Expression of prolactin mRNA and of prolactin-like proteins in endothelial cells: evidence for autocrine effects

C Clapp, F J López-Gómez, G Nava, A Corbacho, L Torner, Y Macotela, Z Dueñas, A Ochoa, G Noris, E Acosta, E Garay and G Martínez de la Escalera

Centro de Neurobiología, Universidad Nacional Autónoma de México, 76001 Querétaro, Qro, México

(Requests for offprints should be addressed to Dr Carmen Clapp, Centro de Neurobiología, Universidad Nacional Autónoma de México, Apartado Postal 1-1141, 76001 Querétaro, Qro, Mexico)

Abstract

Formation of new capillary blood vessels, termed angiogenesis, is essential for the growth and development of tissues and underlies a variety of diseases including tumor growth. Members of the prolactin hormonal family bind to endothelial cell receptors and have direct effects on cell proliferation, migration and tube formation. Because many angiogenic and antiangiogenic factors are produced by endothelial cells, we investigated whether endothelial cells expressed the prolactin gene. Here we show that bovine brain capillary endothelial cells (BBCEC) in culture express the full-length prolactin messenger RNA, in addition to a novel prolactin transcript, lacking the third exon of the gene. In addition cultures of BBCEC synthesize and secrete prolactin-like immunoreactive proteins with apparent molecular masses of 23, 21 and 14 kDa. The

prolactin-like nature of these proteins is supported by the observation that Nb2-cells, a prolactin-responsive cell line, were stimulated to proliferate when co-cultured with endothelial cells and this stimulation was neutralized with prolactin-directed antibodies. Finally, consistent with a possible autocrine effect of endothelial-derived prolactins, polyclonal and monoclonal prolactin antibodies specifically inhibited basal and basic fibroblast growth-factor-stimulated growth of endothelial cells. Taken together, the present findings support the hypothesis of the prolactin gene being expressed in endothelial cells as proteins that could act in an autocrine fashion to regulate cell proliferation.

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Introduction

Angiogenesis is essential for the growth, development and repair of tissues. In the adult mammal, however, angiogenesis is rare. When it occurs, during wound healing or in response to ovulation, it is tightly controlled and delimited (Folkman & Klagsbrun 1987, Polverini 1995). Failure of this control is associated with a variety of diseases dominated by pathologic neovascularization, including neoplasia, diabetic retinopathy, rheumatoid arthritis, psoriasis and arteriosclerosis (Folkman & Klagsbrun 1987, Polverini 1995).

Positive and negative signals regulating angiogenesis act on endothelial cells to stimulate or inhibit their migration, proliferation or association into capillary tubes. Several stimulators and inhibitors of endothelial cell function have been described, most of which are produced by cells within the vicinity of the capillary network (Folkman & Klagsbrun 1987, Klagsbrun & D'Amore 1991, Auerbach & Auerbach 1994, Battagay 1995). Moreover endothelial cells produce and respond to

their own angiogenic or antiangiogenic factors (autocrine regulation). Endothelial peptides with autocrine effects include basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and thrombospondin (McPherson *et al.* 1981, Schweigerer *et al.* 1987, Koyama *et al.* 1994, Nomura *et al.* 1995). Autocrine regulation could help explain the local differences of cell growth and differentiation that must be established in a capillary micro-environment and that are involved, for example, in the selection of a single endothelial cell to initiate a capillary network.

Prolactin (PRL) gene is expressed in the pituitary gland, and at several other sites including the central nervous system and the immune system (Sinha 1995, Ben-Jonathan *et al.* 1996). Products of this gene function as hormones and cytokines regulating reproductive and immunological functions, fluid balance, cellular growth and differentiation (Nicol 1980, Loretz & Bern 1982, Russell 1989, Kelly *et al.* 1991). In addition, members of the PRL family have been implicated recently as potential

regulators of angiogenesis. Fragments of PRL of 16 and 14 kDa bind to endothelial cell receptors and inhibit endothelial cell proliferation and tube formation (Clapp & Weiner 1992, Ferrara *et al.* 1991, Clapp *et al.* 1993, 1994). Likewise, the 16 kDa amino-terminal fragment of PRL inhibits the *in vivo* development of the microvasculature of the chick chorioallantoic membrane (Clapp *et al.* 1993). Furthermore, two PRL-related proteins, proliferin and proliferin-related protein, were shown to stimulate and inhibit endothelial cell migration respectively, and to affect angiogenesis *in vivo* (Jackson *et al.* 1994). Here, we have investigated the hypothesis that the PRL gene could be expressed in endothelial cells, where its products may function as autocrine factors in the regulation of angiogenesis.

