uterine blood flow (McLellan *et al.* 1992, Price *et al.* 1992, Owens *et al.* 1994, Asano *et al.* 1995), hypoxia-induced pulmonary hypertension (Perkett *et al.* 1992, Dempsey *et al.* 1994, Townsend & Stenmark 1995), myocardial ischemia (Reiss *et al.* 1994, Buerke *et al.* 1995, Kluge *et al.* 1995) and acute tubular necrosis (Noguchi *et al.* 1993, Tsao *et al.* 1995). In experimental vascular injury, a context in which initial EC proliferation occurs in relative hypoxia, studies have shown induction of aortic IGF-I mRNA in the vascular wall after balloon denudation (Cercek *et al.* 1990, Khorsandi *et al.* 1992) while immunohistochemical studies have described increased IGF-I and IGF-II protein accumulation during repair of injured arterial intima (Hansson *et al.* 1987, 1989, Levinovitz *et al.* 1992). However, little is known about the effect of hypoxia on the IGF system in vascular EC. This understanding is critical as many clinical situations require ongoing EC proliferation despite adverse hypoxic conditions in order to allow for tissue repair.

Based on observations that exposure to hypoxia alters the expression of the IGF system peptides and our studies demonstrating changes in EC proliferation during exposure to hypoxia (Farber & Rounds 1990, Tucci *et al.* 1997), we hypothesized that hypoxia alters the rate of EC cellular division by altering the expression of IGFs and/or IGFBPs. In the current study, we found that exposure of cultured EC to hypoxia produces alterations in baseline expression of IGFs and IGFBPs that are dependent on the vascular bed from which they originated. These *in vitro* studies carried out in cultured EC eliminate confounding interactions between EC and non-endothelial components, and allow delineation of hypoxia-associated changes in the IGF system in isolated vascular endothelium. The findings suggest that locally expressed IGFs and IGFBPs play a role in the altered proliferation of EC exposed to acute hypoxia, with IGFBPs being the dominant paracrine regulators.

This work was presented in part at the May 1996 meeting of the Pediatric Academic Societies (The American Pediatric Society and the Society for Pediatric

Research) and has been published in abstract form (Tucci *et al.* 1996).

Materials and Methods

Materials

Human recombinant IGF-I was purchased from IMCERA Bioproducts (Terre Haute, IN, USA) and human recombinant IGF-II from Bachem Co. (Torrance, CA, USA). IGF-I and IGF-II were iodinated using the Chloramine-T method as previously described (Hill 1990). Tissue culture plastic ware was obtained from Falcon Plastics (Los Angeles, CA, USA), Costar (Cambridge, MA, USA) and United Scientific (Quincy, MA, USA).

Endothelial cell culture

Bovine aortic and pulmonary arterial endothelial cells (BAEC and BPAEC respectively) were isolated from freshly excised calf aortas and pulmonary arteries as previously described (Zimmerman *et al.* 1991, Graven *et al.* 1993, Tucci *et al.* 1997). Animals used for these experiments were male calves 4–8 weeks of age. EC were obtained by lightly scraping the intimal surface of longitudinally opened vessels and seeded initially into 35 mm plastic Petri dishes with growth media containing minimal essential medium (MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 15% heat-inactivated bovine calf serum (BCS; Hyclone Laboratories, Logan, UT, USA), 1 mM sodium pyruvate (Gibco BRL), 10 000 U/ml penicillin and 10 µg/ml streptomycin (Sigma Chemical Co, St Louis, MO, USA). Cultures were maintained in a humidified incubator at 37 °C in 95% air/5% CO₂. EC purity was assured by the typical cobblestone appearance on phase-contrast microscopy, the uptake of fluorescent acetylated-low density lipoprotein and the presence of immunofluorescence for von Willebrand factor (Farber & Rounds 1990, Zimmerman *et al.* 1991, Graven *et al.* 1993, Tucci *et al.* 1997). BAEC and BPAEC used in these experiments were derived from at least 3 different primary lines subcultured for the study between passages 4 and 12 and were of the same passage number from the same animal for individual experiments.

Exposure to hypoxia

BAEC and BPAEC were seeded into P-100 tissue culture dishes and used at 75–80% confluence. At this time, culture medium was removed and EC were incubated in serum-free medium (SFM) and subjected to the appropriate O₂ condition. Control cells were maintained in 21% O₂ throughout the experimental protocol. All hypoxic cells were exposed to 0% O₂, 5% CO₂, and balanced

N₂ in humidified sealed chambers (Billups-Rothenburg, Del Mar, CA, USA) at 37 °C for 24–72 h. In reoxygenation studies, cells were exposed to 0% oxygen for 24 h, then returned to 21% oxygen for 24 or 48 h. For all hypoxic conditions lasting longer than 24 h, chambers were gassed every 24 h.

At the end of the experimental protocol, conditioned media were collected and centrifuged at 3000 *g* to remove cellular debris. One milliliter aliquots of all samples were stored in –70 °C for IGF RIA and the remaining conditioned media were concentrated by ultrafiltration using Centricon-10 concentrators pre-treated with 5% Tween-20 (Amicon, Beverly, MA, USA). Cells from the same cultures were used for total RNA extraction and DNA quantification.

Western ligand blot analysis

Ligand blot analysis was performed by a modification of the method of Hossenlopp *et al.* (1986) as previously described (Bradshaw & Han 1993). Conditioned medium, 250 µg/lane, was subjected to electrophoresis on a non-reducing 8–12% gradient sodium dodecyl sulfate (SDS) polyacrylamide gel and proteins were then transferred electrophoretically to a 0.2 µm nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA). The membrane was washed in Tris–NaCl pH 7.4, 0.5 mg/ml sodium azide, and 3% Nonidet P-40 (Sigma) and incubated with 400 000 c.p.m. ¹²⁵I-IGF-II in Tris–NaCl pH 7.4, 0.1% Tween 20, and 1% BSA, overnight at 4 °C. The membrane was then washed sequentially in Tris–NaCl pH 7.4 and 0.1% Tween 20 (2 × 15 min) and Tris–NaCl pH 7.4 (3 × 15 min). The membrane was air dried and exposed to X-ray film (XAR, Kodak Laboratories, Rochester, NY, USA) with intensifying screens at –70 °C for 3 to 14 days. Total protein concentrations of the conditioned media were determined by the Bio-Rad Protein assay (Bio-Rad Laboratories Ltd, Richmond, CA, USA). Relative levels of IGFBPs were quantified by densitometry (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA).

