Human umbilical vein endothelial cells express multiple prolactin isoforms

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Abstract

Members of the prolactin (PRL) hormonal family have direct effects on endothelial cell proliferation, migration and tube formation. Moreover, isoforms of PRL may function as autocrine regulators of endothelial cells. Bovine brain capillary endothelial cells (BBCEC) express the PRL gene, while anti-PRL antibodies inhibit BBCEC proliferation. Here, we show the expression of the PRL gene into various PRL isoforms in endothelial cells from the human umbilical vein. Reverse transcription-polymerase chain reaction of total RNA from human umbilical vein endothelial cells (HUVEC) detected the full-length PRL mRNA as well as a 100 bp smaller PRL transcript similar to the one previously reported in BBCEC. HUVEC were positive to PRL immunocytochemistry. In addition, various PRL immunoreactive proteins were detected in HUVEC extracts and HUVEC conditioned media by metabolic labelling immunoprecipitation analysis. These PRL immunorelated proteins had apparent molecular masses of 60, 23, 21, 16 and 14 kDa. In contrast to previous findings in BBCEC, HUVEC conditioned media contained very little PRL bioactivity as determined by the selective bioassay of Nb2 cell proliferation. Moreover, some polyclonal or monoclonal antibodies directed against PRL stimulated HUVEC proliferation, in contrast to the inhibitory effect seen in BBCEC. The present findings extend the previous observations about the expression of PRL gene in endothelial cells from bovine brain capillaries to human cells of the umbilical vein, implicating that endothelium from different types of vessels and species share the expression of PRL gene but may differ in the putative autocrine role of the PRL isoforms expressed.

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Introduction

Angiogenesis, the formation of new blood vessels from pre-existing microvasculature, underlies different physiological processes, which include reproductive events, development and wound repair (Folkman 1995). In the adult, the endothelial cells rarely divide and the vascular network undergoes a very slow remodelling process. However, the lack of a proper spatial and temporal regulation may lead to excessive or deficient angiogenesis, and thus contribute to pathological processes such as cancer and chronic inflammation on the one hand, and impaired wound healing and tissue repair on the other (Folkman 1995). In this regard, knowledge of the molecular mechanisms that regulate angiogenesis can contribute to the development of therapies for the treatment of diseases characterised by abnormal angiogenesis.

Members of the growth hormone (GH)/prolactin (PRL) family have effects on angiogenesis. GH stimulates the proliferation of endothelial cells *in vitro* (Rymaszewky et al. 1991) and GH, PRL and placental lactogen induce angiogenesis of quiescent chick embryo capillaries *in vivo* (Gould et al. 1995, Struman et al. 1999). This family of hormones also exerts anti-angiogenic effects. Molecular fragments of PRL, GH and placental lactogen are inhibitory to angiogenesis. Amino–terminal 16 kDa PRL inhibits the proliferation, tube formation and urokinase activity of endothelial cells in culture, as well as the *in vivo* growth of microvessels in the chick embryo chorioallantoic membrane and in the rat cornea (Clapp et al. 1993, Lee et al. 1998, Dueñas et al. 1999). Likewise, 16 kDa GH and placental lactogen have inhibitory actions on angiogenesis both *in vitro* and *in vivo* (Struman et al. 1999). Furthermore, proliferin and proliferin–related protein, considered members of the PRL family on the basis of primary sequence homology, stimulate and inhibit angiogenesis respectively (Jackson et al. 1994). According to the above data, it has been hypothesised that members of the GH/PRL family through their opposite actions may act as potential modulators of angiogenesis (Jackson et al. 1994, Struman et al. 1999).

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Endothelial cells are known to produce and respond to their own angiogenic and anti-antiangiogenic factors, and thus exert an autocrine control of neovascularisation (McPherson *et al.* 1981, Schweigerer *et al.* 1987, Koyama *et al.* 1994, Nomura *et al.* 1995). Recent work has suggested that PRLs may contribute to this autocrine regulation. Bovine brain capillary endothelial cells (BB-CEC) were found to express the full-length PRL messenger RNA, as well as a novel PRL transcript, lacking the third exon of the gene. Moreover, BBCEC synthesise and secrete PRL immunorelated proteins, and antibodies against PRL inhibit the proliferation of these cells in culture (Clapp *et al.* 1998). Here, we have analysed the possibility that human endothelial cells obtained from the umbilical vein also express the PRL gene and produce and secrete PRL isoforms. Our results extend previous findings with bovine endothelial cells from capillaries, and support the hypothesis that the expression of PRL is common to endothelial cells from different species and types of blood vessels and contributes to the autocrine regulation of angiogenesis.