Materials and Methods

PRL and antibodies

Bovine PRL and ovine PRL (BIO grade) and bovine PRL antiserum (RIA grade) were donated by the National Hormone and Pituitary Program (NHPP, Bethesda, MD, USA). The specificity of the NHPP PRL antiserum has been well characterized as it forms part of the kit distributed for RIA determinations of bPRL. Antisera were also raised locally in rabbits by immunization with the NHPP bovine PRL or ovine PRL. Specificity for bovine PRL of the locally raised antisera was assessed by RIA. At 1 : 50 000 dilution, both antisera reacted with increasing concentrations of bovine PRL, whereas crossreactivity with bovine FSH or bovine LH was less than 0.1%. Bovine PRL monoclonal antibody (5G2) known to crossreact with a specific region of the amino-terminal disulfide loop of bovine PRL (Scammell *et al.* 1992) was kindly provided by J Scammell (University of South Alabama, Mobile, AL, USA). All antibodies were preservative-free.

Endothelial cell culture

Bovine brain capillary endothelial cells (BBCEC) were isolated as described elsewhere (Gospodarowicz *et al.* 1986), grown and serially passaged in BBCEC culture medium (low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% calf serum, 2 mM glutamine, and penicillin/streptomycin (50 U/ml)) as previously reported (Ferrara *et al.* 1991, Clapp *et al.* 1993). Serum was not heat-inactivated. bFGF (1 ng/ml; Gibco BRL) was added to the cultures every other day and the cells were used between passages 5 and 12. Endothelial cells were characterized by their nonoverlapping cobblestone morphology and by the positive immunofluorescence for Von Willebrand factor in more than 95% of cells in the culture (Gerritsen *et al.* 1988).

Figure 1 Identification of PRL mRNAs in BBCEC. (A) Southern blot analysis of PCR from reverse-transcribed total RNA from bovine anterior pituitary (lane 1) or BBCEC (lanes 2–4), amplified with primers bPRL-A and bPRL-2 without (lane 2) or with RT (lanes 3 and 4), and hybridized with a bovine PRL cDNA probe. Lane 4, negative control excluding RNA. (B) Sequencing gel of the 492- and the 384-bp RT-PCR products from BBCEC at the downstream boundary of exon 2 (arrows). The 492-bp product has the sequence of the cloned bPRL cDNA, in which exon 2 is followed by exon 3 (Truong *et al.* 1984). In the 384-bp product, exon 2 is followed by exon 4, denoting the loss of exon 3 of bPRL cDNA.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from semiconfluent BBCEC was isolated and RT-PCR performed essentially as described previously (Clapp *et al.* 1994), using 36 cycles and an annealing temperature of 55 °C. Two primers complementary to bovine PRL cDNA were synthesized: forward primer bPRL-A (5'-GGGCAGTCATGGTGTCCCACTA-3') from exon 2 and downstream primer bPRL-2 (5'-AAGTGTCAATCTTGCTTGAATC-3') from exon 5 (Fig. 1). RT-PCR products were identified by Southern blot using a homologous probe (bovine PRL cDNA, generously supplied by R Maurer, Oregon Health Sciences University, Portland, OR, USA) and performed as previously reported (Clapp *et al.* 1994). PCR transcripts were sequenced by the dideoxy method (Sanger *et al.* 1977) using the AmpliCycle kit (Perkin Elmer, Branchburg, NJ, USA) and α [³²P]dATP (Dupont Co., Wilmington, DE, USA). The sequencing reactions were performed according to the manufacturer's instructions. Aliquots of the sequencing reactions were run on 6% acrylamide gels, vacuum dried, and autoradiographed for at least 18 h.

Metabolic labeling and immunoprecipitation of de novo synthesized proteins

Semiconfluent BBCEC were metabolically labeled for 7 h with [³⁵S]-cysteine and [³⁵S]-methionine (100 μ Ci/ml; Dupont Co.) in 0.2 mg/ml BSA, serum-free, low-glucose DMEM and lysed in 1% NP-40, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 1 μ g/ml aprotinin and 100 μ g/ml

phenylmethylsulfonyl fluoride. BBCEC lysates and concentrated (4X; Centricon 3, Amicon, Beverly, MA, USA) culture media, were incubated overnight with a 1 : 500 dilution of bovine PRL antiserum (NHPP) or normal rabbit serum (NRS) followed by a 1-h incubation with protein A-Sepharose beads (Sigma) as described elsewhere (Sambrook *et al.* 1989). NRS was used for preclearing. Proteins were eluted from the Sepharose beads by boiling in electrophoresis sample buffer (0.4 g SDS, 4 ml β -mercaptoethanol, 2 ml glycerol, 1 mg bromophenol blue, 10 ml water) and resolved in an SDS slab gel (15% acrylamide/bisacrylamide). Gels were fixed, soaked in Enhance (Dupont Co.), dried and autoradiographed.