Western immunoblotting analysis

To identify which IGFBP species was secreted by EC, a modified Western blot method was employed as previously described (Phillips *et al.* 1993). Briefly, conditioned media were separated on 8–12% SDS polyacrylamide gels and transferred to nitrocellulose membranes. After transfer, the nitrocellulose membranes were washed with PBS buffer for 10 min, non-specific binding sites blocked by incubation in PBS containing 5% (w/v) BSA, and then incubated with primary antisera at 4 °C for 18 h. Antibodies used were rabbit polyclonal anti-IGFBP-4 (1:500 dilution; UBI, Lake Placid, NY, USA), rabbit polyclonal anti-human IGFBP-5 (1:500 dilution; UBI), and rabbit

anti-human IGFBP-6 (1:500 dilution; Austral Biologicals, San Ramon, CA, USA). After incubation, the nitrocellulose membranes were washed with PBS buffer and incubated with biotinylated goat anti-rabbit immunoglobulin (1:500 dilution; Vector Laboratories, Burlingame, CA, USA) for 1 h at 23 °C. Following a further wash, the membrane was incubated with avidin and biotinylated peroxidase for 1 h at 23 °C, washed with PBS buffer followed by 50 mmol Tris buffer/l (pH 7.5) at room temperature. Immunoreactive proteins were visualized using freshly prepared 3–3'-diaminobenzidine tetrahydrochloride (1.89 mmol/l) containing 0.03% (v/v) hydrogen peroxide. The reaction was quenched by washing in excess 50 mmol Tris buffer/l.

Immunoprecipitation

For some samples, immunoprecipitation was performed in order to identify IGFBP species with very low expression (Harlow & Lane 1988). Antibodies used were the same as for Western immunoblotting analysis. Briefly, the appropriate lyophilized antibody was resuspended as directed and 1 µl added to 49 µl Hepes binding buffer (1:50 dilution). A 10 µl aliquot of the antibody suspension was added to 90 µl concentrated conditioned media (final antibody dilution of 1:500), vortexed, and incubated overnight at 4 °C to allow antibody–antigen binding. The suspension was then added to 50 µl protein A sepharose suspended in 20 mM sodium phosphate buffer, pH 7.0 (Pharmacia Canada Inc., Baie d'Urfé, Québec, Canada) and incubated at 4 °C for 3–5 h. Sepharose-bound antigen–antibody complexes were spun down at 3000 r.p.m. and eluted from the sepharose by boiling in Laemmli buffer. The supernatant containing the IGFBP of interest was collected and processed for Western ligand blotting as described above.

DNA quantification

DNA quantification was performed by the fluorometric DNA assay described previously (Brunk *et al.* 1979). Briefly, EC monolayers were rinsed with PBS, precipitation was performed with 12.5% trichloroacetic acid (Fisher Scientific, Nepean, Ontario, Canada) and DNA was solubilized in 0.1 M NaOH (Fisher Scientific). Duplicate 100 µl aliquots of DNA suspension were used for quantification after addition of 0.2 M HCl, 2 mM EDTA and HOESCHT 33258 dye (Sigma-Aldrich, Oakville, Ontario, Canada) and measurements were obtained by fluorescence spectrophotometry (Hitachi F-4010 fluorescence spectrophotometer, Tokyo, Japan).

RNA preparation and Northern blot analysis

Total RNA was prepared from EC using the single-step method of RNA isolation by guanidinium

thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi 1987) and Northern blotting was performed as described previously (Han *et al.* 1988). Briefly, total RNA (20 µg/lane) was denatured and subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde. The RNAs were transferred to a Zeta-Probe nylon membrane (Bio-Rad Laboratories) by the capillary transfer technique. Following transfer, the blots were baked and hybridized at 42 °C overnight with ³²P-labeled ovine IGF-I, IGF-II, and IGFBP-1 to -6 cDNA probes (2 × 10⁶ c.p.m./ml buffer containing 5 × SSPE (0.75 M NaCl, 44 mM NaH₂PO₄·2H₂O, 5 mM EDTA, pH 7.4), 7% SDS, and 5 µg/ml denatured salmon sperm DNA). The cDNA inserts were labeled with ³²P-dCTP (ICN, St Laurent, Québec, Canada) to specific activities of 1–2 × 10⁹ c.p.m./µg by the random priming technique using an oligo-labeling kit (Pharmacia Canada Inc.). Blots were washed twice (30 min each) in 1 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0)–0.1% SDS at 42 °C, twice (30 min each) in 0.1 × SSC–0.1% SDS at 42 °C, air-dried and subjected to autoradiography using intensifying screens at –70 °C. Subsequently, blots were stripped between hybridizations by washing twice in 0.01 × SSC and 0.5% SDS for 30 min each at 80 °C. Consistency in loading and transfer of total RNAs in each lane was checked by probing the blots with a radiolabeled cDNA for 18S ribosomal RNA (a gift from Dr David Denhardt, Rutgers University, NJ, USA). Autoradiograms were quantified using densitometry and/or phosphorimaging (Personal Densitometer SI and Phosphorimager SI, Molecular Dynamics, Sunnyvale, CA, USA).

Radioimmunoassay

The IGF-I and IGF-II concentrations in conditioned medium were determined by a modification of a previously described RIA method following separation of IGFs from IGFBPs using acid-gel chromatography (Hill 1990). Briefly, 1 ml samples of conditioned medium were dried in a vacuum centrifuge (SpeedVac Savant concentrator, Savant, Farmingdale, NY, USA), resuspended in 500 µl 0.2 M acetic acid and loaded onto a Sephadex G50 column. One milliliter fractions were collected and IGF-containing fractions were pooled for each conditioned medium sample, lyophilized, resuspended in radioimmunoassay buffer and subjected to RIA. Recovery of IGF-I and IGF-II from the column was determined by the addition of 200 000 c.p.m. ¹²⁵I-IGF-II and was greater than 90%.

Data analysis

All experiments were performed at least three times and each experimental condition performed in triplicate. To compensate for loading differences among RNA samples in Northern blot analyses, densitometric analysis of the

hybridizing mRNA bands were equalized to the corresponding 18S ribosomal RNA bands. In ligand blot analyses, signal intensity of IGFBPs as quantified by densitometry was adjusted per µg DNA. The percentage change in mRNA and protein expression for each hypoxic and reoxygenation condition was then compared with the control condition (21% oxygen) and the data expressed as the percentage change relative to control. All data are presented as the mean ± S.E.M. ANOVA was used for time-course analysis and comparisons performed using the Bonferroni multiple comparisons test. Differences were considered significant at *P* < 0.05.