Materials and Methods

PRL and antibodies

Biological grade human 23 kDa PRL was donated by the National Hormone and Pituitary Program (NHPP, Torrance, CA, USA). ^{125}I-Labelled human (h) PRL was purchased from DuPont NEN (Boston, MA, USA). Human PRL antiserum was obtained from Dr J Parlow (NHPP) (Harbor-UCLA Medical Center, Torrance, CA, USA) or generated (local) in our laboratory by the immunisation of rabbits with NHPP human 23 kDa PRL. Specificity of the locally raised antiserum was assessed by RIA. At 1:100 000 dilution, the antiserum reacted with increasing concentrations of human PRL, whereas cross-reactivity with human follicle-stimulating hormone or human luteinising hormone was less than 0·1%. Human PRL polyclonal antibodies and a monoclonal anti-human PRL antibody (MIP0202 Mab 14120) were purchased from Genzyme (Genzyme Diagnostics, San Carlos, CA, USA). The human PRL monoclonal antibody (INN-hPRL-219) (Staindl *et al.* 1987) was kindly provided by P Berger (Institute for Biomedical Aging Research, Innsbruck, Austria). Polyclonal antibody against Von Willebrand antigen was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY, USA).

Purification of immunoglobulin G (IgG)

Antibodies from antisera, non-immune serum or ascites fluid were purified on a protein-A-Sepharose column (Sigma, St Louis, MO, USA) as described (Baglia *et al.* 1991). Briefly, samples were added to columns equilibrated and washed with phosphate-buffered saline (pH 8). Antibodies were eluted out with 0·1 M sodium acetate, pH 3, into tubes with 1·0 M Tris–hydrochloride (pH 8) to neutralise the acid. The concentration of antibodies was determined by the Bradford method (Bio-Rad, Richmond, CA, USA).

Human umbilical vein endothelial cells (HUVEC)

HUVEC were isolated from fresh human umbilical cords following the method previously described (Olsen 1994). Briefly, the umbilical vein was cannulated and washed with Hank's salt solution (Gibco BRL, Gaithersburg, MD, USA) and filled with 0·05% trypsin-EDTA-Hank's salt solution. After incubation for 15 min at 37 °C, the cell suspension was centrifuged and the cell pellet resuspended in culture medium (F12K medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml porcine heparin (Gibco BRL), 50 U/ml penicillin/streptomycin (Gibco BRL), and 25 µg/ml Endothelial Cell Growth Supplement (Sigma)). A serum-free defined medium (Human Endothelial-Serum Free Medium System; Gibco BRL) was supplemented with epidermal growth factor (EGF, 10 ng/ml; Gibco BRL) and antibiotics. Cells were cultured on 100-mm plastic dishes (Corning Costar Corp., Cambridge, MA, USA) previously coated with human plasma fibronectin (10 µg/ml; Gibco BRL). Media were changed every other day and the cells were used between passages 3 and 7. Endothelial cells were characterised by their non-overlapping cobblestone morphology, proliferation in response to vascular endothelial growth factor and positive immunofluorescence for Factor VIII-related antigen (Gerritsen *et al.* 1988).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from non-confluent HUVEC (1·5–2 × 10^6 cells/100 mm well) was isolated and RT-PCR performed essentially as described by Clapp *et al.* (1998), using 40 cycles and an annealing temperature of 55 °C. Two primers complementary to human PRL cDNA were synthesised: upstream primer from exon 2 (5′-GCAGTT GTTGTTGTGGATGATT-3′) and downstream primer from exon 5 (5′-GATGCCAGGTGACCCTTCGAGA-3′). RT-PCR products were identified by Southern blot using an homologous probe (human cDNA, American Type Culture Collection, Manassas, VA, USA) and a previously reported procedure (Clapp *et al.* 1998). PCR transcripts were sequenced by the dideoxy method (Sanger *et al.* 1977) with the AmpliCycle kit (Perkin Elmer, Branchburg, NJ, USA) and [^{32}P]dATP (DuPont NEN), according to the manufacturer's instructions. Aliquots of the sequencing reactions were run on 8% acrylamide gels, vacuum dried, and autoradiographed for at least 18 h.

Metabolic labelling and immunoprecipitation

Non-confluent HUVEC were metabolically labelled for 7 h with ^{35}S-methionine and ^{35}S-cysteine (100 µCi/ml; DuPont NEN) in 0·2 mg/ml BSA, serum-free, low-glucose Dulbecco's modified Eagle's medium without methionine and cysteine (DMEM − cys, − met; Gibco BRL). Cells were lysed in 1% Nonidet P-40, 0·1% SDS, 50 mM Tris, 150 mM NaCl, 1 µg/ml aprotinin and 100 µg/ml phenylmethylsulphonyl fluoride (Sigma). HUVEC lysates and concentrated (3 ×; Centricon 10, Amicon, Beverly, MA, USA) conditioned media were incubated overnight with anti-PRL antisera (1:500), anti-PRL monoclonal antibodies (1 µg/ml), normal rabbit serum (NRS) (1:500), or mouse preimmune antibodies (2 µg/ml) followed by a 1-h incubation with protein-A Sepharose beads (25 µl/ml, Sigma) as described elsewhere (Sambrook *et al.* 1989). NRS was used for preclearing. Proteins were eluted by boiling in electrophoresis sample buffer (Laemmli 1970) and resolved in an SDS slab gel (15% acrylamide/bisacrylamide). Gels were fixed, soaked in Enhance (Dupont NEN), dried and autoradiographed for 1–2 weeks at −70 °C. Relative molecular masses of labelled proteins were estimated by their electrophoretic mobility with respect to standard molecular weight markers (Bio Rad, Hercules, CA, USA).