Results

IGF-I and IGF-II expression and release

IGF-I mRNA levels in both BAEC and BPAEC exposed to 21% or 0% oxygen were low. IGF-I mRNA was relatively more abundant in BPAEC than BAEC with regard to the expression of 9.0 kilobase (kb), 8.0 kb and 4.2 kb IGF-I transcripts. The 8.0 kb transcript decreased (to 25% of control level) with exposure to hypoxia and increased within 24 h of reoxygenation (to 50% of control level) in BPAEC. Although the mRNA levels were lower, a similar modulation occurred in BAEC (Fig. 1). The expression of IGF-II mRNAs was lower than that of IGF-I in both cell types (data not shown).

IGF-I was detectable only in the conditioned media of normoxic and reoxygenated EC (levels were below 1 ng/ml); IGF-I was not detectable in the conditioned media of hypoxic EC. IGF-II was also detectable in some conditioned media used for these studies; its expression was low (below 2 ng/ml) and showed no variation with exposure to hypoxia.

IGFBP expression and secretion

Ligand blot analyses demonstrated that BAEC and BPAEC secreted four IGFBP species (Fig. 2): the 24 kDa protein was confirmed as IGFBP-4, the 43 kDa and 48 kDa proteins were unglycosylated and glycosylated IGFBP-3, and the 30 kDa protein was consistent with either IGFBP-5 and/or IGFBP-6. IGFBP-4 was the most abundant IGFBP in both BAEC and BPAEC. The identity of IGFBP-3 and IGFBP-4 was confirmed by Western immunoblotting with specific antisera (data not shown); IGFBP-5 identity was confirmed by immunoprecipitation (Fig. 3). Northern blots probed sequentially with IGFBP-1 to -6 cDNAs demonstrated expression of IGFBP-3, -4, -5 and -6 mRNAs. Neither the protein, nor the mRNA for IGFBP-1 and IGFBP-2 was detected (Figs 4 and 5).

IGFBP-3 Both cell types consistently expressed two glycosylation variants of IGFBP-3 (calculated sizes 43 kDa

Figure 1 Northern analysis of total RNA (20 µg/lane) extracted from BAEC (lanes 1–6) and BPAEC (lanes 7–12) maintained in 21% oxygen, exposed to hypoxia (0% oxygen) or exposed to hypoxia followed by reoxygenation. Panel A: representative Northern blot of total RNA from EC cultures exposed to control 21% oxygen (C), to 0% oxygen for 24 h, 48 h and 72 h, and to reoxygenation for 24 h (R24) and 48 h (R48) after exposure to 0% oxygen for 24 h. Fetal sheep liver RNA is used as a positive control (+C). Blots were probed sequentially with ³²P-labeled ovine IGF-I and 18S rRNA cDNAs. IGF-I blots were exposed to X-ray film for 14 days. Panel B: relative levels of IGF-I mRNA expression for the 8.0 kb transcript were quantified by densitometry, normalized by comparison with 18S and expressed as the change relative to control (21% oxygen) for each cell type. **P* < 0.05 (compared to controls 21% oxygen).

and 48 kDa); however, IGFBP-3 levels were greater in normoxic BPAEC than BAEC conditioned media (Fig. 3). IGFBP-3 release into the media of BPAEC increased 2- to 3-fold within 24 h of exposure to hypoxia while, in BAEC, no significant change occurred (Fig. 3). A single 2.4 kb IGFBP-3 mRNA was consistently expressed by both cell types; with exposure to hypoxia, its expression increased 3- to 4-fold in BPAEC while it decreased in BAEC (Fig. 4).

IGFBP-4 The production of IGFBP-4 in both BAEC and BPAEC remained unchanged with exposure to hypoxia (Fig. 2). Northern analysis revealed a similar

abundance of IGFBP-4 (2.3 kb) mRNA expression in both cell types during normoxia. With exposure to hypoxia, IGFBP-4 mRNA abundance decreased significantly in BAEC, while no change was observed in BPAEC (Fig. 4). During reoxygenation, BAEC IGFBP-4 mRNA levels returned to control levels within 24 h, while the mRNA levels in BPAEC remained the same.

IGFBP-5 Ligand blot analyses of conditioned media from both BAEC and BPAEC revealed equivalent amounts of an approximately 30 kDa IGFBP consistent with either IGFBP-5 or -6 (Fig. 2). Immunoblotting and immunoprecipitation using antihuman IGFBP-5 antiserum confirmed that this 30 kDa IGFBP is IGFBP-5 (Fig. 3). In addition, the fact that IGFBP bound ¹²⁵I-IGF-I and ¹²⁵I-IGF-II with similar affinity in ligand blots indicated that this IGFBP is unlikely to be IGFBP-6. A single IGFBP-5 mRNA (6.0 kb) was expressed in both normoxic cell types but was less abundant in BPAEC than in BAEC. Interestingly, the IGFBP-5 mRNA abundance was significantly diminished in the more confluent cultures. With exposure to hypoxia, the expression of IGFBP-5 mRNA decreased in BAEC and returned to control levels with reoxygenation (Fig. 5). The IGFBP-5 levels were low to undetectable in BPAEC.

IGFBP-6 Whether a component of the 30 kDa IGFBP constitutes IGFBP-6 could not be confirmed by immunoblotting because of the lack of a specific antiserum. Northern analysis for IGFBP-6 revealed weak expression of a 1.3 kb hybridizing band in both cell types. With exposure to hypoxia, IGFBP-6 mRNA expression exhibited a 2- to 3-fold increase that returned to control levels with reoxygenation (Fig. 5).

Discussion

In this study we have demonstrated that exposure to acute hypoxia causes cultured vascular EC to alter the expression of IGF and IGFBP genes. These include (a) a reduction in IGF-I gene expression, (b) EC type-specific changes in response to hypoxia for some IGFBPs and (c) the reversibility of hypoxia-induced changes of both IGF and IGFBPs. Since IGFs and IGFBPs interact with other components of the vasculature including mural cells such as smooth muscle cells, pericytes and fibroblasts, a paracrine interaction between EC and mural cells via IGF and IGFBP may be an important component of vascular endothelial and mural cell response to hypoxia.

IGF-I is secreted by many cell types including vascular EC (Kern *et al.* 1989, Delafontaine *et al.* 1991, Gajdusek *et al.* 1993). While some have reported that the major portion of EC IGF-I is derived from exogenous sources (Gajdusek *et al.* 1993), several have also demonstrated the existence of a low *de novo* synthesis of IGF-I by vascular

Figure 2 Western ligand blot analysis of conditioned media from BAEC (lanes 1–6) and BPAEC (lanes 7–12) maintained in 21% oxygen, exposed to hypoxia (0% oxygen) or exposed to hypoxia followed by reoxygenation. (a) Autoradiograph of a representative Western ligand blot showing control 21% oxygen (C), exposure to 0% oxygen for 24 h, 48 h and 72 h, and reoxygenation for 24 h (R24) and 48 h (R48) after exposure to 0% oxygen for 24 h. (b) IGFBP-3, 30 kDa (IGFBP-4), 43 and 48 kDa (IGFBP-5 and/or -6), 43 and 48 kDa (IGFBP-3 glycosylation variants). (c) Densitometric analysis (mean from three separate experiments performed in triplicate) of changes in IGFBP-3 levels in conditioned medium. Relative levels of IGFBP-3 were quantified by densitometry, normalized per μg DNA and are expressed as the change relative to control (21% oxygen) for each cell type. (d) Similar analysis for the 30 kDa ligand (IGFBP-5 and/or -6). (e) Similar analysis for IGFBP-4. * $P < 0.05$ (compared to controls 21% oxygen).

Figure 3 Analysis of conditioned media from BAEC (A and B) and BPAEC (C and D) processed for Western ligand blotting (A and C) and for immunoprecipitation (B and D). Panels A and C show four bands with the calculated molecular weights described previously. Panels B and D show the same conditioned media after immunoprecipitation with anti-human IGFBP-5 antibody and demonstrate a single 30 kDa band.

EC, as we have shown in this study (Kern *et al.* 1989, Delafontaine *et al.* 1991, Gajdusek *et al.* 1993). Cultured EC express high-affinity IGF receptors which are probably involved in the processing of IGF-I by vascular endothelium (Bar & Boes 1984, Bar *et al.* 1989, Boes *et al.* 1991). In both EC types, exposure to hypoxia caused downregulation of IGF-I mRNA expression. As IGF-I is a mitogen for EC and other vascular components (King *et al.* 1985, Hansson *et al.* 1987, 1989, Khorsandi *et al.* 1992, Nakao *et al.* 1992, Grant *et al.* 1993), this reduced IGF-I synthesis may affect autocrine/paracrine regulation of endothelium integrity and regeneration as well as regulation of IGF-I availability within mural components of the vasculature. Interestingly, baseline IGF-I expression was more pronounced in BPAEC than BAEC, an observation which is not attributable to differences in confluency (Gajdusek *et al.* 1993) and which suggests differential processing and/or utilization of IGF-I by EC from different vascular origins.

The biological actions of endogenous or exogenous IGF-I are modulated by EC IGFBPs and alterations in the biosynthesis of these IGFBPs will influence IGF actions. Cultured EC express mRNAs for IGFBP-2 through -6 and secrete substantial quantities of IGFBPs into the culture medium (Boes *et al.* 1992, Moser *et al.* 1992, Yang *et al.* 1993). In addition, IGFBPs synthesized by EC are involved in the mobilization and transfer of IGFs across the EC barrier (Gajdusek *et al.* 1993, Taylor *et al.* 1993). Marked variability has been described in the expression of the various IGFBPs that is dependent on numerous factors including the vascular origin of the EC (Moser *et al.* 1992), the culture milieu and the confluency of the cell cultures used (Hayzer *et al.* 1995, Delafontaine *et al.* 1996). The IGFBP profile of EC in this study is similar to that described previously. To date IGFBP-1 has not been

shown to be expressed in macrovascular EC (Moser *et al.* 1992), nor in our BAEC and BPAEC. IGFBP-2 mRNA is detectable in large vessel EC using reverse transcription-PCR (Bar *et al.* 1987), but neither IGFBP-2 mRNA nor protein was detected in our cells. Both EC types expressed IGFBP-3 as described (Hayzer *et al.* 1995). IGFBP-4 was the predominant IGFBP expressed by both types of EC (Boes *et al.* 1992, Moser *et al.* 1992, Yang *et al.* 1993). Although a small amount of a 30 kDa glycosylation variant of IGFBP-4 has been described (Moser *et al.* 1992), no 30 kDa band corresponding to IGFBP-4 was seen in either BAEC or BPAEC. Low expression of IGFBP-5 and -6 has already been reported in large vessel EC; their expression is apparently higher in microvessels (Moser *et al.* 1992). Our findings concur with this observation and demonstrate low expression of both these binding proteins in vascular EC.

Under normoxic conditions, IGFBP-3 inhibits growth of actively proliferating EC; confluent growth-arrested (contact-inhibited) EC show marked induction of IGFBP-3 gene expression, while actively proliferating EC have lesser IGFBP-3 gene expression and protein production (Delafontaine *et al.* 1996). In our study, hypoxic BPAEC, despite subconfluent cell densities, increased IGFBP-3 gene expression whereas BAEC at the same densities did not. This vascular bed-dependent response suggests that BPAEC, which are exposed to relatively lower oxygen tension *in vivo* than BAEC, are perhaps more hypoxia tolerant and need a lesser proliferative impetus under hypoxic conditions than their aortic counterparts.

With exposure to hypoxia, IGFBP-4 protein levels were unchanged in both cell types while mRNA levels showed a striking decrease in BAEC. That IGFBP-4 gene expression exhibits an origin-dependent differential response to hypoxia suggests that EC from systemic (aortic) vascular beds are more susceptible to downregulate this generally inhibitory IGFBP in order to promote IGF-I action. In contrast, pulmonary EC, which may be more hypoxia tolerant given their *in vivo* environment, require a lesser proliferative stimulus. Interestingly, while total protein production in EC decreases by up to 75% with exposure to hypoxia (Farber & Rounds 1990), levels of IGFBP-4 protein remained unchanged in both EC types suggesting that this binding protein plays an important role within the vasculature. A potential explanation for unchanged protein synthesis despite decreased mRNA expression is that hypoxia alters proteolytic processing of IGFBP-4 by various proteases (Kamyar *et al.* 1994, Gockerman & Clemmons 1995, Parker *et al.* 1995) leading to increased stability of secreted IGFBP-4 or that hypoxic modulation of one IGFBP may be regulating the availability of other IGFBPs (Fowlkes *et al.* 1995, Boes *et al.* 1996, Donnelly & Holly 1996, Noll *et al.* 1996).

IGFBP-5 has the unique property of adhering tightly to fibroblast extracellular matrix and may be an important factor in regulating IGF-I actions (Parker *et al.* 1996).