Immunocytochemistry

Endothelial cells grown in the absence of serum on glass cover slips previously coated with fibronectin were washed with PBS and fixed in 4% formaldehyde in PBS for 10 min at room temperature (RT). Subsequently, cells were incubated with 10% normal goat serum (Sigma) in 0·3% Triton-PBS for 2 h, followed by an overnight incubation at RT with anti-PRL antiserum (NHPP) or with the monoclonal antibody (INN-hPRL-219). Preparations were then incubated with a 1:100 dilution of goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate (FITC, Jackson Immunoresearch Laboratories Inc., Pennsylvania, PA, USA) or with goat anti-mouse antibodies coupled to tetramethyl rhodamine isothiocyanate (TRITC, Jackson Immunoresearch Laboratories) for 1 h at RT. For co-localisation experiments, cells were double labelled for PRL and the Factor VIII-related antigen, an endothelial cell marker. For this purpose, overnight incubation with (1 µg/ml) anti-PRL monoclonal antibody (INN-hPRL-219) was followed by incubation with secondary antibodies coupled to TRITC. Cells were thoroughly washed, incubated overnight with 1:50 anti-Von Willebrand antigen antibodies and then with secondary antibodies coupled to FITC. Omission or substitution of primary antibodies with NRS or preimmune mouse antibodies confirmed the specificity of the reaction. Cells were coverslipped using an anti-fade kit (Molecular Probes Inc., Eugene, OR, USA) and examined under an epifluorescence microscope (Nikon Optiphot, Nikon Inc. Melville, NY, USA) equipped with filters for TRITC and FITC fluorescence. Selected areas of double labelled studies were also viewed with a 60 × oil immersion objective on a Nikon microscope with an attached confocal system (BioRad MRC600, México City, México).

Nb2 cell bioassay

Nb2 cells, a PRL-responsive cell line derived from a rat T cell lymphoma (Tanaka *et al.* 1980) were kindly provided by P Gout (British Columbia Cancer Agency, Vancouver, BC, Canada). Nb2 cells were grown in high-glucose DMEM supplemented with 10% horse serum, 10% FBS, 50 U/ml penicillin/streptomycin and 10^{-4} M β-mercaptoethanol as described (Tanaka *et al.* 1980). To analyse the bioactivity of PRL-like proteins in HUVEC, Nb2 cells ($2·5 \times 10^4$ cells/15 mm well) were co-cultured with HUVEC at various cellular densities (0·625, 1·25 or $2·5 \times 10^5$ cells/15 mm well) following the procedure previously described for bovine capillary endothelial cells (Clapp *et al.* 1998). After 60 h, [^{3}H]thymidine was added to co-cultures for 12 h. Because Nb2 cells grow in suspension, [^{3}H]thymidine incorporation into Nb2 cells was assayed in co-culture supernates after collecting the cells by centrifugation. Values were corrected by subtracting the c.p.m. present in the supernates of HUVEC cultured in the absence of Nb2 cells. [^{3}H]Thymidine incorporation was assayed essentially as described by Ferrara *et al.* (1991).

To further analyse the presence of bioactive PRL in the culture medium of HUVEC, Nb2 cells (15×10^4 cells/ml) were cultured for 60 h with serial dilutions of concentrated HUVEC conditioned or non-conditioned media. HUVEC conditioned medium was obtained by incubating non-confluent HUVEC ($1·5–2·5 \times 10^6$ cells/100 mm) for 7 h in serum-free F12K medium supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (Scious, Mountain View, CA, USA) and 100 µg/ml heparin. Conditioned media were clarified by centrifugation (10 min at 1200 **g**) and concentrated (500 ×; Centricon 10). Cultures were carried out in the absence or presence of a 1:500 dilution of the locally produced PRL antiserum. Nb2 cell proliferation and viability were measured by reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; Sigma) as previously described (Green *et al.* 1984). Briefly, cells were incubated with MTT (500 mg/ml) at 37 °C for 4 h, formazan precipitates were solubilised with 0·4 M HCl–10% SDS for 30 min at RT and their absorbance evaluated at 590 nm.

Endothelial cell growth

To assess cell proliferation, HUVEC ($2·5 \times 10^4$ cells/ 15 mm wells) were cultured on fibronectin-coated wells
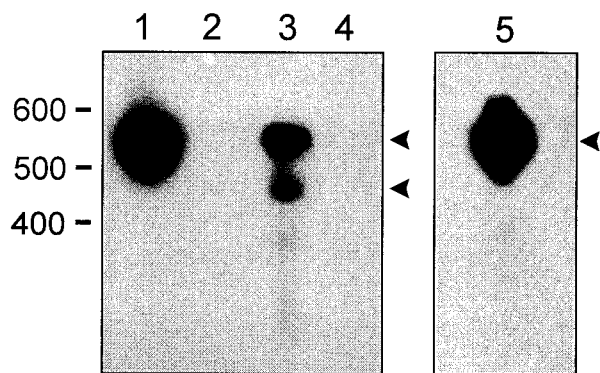
Figure 1 Detection of PRL mRNA in HUVEC. Southern blot analysis of PCR from human PRL cDNA (lane 1) or from reverse-transcribed total RNA from HUVEC (lane 2–4) or placenta (lane 5). Total RNA was processed in the absence (lane 2) or presence (lanes 3, 4, 5) of reverse transcriptase, amplified with specific primers and hybridised with the human PRL cDNA probe. Lane 4, negative control without RNA. PRL transcripts amplified are indicated (black arrowheads).