Figure 4 Northern analysis of total RNA (20 µg/lane) extracted from BAEC (panels A–C) and BPAEC (panels D–F) maintained in 21% oxygen, exposed to hypoxia (0% oxygen) or exposed to hypoxia followed by reoxygenation. All blots were probed sequentially with ³²P-labeled bovine IGFBP-3, ovine IGFBP-4 and 18S rRNA cDNAs. All samples were analyzed in duplicate. Fetal sheep liver RNA is used as a positive control (+C). Panel A: representative Northern blot of total RNA from BAEC cultures exposed to 0% oxygen (C), to 0% oxygen for 24 h, 48 h and 72 h, and to reoxygenation for 24 h (R24) and 48 h (R48) after exposure to 0% oxygen for 24 h. Panel B: densitometric analysis (mean from three separate experiments performed in triplicate) of changes in IGFBP-3 mRNA in BAEC. Relative levels of IGFBP-3 mRNA expression were quantified by densitometry, normalized by comparison with 18S and expressed as the change relative to control (21% oxygen). Panel C: similar analysis for IGFBP-4 in BAEC. Panel D: representative Northern blot of total RNA from BPAEC cultures exposed to the same conditions as panel A. All blots were probed sequentially with the same ³²P-labeled used for BAEC in panel A. Panel E: analysis for IGFBP-3 in BPAEC similar to that performed in BAEC. Panel F: analysis for IGFBP-4 in BPAEC similar to that performed in BAEC. **p* < 0.05 (compared to controls 21% oxygen).

Figure 5 Northern analysis of total RNA (20 µg/lane) extracted from BAEC (lanes 1–6) and BPAEC (lanes 7–12) maintained in 21% oxygen, exposed to hypoxia (0% oxygen) or exposed to hypoxia followed by reoxygenation. Blots were probed sequentially with 32 P-labeled ovine IGFBP-5, ovine IGFBP-6 and 18S rRNA cDNAs. The estimated sizes of specific mRNAs in BAEC and BPAEC calculated from standards of known size were 6.0 kb for IGFBP-5 and 1.3 kb for IGFBP-6. Panel A: representative Northern blot of total RNA from EC cultures exposed to control 21% oxygen (C), to 0% oxygen for 24 h, 48 h and 72 h, and to reoxygenation for 24 h (R24) and 48 h (R48) after exposure to 0% oxygen for 24 h. Fetal sheep liver RNA (+C) is used as a positive control. Panel B: densitometric analysis (mean from three separate experiments performed in triplicate) of changes in IGFBP-5 mRNA. Relative levels of IGFBP-5 mRNA expression were quantified by densitometry, normalized by comparison with 18S and are expressed as the change relative to control (21% oxygen) for each cell type. Quantification was not performed in BPAEC for IGFBP-5. Panel C: similar analysis for IGFBP-6. * $P < 0.05$ (compared to controls 21% oxygen). For experiments performed two times or less, no error bars and S.E.M. are shown.

Moreover, in other cell types IGFBP-5 mRNA expression is enhanced by ischaemia/reperfusion in myocardium (Kluge *et al.* 1995) and is upregulated coordinately with IGF-I expression in injured astrocytes after hypoxic-ischemic brain injury (Lee *et al.* 1996). IGF-I upregulates IGFBP-5 synthesis by transcriptionally activating the IGFBP-5 gene in aortic smooth muscle cells (Duan *et al.* 1996) or by protecting IGFBP-5 from proteolysis (Cheng *et al.* 1996). In our study, we have observed that the capacity to synthesize IGFBP-5 differed according to the vascular bed of origin; although IGFBP-5 protein levels in the conditioned media were comparable in both cell types, IGFBP-5 mRNA expression was markedly lower in BPAEC suggesting that the latter cells may have either more efficient translation of IGFBP-5 mRNA or less IGFBP-5 degradation. IGFBP-5 mRNA expression decreased in BAEC with exposure to hypoxia. Nonetheless, its predominance in BAEC and its decrease with exposure to hypoxia in both cell types taken with its

demonstrated upregulation in non-endothelial cell types exposed to hypoxic stress (Kluge *et al.* 1995, Lee *et al.* 1996) suggests a potential role for IGFBP-5 in the transport of IGFs from the vascular compartment to subendothelial target tissues.

Little is known about IGFBP-6 expression in the vasculature. While some have described IGFBP-6 production in vascular smooth muscle cells (Boes *et al.* 1996), no studies have reported hypoxia-induced IGFBP-6 modulation. In both our EC types, IGFBP-6 mRNA expression was very low and increased with exposure to hypoxia. Potential explanations for this observation include modulation of endogenous IGFBP-6 gene expression and/or altered mRNA stability with exposure to hypoxia.

While biosynthesis of IGFBPs is affected by exposure to hypoxia in other cell types or biological systems, no previous study has described IGFBP production by the vascular endothelium during hypoxia. In the ovine fetus subjected to hypoxia, IGF and IGFBP levels in the serum

and their gene expression in various tissues are altered (McLellan *et al.* 1992, Price *et al.* 1992, Owens *et al.* 1994, Asano *et al.* 1995). After hypoxic-ischemic injury in the central nervous system, IGF-I, IGFBP-2, IGFBP-3 and IGFBP-5 are induced at sites of injury and recovery (Lee *et al.* 1992, Klempt *et al.* 1992, 1993, Beilharz *et al.* 1993, Lee & Bondy 1993, Stephenson *et al.* 1995, Johnston *et al.* 1996, Sandberg *et al.* 1996). The involvement of the IGF system has also been described in hypoxia-induced pulmonary hypertension studies in the newborn (Perkett *et al.* 1992, Dempsey *et al.* 1994, Townsend & Stenmark 1995), in acute tubular necrosis (Noguchi *et al.* 1993, Tsao *et al.* 1995) and in ischemic myocardium (Reiss *et al.* 1994, Buerke *et al.* 1995, Kluge *et al.* 1995, Moromisato *et al.* 1996). While most of these studies demonstrate hypoxia-induced alterations of the IGF system in organ systems, it is critical to understand how individual components within these systems and more particularly within the vasculature are affected by hypoxia in order to delineate how they might then interact *in vivo*.

Our study showed that hypoxia modulated IGF and IGFBP gene expression in EC and that, for IGF-I and some components of the IGFBP system, this modulation was vascular bed dependent. The fact that IGFBPs were affected more than the IGFs during exposure to hypoxia suggests that any proliferative/regenerative EC response is more dependent on IGFBPs. More importantly, given that IGF-I is a progression factor exerting its control at stage G1 of the cell cycle (O'Keefe & Pledger 1983, Pardee 1989), hypoxia-induced modulation of the IGFBPs which alter IGF bioavailability, might influence EC proliferation and regeneration after injury. Although we did not demonstrate a direct link between the modulation of the IGF system and EC hypoxia tolerance, our findings suggest that IGF/IGFBPs are involved in the maintenance of vascular endothelium integrity and may contribute to endothelium regeneration within the pulmonary and systemic circulations after cellular injury. IGF and IGFBPs may also originate from subendothelial components such as smooth muscle cells and fibroblasts. Therefore, the maintenance of endothelial integrity during hypoxia may be mediated by EC IGFBPs or by IGF/IGFBPs from other components of the vasculature.

Acknowledgements

M T was supported by a Research Fellowship bursary from the Medical Research Council of Canada and by the Pooled Research Trust Fund of the Lawson Research Institute. H W F was supported by a Career Investigator Award from the American Lung Association and a grant from the National Institutes of Health (HL-45537). V K M H and D J H were supported by a Group grant in Fetal and Neonatal Health and Development from the Medical Research Council of Canada. This work was in

part supported by the grant from the Ontario Thoracic Society. We wish to thank Michelle Gregory for her expert technical assistance.

References

- Asano H, Richardson BS & Han VK 1995 IGF growth factors and their binding proteins in the near term ovine fetus in response to sustained hypoxemia. *Journal of the Society for Gynecological Investigation* (Abstract) **2** 413.
- Bar RS & Boes M 1984 Distinct receptors for IGF-I, IGF-II, and insulin are present on bovine capillary endothelial cells and large vessel endothelial cells. *Biochemical and Biophysical Research Communications* **124** 203–209.
- Bar RS, Dake BL & Stueck S 1987a Stimulation of proteoglycans by IGF-I and -II in microvessel and large vessel endothelial cells. *American Journal of Physiology* **253** E21–E27.
- Bar RS, Harrison LC, Baxter RC, Boes M, Dake BL, Booth B & Cox A 1987b Production of IGF-binding proteins by vascular endothelial cells. *Biochemical and Biophysical Research Communications* **148** 734–739.
- Bar RS, Boes M, Dake BL, Booth BA, Henley SA & Sandra A 1988a Insulin, insulin-like growth factors, and vascular endothelium. *American Journal of Medicine* **85** 59–70.
- Bar RS, Siddle K, Dolash S, Boes M & Dake B 1988b Actions of insulin and insulin-like growth factors-I and -II in cultured microvessel endothelial cells from bovine adipose tissue. *Metabolism* **37** 714–720.
- Bar RS, Booth BA, Boes M & Dake BL 1989 Insulin-like growth factor-binding proteins from vascular endothelial cells: purification, characterization, and intrinsic biological activities. *Endocrinology* **125** 1910–1920.
- Beilharz EJ, Klempt ND, Klempt M, Sirimanne E, Dragunow M & Gluckman PD 1993 Differential expression of insulin-like growth factor binding proteins (IGFBP)-4 and -5 mRNA in the rat brain after transient hypoxic-ischemic injury. *Brain Research and Molecular Brain Research* **18** 209–215.
- Boes M, Dake BL & Bar RS 1991 Interactions of cultured endothelial cells with TGF-beta, bFGF, PDGF and IGF-I. *Life Sciences* **48** 811–821.
- Boes M, Booth BA, Sandra A, Dake BL, Bergold A & Bar RS 1992 Insulin-like growth factor binding protein (IGFBP)-4 accounts for the connective tissue distribution of endothelial cell IGFBPs perfused through the isolated heart. *Endocrinology* **131** 327–330.
- Boes M, Booth BA, Dake BL, Moser DR & Bar RS 1996 Insulin-like growth factor binding protein production by bovine and human vascular smooth muscle cells: production of insulin-like growth factor binding protein-6 by human smooth muscle. *Endocrinology* **137** 5357–5363.
- Bradshaw SL & Han VK 1993 Hormonal regulation of astroglial insulin-like growth factor (IGF)-binding protein gene expression by IGFs and insulin. *Endocrinology* **133** 1767–1777.
- Brunk CF, Jones KC & James TW 1979 Assay for nanogram quantities of DNA in cellular homogenates. *Analytical Biochemistry* **92** 497–500.
- Buerke M, Murohara T, Skurk C, Nuss C, Tomaselli K & Lefer AM 1995 Cardioprotective effect of insulin-like growth factor-I in myocardial ischemia followed by reperfusion. *Proceedings of the National Academy of Sciences of the USA* **92** 8031–8035.
- Cercek B, Fishbein MC, Forrester JS, Helfant RH & Fagin JA 1990 Induction of insulin-like growth factor-I messenger RNA in rat aorta after balloon denudation. *Circulation Research* **66** 1755–1760.
- Cheng HL, Randolph A, Yee D, Delafontaine P, Tennekoon G & Feldman EL 1996 Characterization of insulin-like growth factor-I and its receptor and binding proteins in transected nerves and cultured Schwann cells. *Journal of Neurochemistry* **66** 525–536.

- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Clemmons DR 1991 Insulin-like growth factor binding proteins: roles in regulating IGF physiology. *Journal of Developmental Physiology* **15** 105–110.
- Cohick WS & Clemmons DR 1993 The insulin-like growth factors. *Annual Review of Physiology* **55** 131–153.
- Delafontaine P, Bernstein KE & Alexander RW 1991 Insulin-like growth factor-I gene expression in vascular cells. *Hypertension* **17** 693–699.
- Delafontaine P, Ku L, Anwar A & Hayzer DJ 1996 Insulin-like growth factor-I binding protein-3 synthesis by aortic endothelial cells is a function of cell density. *Biochemical and Biophysical Research Communications* **222** 478–482.
- Dempsey EC, Badesch DB, Dobyns EL & Stenmark KR 1994 Enhanced growth capacity of neonatal pulmonary artery smooth muscle cells *in vitro*: dependence on cell size, time from birth, insulin-like growth factor-I, and auto-activation of protein kinase C. *Journal of Cell Physiology* **160** 469–481.
- Donnelly MJ & Holly JM 1996 The role of IGFBP-3 in the regulation of IGFBP-4 proteolysis. *Journal of Endocrinology* **149** 1–7.
- Drop SLS, Brinkman A, Kortleve DJ, Groffen CAH, Schuller A & Zwarthoff EC 1991 The evolution of the insulin-like growth factor binding protein family. In *Modern Concepts of Insulin-like Growth Factors*, pp 311–328. Ed EM Spencer. New York: Elsevier.
- Duan C, Hawes SB, Prevette T & Clemmons DR 1996 Insulin-like growth factor-I (IGF-I) regulates IGF-binding protein-5 synthesis through transcriptional activation of the gene in aortic smooth muscle cells. *Journal of Biological Chemistry* **271** 4280–4288.
- Farber HW & Rounds S 1990 Effect of long-term hypoxia on cultured aortic and pulmonary arterial endothelial cells. *Experimental Cell Research* **191** 27–36.
- Fowlkes JL, Serra DM, Rosenberg CK & Thraill KM 1995 Insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) functions as an IGF-reversible inhibitor of IGFBP-4 proteolysis. *Journal of Biological Chemistry* **270** 27481–27488.
- Gajdusek CM, Luo Z & Mayberg MR 1993 Sequestration and secretion of insulin-like growth factor-I by bovine aortic endothelial cells. *Journal of Cell Physiology* **154** 192–198.
- Gockerman A & Clemmons DR 1995 Porcine aortic smooth muscle cells secrete a serine protease for insulin-like growth factor binding protein-2. *Circulation Research* **76** 514–521.
- Grant MB, Mames RN, Fitzgerald C, Ellis EA, Caballero S, Chegini N & Guy J 1993 Insulin-like growth factor-I as an angiogenic agent. *In vivo* and *in vitro* studies. *Annals of the New York Academy of Sciences* **692** 230–242.
- Graven KK, Zimmerman LH, Dickson EW, Weinhouse GL & Farber HW 1993 Endothelial cell hypoxia associated proteins are cell and stress specific. *Journal of Cell Physiology* **157** 544–554.
- Han VK, Lund PK, Lee DC & D'Ercole AJ 1988 Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization, and tissue distribution. *Journal of Clinical Endocrinology and Metabolism* **66** 422–429.
- Hansson HA, Jennische E & Skottner A 1987 Regenerating endothelial cells express insulin-like growth factor-I immunoreactivity after arterial injury. *Cell and Tissue Research* **250** 499–505.
- Hansson HA, Brandsten C, Lossing C & Petruson K 1989 Transient expression of insulin-like growth factor-I immunoreactivity by vascular cells during angiogenesis. *Experimental Molecular Pathology* **50** 125–138.
- Harlow E & Lane D 1988 Immunoprecipitation. In *Antibodies - A Laboratory Manual*, First edn, pp 421–470. Eds E Harlow & D Lane. New York: Cold Spring Harbor Laboratory.
- Hayzer D, Anwar A, Bernstein K & Delafontaine P 1995 Insulin-like growth factor binding protein-3 synthesis by bovine aortic endothelial cells is induced by cell quiescence and contact inhibition. *FASEB Journal* **9** A872.
- Hill DJ 1990 Relative abundance and molecular size of immunoreactive insulin-like growth factors-I and -II in human fetal tissues. *Early Human Development* **21** 49–58.
- Hossenlopp P, Seurin D, Segovia QB, Hardouin S & Binoux M 1986 Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding studies. *Analytical Biochemistry* **154** 138–143.
- Johnston BM, Mallard EC, Williams CE & Gluckman PD 1996 Insulin-like growth factor-I is a potent neuronal rescue agent after hypoxic-ischemic injury in fetal lambs. *Journal of Clinical Investigation* **97** 300–308.
- Jones JI & Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* **16** 3–34.
- Kamrath A, Pirola CJ, Wang HM, Sharifi B, Mohan S, Forrester JS & Fagin JA 1994 Expression and insulin-like growth factor-dependent proteolysis of insulin-like growth factor-binding protein-4 are regulated by cell confluence in vascular smooth muscle cells. *Circulation Research* **74** 576–585.
- Kern PA, Svoboda ME, Eckel RH & Van WJ 1989 Insulin-like growth factor action and production in adipocytes and endothelial cells from human adipose tissue. *Diabetes* **38** 710–717.
- Khorsandi MJ, Fagin JA, Giannella ND, Forrester JS & Cercek B 1992 Regulation of insulin-like growth factor-I and its receptor in rat aorta after balloon denudation. Evidence for local bioactivity. *Journal of Clinical Investigation* **90** 1926–1931.
- King GL, Goodman AD, Buzney S, Moses A & Kahn CR 1985 Receptors and growth-promoting effects of insulin and insulin-like growth factors on cells from bovine retinal capillaries and aorta. *Journal of Clinical Investigation* **75** 1028–1036.
- Klempt M, Klempt ND & Gluckman PD 1993 Hypoxia and hypoxia/ischemia affect the expression of insulin-like growth factor binding protein-2 in the developing rat brain. *Brain Research and Molecular Brain Research* **17** 347–350.
- Klempt ND, Klempt M, Gunn AJ, Singh K & Gluckman PD 1992 Expression of insulin-like growth factor-binding protein-2 (IGF-BP 2) following transient hypoxia-ischemia in the infant rat brain. *Brain Research and Molecular Brain Research* **15** 55–61.
- Kluge A, Zimmermann R, Munkel B, Verdouw PD, Schaper J & Schaper W 1995 Insulin-like growth factor-II is an experimental stress inducible gene in a porcine model of brief coronary occlusions. *Cardiovascular Research* **29** 708–716.
- Lee WH & Bondy C 1993 Insulin-like growth factors and cerebral ischemia. *Annals of the New York Academy of Sciences* **679** 418–422.
- Lee WH, Javedan S & Bondy CA 1992 Coordinate expression of insulin-like growth factor system components by neurons and neuroglia during retinal and cerebellar development. *Journal of Neuroscience* **12** 4737–4744.
- Lee WH, Wang GM, Seaman LB & Vannucci SJ 1996 Coordinate IGF-I and IGFBP-5 gene expression in perinatal rat brain after hypoxia-ischemia. *Journal of Cerebral Blood Flow and Metabolism* **16** 227–236.
- Levinovitz A, Jennische E, Oldfors A, Edwall D & Norstedt G 1992 Activation of insulin-like growth factor-II expression during skeletal muscle regeneration in the rat: correlation with myotube formation. *Molecular Endocrinology* **6** 1227–1234.
- McLellan KC, Hooper SB, Bocking AD, Delhanty PJ, Phillips ID, Hill DJ & Han VK 1992 Prolonged hypoxia induced by the reduction of maternal uterine blood flow alters insulin-like growth factor-binding protein-1 (IGFBP-1) and IGFBP-2 gene expression in the ovine fetus. *Endocrinology* **131** 1619–1628.
- Moromisato DY, Moromisato MY, Zancanato S & Roberts CJ 1996 Effect of hypoxia on lung, heart, and liver insulin-like growth factor-I gene and receptor expression in the newborn rat. *Critical Care Medicine* **24** 919–924.