Figure 2 Autoradiographs of metabolically labelled HUVEC lysate (lanes 2 and 3) and culture medium (lanes 5 and 6) immuno-precipitated with anti-PRL antiserum (NHPP) (lanes 2, 5) or normal rabbit serum (NRS) (lanes 3, 6). Standard PRL added to sample in lane 5 and revealed by Coomassie Blue stain (lane 4) and ^{125}I-labelled PRL (lane 1) mark the 23 kDa molecular mass position as indicated (open arrowheads). Immuno-precipitated PRL-like proteins and their relative molecular masses are indicated (black arrows). Numbers at left indicate relative mobility of molecular weight markers.

with Human Endothelial–Serum Free Medium System (Gibco) supplemented with EGF (10 ng/ml) in the absence or presence of bFGF (20 ng/ml) as indicated (Battista 1994). Incubations were for 60 h with antibodies added twice, once at the time of seeding the cells and again 48 h later. At this time, cells were pulsed with 0·6 μCi [^{3}H]thymidine/15 mm well for 12 h and [^{3}H]thymidine incorporation into DNA was measured as an index of cell proliferation (Ferrara *et al.* 1991).

Statistical analysis

The data were analysed for statistical significance by Student's *t*-test. Results are expressed as the means ± standard error of the mean of triplicate determinations. Results were replicated in three or more experiments.

Results

PRL mRNA

Total RNA from HUVEC was reverse-transcribed and amplified by PCR using primers with annealing sites within exons 2 and 5 of the human PRL cDNA. Southern blot analysis showed two amplification products, one around 560 bp and the other about 100 bp smaller (Fig. 1, lane 3). The bigger transcript was of a similar size to that amplified from PRL cDNA cloned from the human pituitary (Fig. 1, lane 1), and reversed-transcribed and amplified from human placenta (Fig. 1, lane 5). No positive signal was detected in the absence of reverse transcriptase (Fig. 1, lane 2) nor in the negative control without RNA (Fig. 1, lane 4). The 560 bp transcript
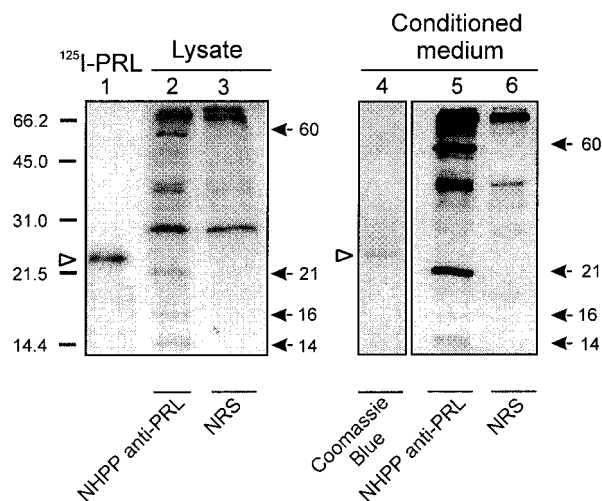
showed identical sequence homology with human pituitary PRL throughout an analysed region of 105 bp comprising codons encoding for amino acids 47 to 81 (not shown). No sequence information was obtained for the smaller transcript.

PRL immunorelated proteins in HUVEC

Metabolic labelling and immunoprecipitation of *de novo* synthesised proteins To assay for the translational products of PRL transcripts in HUVEC, protein synthesis was metabolically labelled with ^{35}S-cysteine and ^{35}S-methionine and the ^{35}S-labelled proteins were immuno-precipitated with anti-PRL antiserum (NHPP) (Fig. 2). PRL-like immunoreactive proteins with apparent molecular masses of 60, 21, 16 and 14 kDa were detected in lysates from HUVEC, and all of them appeared to be secreted by the endothelial cells since they were also detected in HUVEC conditioned media (Fig. 2, lanes 2 and 5). Specificity of antiserum reaction was indicated by the lack of detection of the PRL-like antigens when PRL antiserum was substituted by normal rabbit serum (NRS; Fig. 2, lanes 3 and 6). Native 23 kDa PRL added to HUVEC conditioned media (of the same sample shown in lane 5) migrated as a band distinct from that of the 21 kDa PRL-like antigen, as indicated by Coomassie blue staining (Fig. 2, lane 4). Thus, the different migration rate between the 21 kDa PRL-like protein and native 23 kDa PRL
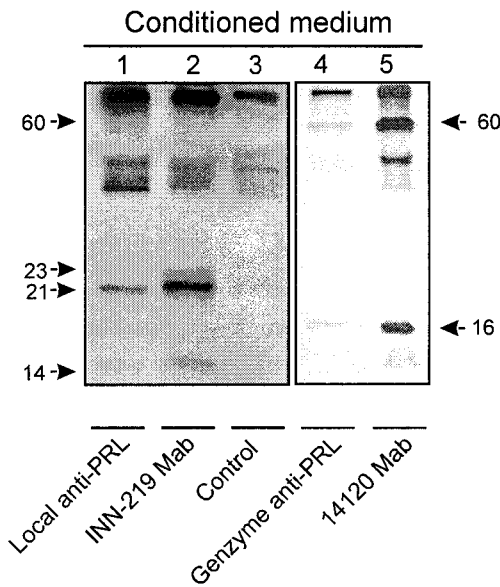
Figure 3 Autoradiographs of metabolically labelled HUVEC culture media immuno-precipitated with an anti-PRL antiserum produced in our laboratory (local, lane 1), anti-PRL polyclonal antibodies from Genzyme (lane 4) and anti-PRL monoclonal antibodies (Mab) INN-219 (lane 2) and 14120 (lane 5). Control is with pre-immune mouse IgG (lane 3). Immuno-precipitated PRL-like proteins and their relative molecular masses are indicated (black arrows).

appears to be due to the different size of the proteins and cannot be ascribed to an artefact in protein mobility due to interference with material from HUVEC samples.

The PRL–like antigens of 60 and 21 kDa appeared to be more abundant in the conditioned media than in cell lysates (Fig. 2). However discrepancies were noted in the relative abundance of these PRL antigens when other anti–PRL antibodies were used (Fig. 3). An anti–PRL antiserum produced in our laboratory (local; Fig. 3, lane 1) and an anti–PRL monoclonal antibody (INN–hPRL–219 Mab; Fig. 3, lane 2) reacted with the proteins of 21 and 14 kDa, but not with those of 60 and 16 kDa. Also, both types of antibodies (local and 219 Mab) reacted with a

23 kDa protein not detected with the NHPP anti–PRL antiserum (Fig. 3, lanes 1 and 2 vs Fig. 2, lane 5). Furthermore, an anti–PRL polyclonal antibody (Genzyme; Fig. 3, lane 4) and another anti–PRL monoclonal antibody (14120 Mab; Fig. 3, lane 5) reacted with the 60 and 16 kDa proteins but not with the 21 and 14 kDa PRL–like antigens. In no case were any of the labelled proteins precipitated by preimmune rabbit serum (Fig. 2, lane 6) or mouse preimmune IgG (Fig. 3, lane 3).

Immunocytochemical detection of PRL immuno-related proteins HUVEC cells were grown on cover-slips in the absence of serum and processed for double labelled immunofluorescence confocal laser scanning microscopy using polyclonal antibodies to Factor VIII-related antigen, an endothelial cell marker (Fig. 4A), and the anti–PRL 219 Mab (Fig. 4B). Both antibodies reacted with the same cells and labels were located inside the cytoplasm, as revealed by serial optical sectioning (not shown). Specificity of both reactions was confirmed by the lack of visible spillover between the two emissions used in the absence of both or either one of the primary antibodies. No staining followed the use of preimmune rabbit nor mouse antibodies (not shown). Moreover, the NHPP anti–PRL antiserum coupled to light immuno–fluorescence-labelled HUVEC (Fig. 4C) and no positive cells were found with NRS (not shown). HUVEC adopt various morphologies within the same culture; thus apparent differences in morphology are circumstantial and do not represent differences in cell type nor in antigen location. The distribution of PRL antigens revealed by the NHPP anti–PRL antiserum was similar to that found with the anti–PRL 219 Mab in that it stained the cytoplasm of nearly all (>95%) HUVEC.

Nb2 cell proliferation

To analyse the PRL nature of endothelial proteins, HU-VEC were seeded at various densities and co-cultured with PRL responsive Nb2 rat lymphoma cells. In no case did co-culture with HUVEC modify Nb2 cell
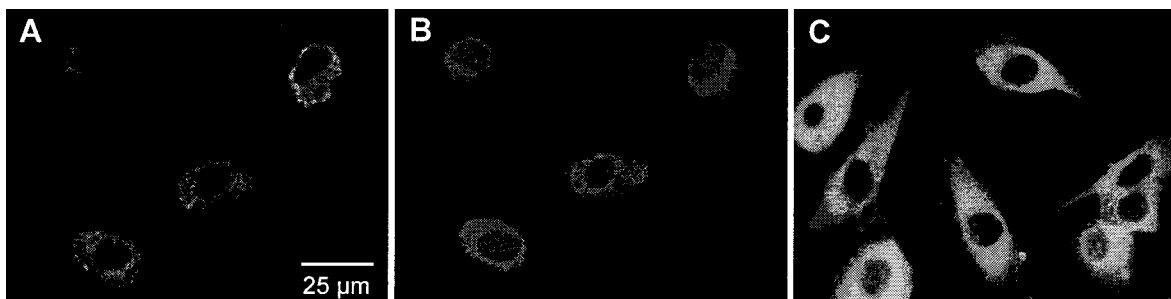