- Moser DR, Lowe WJ, Dake BL, Booth BA, Boes M, Clemmons DR & Bar RS 1992 Endothelial cells express insulin-like growth factor-binding proteins-2 to -6. *Molecular Endocrinology* **6** 1805–1814.
- Nakao HJ, Ito H, Kanayasu T, Morita I & Moruta S 1992 Stimulatory effects of insulin and insulin-like growth factor-I on migration and tube formation by vascular endothelial cells. *Atherosclerosis* **92** 141–149.
- Nicosia RF, Nicosia SV & Smith M 1994 Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-I promote rat aortic angiogenesis *in vitro*. *American Journal of Pathology* **145** 1023–1029.
- Noguchi S, Kashiwara Y, Ikegami Y, Morimoto K, Miyamoto M & Nakao K 1993 Insulin-like growth factor-I ameliorates transient ischemia-induced acute renal failure in rats. *Journal of Pharmacology and Experimental Therapeutics* **267** 919–926.
- Noll K, Wegmann BR, Havemann K & Jaques G 1996 Insulin-like growth factors stimulate the release of insulin-like growth factor-binding protein-3 (IGFBP-3) and degradation of IGFBP-4 in non-small cell lung cancer cell lines. *Journal of Clinical Endocrinology and Metabolism* **81** 2653–2662.
- Ogawa S, Shreeniwas R, Brett J, Clauss M, Furie M & Stern DM 1990 The effect of hypoxia on capillary endothelial cell function: modulation of barrier and coagulant function. *British Journal of Haematology* **75** 517–524.
- O'Keefe EJ & Pledger WJ 1983 A model of cell cycle control: sequential events regulated by growth factors. *Molecular and Cellular Endocrinology* **31** 167–186.
- Owens JA, Kind KL, Carbone F, Robinson JS & Owens PC 1994 Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *Journal of Endocrinology* **140** 5–13.
- Pardee AB 1989 G1 events and regulation of cell proliferation. *Science* **246** 603–608.
- Parker A, Gockerman A, Busby WH & Clemmons DR 1995 Properties of an insulin-like growth factor-binding protein-4 protease that is secreted by smooth muscle cells. *Endocrinology* **136** 2470–2476.
- Parker A, Clarke JB, Busby WJ & Clemmons DR 1996 Identification of the extracellular matrix binding sites for insulin-like growth factor-binding protein-5. *Journal of Biological Chemistry* **271** 13523–13529.
- Perkett EA, Badesch DB, Roessler MK, Stenmark KR & Meyrick B 1992 Insulin-like growth factor-I and pulmonary hypertension induced by continuous air embolization in sheep. *American Journal of Respiratory Cell and Molecular Biology* **6** 82–87.
- Phillips ID, Arany E, Strain AJ, Han VK & Hill DJ 1993 Rapid clearance of insulin-like growth factor (IGF)-binding protein species from blood and an associated fall in circulating IGF-I following partial hepatectomy in the rat. *Journal of Endocrinology* **137** 271–280.
- Price WA, Rong L, Stiles AD & D'Ercole AJ 1992 Changes in IGF-I and -II, IGF binding protein, and IGF receptor transcript abundance after uterine artery ligation. *Pediatric Research* **32** 291–295.
- Rechler MM & Nissley SP 1990 Insulin-like growth factors. In *Peptide Growth Factors and their Receptors*, pp 263–367. Eds MB Sporn & AB Roberts. Berlin: Springer-Verlag.
- Reiss K, Kajstura J, Zhang X, Li P, Szoke E, Olivetti G & Anversa P 1994 Acute myocardial infarction leads to upregulation of the IGF-1 autocrine system, DNA replication, and nuclear mitotic division in the remaining viable cardiac myocytes. *Experimental Cell Research* **213** 463–472.
- Sandberg NA, von Holst H, Holmin S, Sara VR, Bellander BM & Schalling M 1996 Increase of insulin-like growth factor (IGF)-I, IGF binding protein-2 and -4 mRNAs following cerebral contusion. *Brain Research and Molecular Brain Research* **38** 285–293.
- Shimasaki S & Ling N 1991 Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5, and -6). *Progress in Growth Factor Research* **3** 243–266.
- Shreeniwas R, Ogawa S, Cozzolino F, Torcia G, Braunstein N, Butura C, Brett J, Lieberman HB, Furie MB, Joseph SJ *et al.* 1991 Macrovascular and microvascular endothelium during long-term hypoxia: alterations in cell growth, monolayer permeability, and cell surface coagulant properties. *Journal of Cell Physiology* **146** 8–17.
- Stephenson DT, Rash K & Clemens JA 1995 Increase in insulin-like growth factor-II receptor within ischemic neurons following focal cerebral infarction. *Journal of Cerebral Blood Flow and Metabolism* **15** 1022–1031.
- Taylor WR, Nerem RM & Alexander RW 1993 Polarized secretion of IGF-I and IGF-I binding protein activity by cultured aortic endothelial cells. *Journal of Cell Physiology* **154** 139–142.
- Townsend SF & Stenmark KR 1995 Developmental and hypoxic regulation of pulmonary artery adventitial fibroblast insulin-like growth factor binding proteins (IGFBPs). *Pediatric Research* **37** 71A.
- Tsao T, Wang J, Fervenza FC, Vu TH, Jin IH, Hoffman AR & Rabkin R 1995 Renal growth hormone–insulin-like growth factor-I system in acute renal failure. *Kidney International* **47** 1658–1668.
- Tucci M, Hammerman SI, Furfaro S, Saukonnen JJ, Conca TJ & Farber HW 1997 Distinct effect of hypoxia on endothelial cell proliferation and cycling. *American Journal of Physiology* **272** C1700–C1708.
- Tucci M, McDonald RJ, Aaronson RA, Graven KK & Farber HW 1996 Specificity and uniqueness of endothelial cell stress responses. *American Journal of Physiology* **271** L341–L348.
- Tucci M, Nygard K, Farber HW & Han VKM 1996 Modulation of IGF and IGF binding protein expression by hypoxia in cultured vascular endothelial cells (EC). *Pediatric Research* (Abstract 2333) **39** 392A.
- Yang YW, Pioli P, Fiorelli G, Brandi ML & Rechler MM 1993 Cyclic adenosine monophosphate stimulates insulin-like growth factor binding protein-4 and its messenger ribonucleic acid in a clonal endothelial cell line. *Endocrinology* **133** 343–351.
- Zimmerman LH, Levine RA & Farber HW 1991 Hypoxia induces a specific set of stress proteins in cultured endothelial cells. *Journal of Clinical Investigation* **87** 908–914.

Received 30 April 1997

Revised manuscript received 22 September 1997

Accepted 22 October 1997