Figure 4 Immuno-fluorescence localisation of PRL in HUVEC. Confocal analysis of double immunostaining using an anti-Factor VIII-related antigen antiserum (A) and a monoclonal antibody against PRL (INN-hPRL-219) (B). Light immuno-fluorescence of another HUVEC culture using the anti-PRL antiserum from NHPP (C).
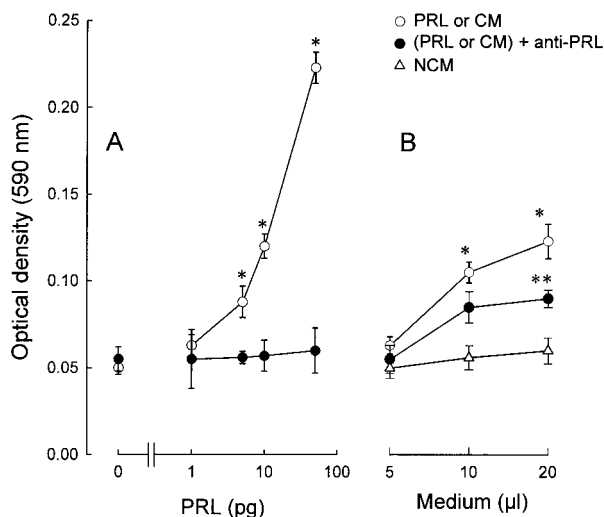
Figure 5 (A) Proliferation of Nb2 cells cultured in the absence or the presence of increasing concentrations of PRL alone or together with the anti-PRL antiserum raised in our laboratory. (B) Proliferation of Nb2 cells cultured in the presence of increasing amounts of concentrated (500 ×) HUVEC conditioned medium, alone (CM) or together with a 1:500 dilution of the anti-PRL antiserum raised in our laboratory. Concentrated (500 ×) non-conditioned medium (NCM) was included as a negative control. Reduction of MTT and measurement of reaction product absorbance at 595 nm determined proliferation. *$P<0.05$ vs cells without PRL or CM. **$P<0.05$ vs respective value of CM and NCM groups.

proliferation (not shown). In order to increase detection of PRL-like bioactivity in the medium of HUVEC, serum-free medium conditioned by HUVEC was concentrated 500 times and tested on Nb2 cells. HUVEC conditioned medium, but not non-conditioned medium, stimulated Nb2 cell proliferation in a dose-dependent manner (Fig. 5). The stimulatory effect of the conditioned medium was only partially blocked by a PRL antiserum able to completely neutralise the mitogenic action of the PRL standard on Nb2 cells (Fig. 5). The level of activity neutralised in HUVEC conditioned medium was equivalent to 1 pg/ml pituitary PRL, as estimated by the serial dose–response effect of the PRL standard (Fig. 5).

Effect of anti-PRL antibodies on endothelial cell proliferation

To investigate a possible autocrine effect of HUVEC PRL-like proteins on cell growth, we analysed the proliferation of HUVEC in the presence of PRL-directed antibodies in an attempt to neutralise the putative action of endogenous PRL. Purified IgG from two anti-PRL antisera failed to alter HUVEC proliferation (Fig. 6). Conversely, other polyclonal anti-PRL antibodies (Genzyme) and the 14120 anti-PRL Mab stimulated the proliferation of HUVEC (Fig. 6). Both stimulatory antibodies, but not the inactive ones, shared a preference to react with the 60 kDa and the 16 kDa PRL-like proteins present in HUVEC conditioned medium (Fig. 3, lanes 4, 5). No effect followed incubation of HUVEC cells with preimmune antibodies.

Discussion

The endothelium is the production site for several factors with the ability to stimulate or inhibit endothelial cell function (McPherson *et al.* 1981, Schweigerer *et al.* 1987,
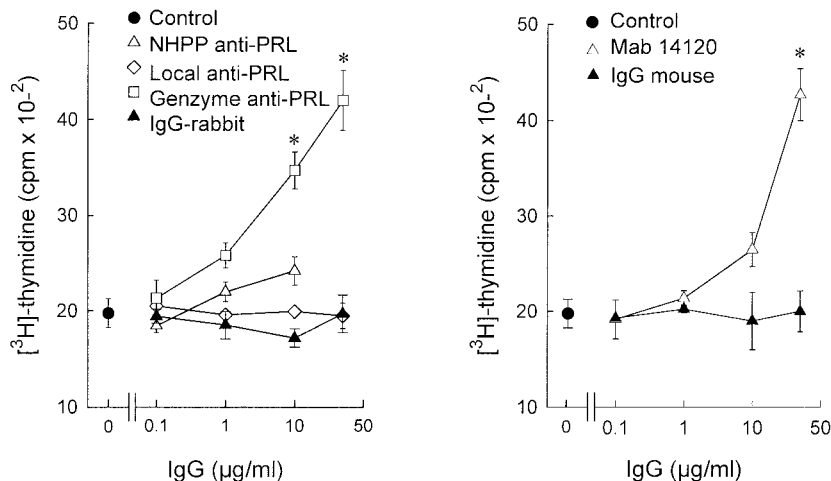

Figure 6 Effect of anti-PRL antibodies on HUVEC proliferation. HUVEC were incubated without (controls) or with increasing concentrations of anti-PRL polyclonal antibodies (isolated from antisera, NHPP or locally raised, or obtained from Genzyme), anti-PRL monoclonal antibody (Mab 14120) or preimmune rabbit or mouse immunoglobulins. [3H]Thymidine was added to the cultures for 12 h. *$P<0.05$ vs non-treated control.

Koyama *et al.* 1994, Nomura *et al.* 1995). Autocrine regulation provides an insight into the mechanism by which local differences of cell growth and differentiation are established within a capillary microenvironment that would determine, for example, the selection of a single endothelial cell to initiate a new capillary network.

Recent work has suggested that PRL-related proteins may contribute to the autocrine regulation of angiogenesis. Members of the PRL family have direct effects on endothelial cells and affect angiogensis both *in vivo* and *in vitro* (Clapp *et al.* 1993, 1994, Dueñas *et al.* 1999, Struman *et al.* 1999). Endothelial cells from bovine brain capillaries express the PRL gene and synthesise and secrete PRL-like proteins (Clapp *et al.* 1998). Moreover, antibodies directed against PRL inhibit BBCEC growth (Clapp *et al.* 1998). The present report extends and strengthens these findings by showing that endothelial cells from human umbilical veins also express the PRL gene, and synthesise and secrete PRL immunorelated proteins. However, in contrast to the effect on bovine capillary cells, anti-PRL antibodies stimulate the proliferation of human endothelial cells from veins. Differences between both endothelial cell types suggest that the autocrine action of PRL could be a function of the endothelial cell phenotype that results from vascular bed and/or species variations.

Expression of the PRL gene in HUVEC is indicated by RT-PCR amplification of a PRL transcript with the size and sequence of cloned full-length 23 kDa PRL. Also, a 100 bp smaller RT-PCR product was detected. Although no sequence information was obtained for this smaller transcript, its relative size resembles that of an alternatively spliced PRL mRNA with a third exon deletion amplified in BBCEC (Clapp *et al.* 1998). This deletion comprises codons encoding for 35 amino acids and would predict a PRL with a molecular mass of about 20 kDa (Truong *et al.* 1984).

In support of the translation of PRL transcripts, HUVEC synthesised and secreted PRL-like antigens with apparent molecular masses of 60, 23, 21, 16 and 14 kDa. Consistent with their PRL-like nature, these proteins reacted with various polyclonal and monoclonal anti-PRL antibodies, but not with pre-immune antibodies. The 23, 21 and 14 kDa proteins are similar to those synthesised and secreted by BBCEC (Clapp *et al.* 1998). However, in contrast to bovine capillary cells, HUVEC also produce and release 60 and 16 kDa PRL-like proteins. The sizes of the 23 and 21 kDa proteins paralleled those expected for the translational products of the two PRL transcripts detected in HUVEC – that is, the full-length PRL mRNA and the smaller RT-PCR product related in size to the third-exon-deleted PRL mRNA found in BBCEC. However, postranslational modification of native 23 kDa PRL encoded by full-length PRL mRNA could account for all PRL-like isoforms detected in HUVEC, including the 21 kDa protein. For example, in the anterior pituitary

gland a PRL product of 22 kDa results from 23 kDa PRL proteolysis by kallikrein (Anthony *et al.* 1993). Likewise, the 16 kDa and the 14 kDa proteins found in HUVEC are similar in size to PRL fragments generated after cleavage of PRL by a cathepsin D-like protease (Baldocchi *et al.* 1993). Finally, a 60 kDa PRL similar to that found in HUVEC is present in human peripheral blood mononuclear cells and may result from monomer aggregation (Sabharwal *et al.* 1992). Although final identification of these proteins remains to be determined, their synthesis and release by HUVEC is consistent with the expression of the PRL gene by human endothelial cells and its processing into various PRL isoforms.

The amount of each PRL-like protein produced and released by HUVEC is uncertain, since discrepancies were noted in the cross-reactivity of these proteins with various anti-PRL antibodies. The 21 kDa PRL-like protein was predominant in HUVEC conditioned media after immunoprecipitation with some anti-PRL antibodies (NHPP-, locally produced-antiserum and 219 monoclonal). However, with other antibodies (Genzyme polyclonal and 14120 monoclonal), a 16 kDa PRL-like protein appeared to predominate. These discrepancies may reflect differences in antibody binding affinities and/or in the nature of the epitopes recognised by the antibodies.

Additional support for the expression of PRL-like antigens in HUVEC was obtained through fluorescence immunocytochemistry using anti-PRL polyclonal and monoclonal antibodies. Dual-label experiments indicated that cells positively stained for the endothelial cell marker Von Willebrand factor were co-stained for PRL. Since these cultures were kept in defined medium, serum PRL cannot be the source of these PRL-like antigens, a result consistent with the proposed endothelium origin of these proteins.

To investigate whether the PRL-like proteins in HUVEC media had PRL-like bioactivity, HUVEC were co-cultured with the pre-T rat lymphoma Nb2 cells. Mitogenesis of Nb2 cells is highly dependent on lactogenic hormones (Shiu *et al.* 1983). HUVEC did not alter Nb2 cell proliferation at any of the endothelial cell densities tested. This result contrasts with previous findings in which co-cultures with BBCEC stimulated Nb2 cell growth, with potencies directly related to BBCEC number (Clapp *et al.* 1998). To favour detection of PRL-like bioactivity, HUVEC conditioned medium was concentrated 500 times and tested on Nb2 cell proliferation. Under these conditions a slight stimulation was detected, which was equivalent to a concentration of PRL lower than that measured in the media of BBCEC (1 vs 30 pg/ml respectively). This difference in PRL bioactivity may be related to the type of PRL isoform secreted by each endothelial cell type. For example, in the conditioned media of HUVEC the antibodies detect a much smaller proportion of the 23 kDa protein than of the 21 kDa PRL antigen. Conversely, similar amounts of both proteins

were found in the media of bovine cells (Clapp *et al.* 1998). The 23 kDa PRL is the well-accepted ligand to activate signal transduction by the PRL receptor (Bole-Feysot *et al.* 1998), while the other PRL isoforms synthesised and released by HUVEC may not be as efficient. The 21 kDa PRL could be lacking the region encoded by exon 3 known to be needed to activate the PRL receptor (Goffin *et al.* 1995). Likewise, PRL fragments of 16 and 14 kDa and PRL aggregates, such as the presumed 60 kDa PRL-like protein, are known to have reduced binding and biological activity via the PRL receptor (Clapp *et al.* 1988, Lopez-Gomez *et al.* 1995, Sinha 1995). Therefore, the nearly absent PRL-like bioactivity of HUVEC media would result from reduced levels of 23 kDa PRL and from PRL isoforms with reduced ability to activate the classic (cloned) PRL receptor.

The putative autocrine effects of PRL appear to be mediated by a novel receptor, distinct from the cloned PRL receptor. Ligand binding and chemical cross-linking studies showed that 16 kDa PRL, but not 23 kDa PRL, binds to high-affinity, saturable sites in endothelial cell membranes that are different in size from the classic PRL receptor (Clapp & Weiner 1992). To investigate a possible autocrine effect of PRL-like isoforms on HUVEC growth, we studied the proliferation of HUVEC in the presence of PRL-directed antibodies in an attempt to immunosequester the secreted PRL-like proteins. Polyclonal (Genzyme) and monoclonal (14120) anti-PRL antibodies stimulated HUVEC proliferation in a dose-dependent manner. This result is consistent with HUVEC PRL acting in an autocrine manner to inhibit cell proliferation. However, other PRL-directed antibodies (NHPP or produced in our laboratory) did not modify the proliferation of HUVEC. One possible explanation for this discrepancy relates to the different cross-reactivities between the antibodies used and the PRL-like proteins present in HUVEC media. For example, the antibodies that stimulate HUVEC proliferation reacted with the 60 and the 16 kDa proteins, but not with the other PRL-like proteins. Conversely, the antibodies displaying no proliferative effect reacted with the other PRL-like proteins but showed little or no cross-reactivity with the 16 kDa protein. As already noted, the 16 kDa protein has the size of an N-terminal fragment of PRL that has anti-angiogenic effects. The 16 kDa PRL inhibits the proliferation, tube formation and urokinase activity of endothelial cells in culture (Clapp *et al.* 1993, Lee *et al.* 1998). Because the antibodies which are able to stimulate HUVEC proliferation immuno-precipitated higher amounts of the 16 kDa protein than the antibodies with no effect, it could be reasoned that stimulation of HUVEC growth resulted from an efficient immuno-sequestration of anti-angiogenic 16 kDa by antibodies with higher affinity for this PRL fragment.

These considerations imply that HUVEC-derived PRL isoforms (presumably 16 kDa PRL) act in an autocrine manner to inhibit cell proliferation. This conclusion is opposite to findings in BBCEC, where passive immunisation with anti-PRL antibodies inhibited endothelial cell growth (Clapp *et al.* 1998). In bovine cells, the inhibition was associated with immuno-sequestration of PRL-like antigens of 23, 21 and 14 kDa which, in turn, would be acting as stimulators of endothelial cell growth. These three PRL-like proteins are also released by HUVEC, and were immunoprecipitated by antibodies that showed no effect on HUVEC proliferation. Thus, it is possible that the lack of effect of these antibodies could have resulted from both the immuno-sequestration of angiogenic PRL isoforms, and from the neutralisation of PRL variants with anti-angiogenic actions. Accordingly, we speculate that HUVEC produce and release PRL isoforms that stimulate and inhibit endothelial cell growth.

The present results extend the expression of PRL gene and its derived products from endothelium of bovine capillaries to endothelial cells lining the veins of humans, and thus suggest that the expression of PRL is common to endothelial cells from the macro- and micro-circulation of different species. The present work also substantiates the heterogeneity of endothelial cells isolated from the various vascular beds and species. Functional dissimilarities between various endothelial cell phenotypes have been postulated both *in vivo* and *in vitro* (Lelkes *et al.* 1996). Endothelial cells derived from various anatomical locations differ in their ability to perceive, transduce and respond to angiogenic signals (Lelkes *et al.* 1996). Likewise, endothelial cells from the microcirculation differ from those of large vessels in the expression and activation of numerous cell surface antigens, such as cell adhesion molecules (Swerlick *et al.* 1992). Here, we show that endothelial cells from human veins differ from those of bovine capillaries in the type and putative function of the PRL released. Endothelial cells from human veins release PRL isoforms with stimulatory and inhibitory autocrine effects on cell proliferation, while PRL variants from bovine capillary endothelium are predominantly stimulatory. Future studies need to be conducted to characterise these differences further and to help establish the functional significance of endothelial-derived PRL isoforms as a function of endothelial cell phenotype.

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