Nb2 cell growth

Nb2 cells, a PRL-responsive rat lymphoma cell line (kindly provided by P Gout, British Columbia Cancer Agency, Vancouver, BC, Canada), were grown in suspension in high-glucose DMEM containing 2 β -mercaptoethanol (10^{-4} M) and supplemented with 10% horse serum, 10% fetal bovine serum and antibiotics as described by Tanaka *et al.* (1980). Sera were not heat-inactivated. For individual experiments, Nb2 cells (2.5×10^4 cells/15-mm well) were co-cultured with BBCEC previously attached to the wells at different (0.625 , 1.25 or 2.5×10^5) cellular densities, in the above incubation medium but without fetal calf serum. After 60 h, [^{3} H]thymidine was added to co-cultures for 4 or 12 h. Because Nb2 cells grow in suspension, [^{3} H]thymidine incorporation into Nb2 cells was assayed in co-cultures supernates after collecting the cells by centrifugation. Values were corrected by subtracting the c.p.m. present in supernates of BBCEC cultured in the absence of Nb2 cells. [^{3} H]Thymidine incorporation was assayed essentially as previously described (Ferrara *et al.* 1991). Co-cultures were carried out in the absence or presence of a 1 : 500 dilution of bovine PRL antisera (NHPP) or locally produced anti-bovine PRL or anti-ovine PRL antisera or NRS.

Endothelial cell growth

To determine cell proliferation, BBCEC were plated (10^4 cells/35-mm well or 2.5×10^3 cells/15-mm well) and cultured in calf serum (5% or 0.1%), or in 0.2 mg/ml BSA, serum-free, low-glucose DMEM. Incubations were for 60 h with bFGF and antibodies added twice, once at the time of seeding the cells, and once again, 48 h later. At the end of incubations the number of viable cells was estimated by the dye exclusion method (Harlow & Lane 1988) and cell proliferation assayed either by cell number (hemocytometer) or by measuring [^{3} H]thymidine incorporation into cells. BBCEC were cultured in the absence or presence of bFGF (1 ng/ml) alone or together with different concentrations of bovine PRL antiserum

(NHPP), bovine PRL monoclonal antibody, NRS or preimmune mouse IgGs (Sigma Chemical Co., St Louis, MO, USA). For neutralization experiments, cells were cultured with bFGF and PRL antibodies in combination with bovine PRL (100 nM). Specificity to endothelial cells was analyzed by testing the effect of PRL monoclonal antibody on NIH/3T3 cells. NIH/3T3 cells obtained from American Type Culture Collection (Rockville, MD, USA) were maintained in 5% calf serum, BBCEC culture medium and the mitogenic effects of bFGF alone or in combination with the PRL monoclonal antibody were analyzed as described for BBCEC. Detection and quantitation of endotoxin in all PRL antibodies was determined by the Limulus amoebocyte lysate assay using the TOXATE kit (Sigma) as previously reported (Clapp *et al.* 1993).

Statistical analysis

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

Results

Identification of PRL gene transcripts by RT-PCR and sequence analysis

Total RNA from BBCEC was reverse-transcribed and amplified by PCR using primers with annealing sites within exons 2 and 5 of the bovine PRL cDNA. Southern blot analysis showed a PCR product of 492 bp, which corresponded in size to that amplified from bovine anterior pituitary RNA and consistent with the predicted size for the cloned full-length 23-kDa PRL mRNA (Fig. 1A). Smaller size PCR fragments were also amplified from BBCEC: the two most abundant were of about 430 and 384 bp. No positive signal was detected in the absence of reverse transcriptase or in the negative control without RNA (Fig. 1A). Similarly, no signal was observed in NIH/3T3 fibroblasts (not shown). The amplified 492-bp product showed identical sequence homology with bovine pituitary PRL throughout an analyzed region of 181 bp comprising codons encoding for amino acids 31–91 (Fig. 1B). Direct sequencing of the 384-bp PCR product showed total sequence identity with pituitary PRL over an analyzed region of 157 bp, corresponding to codons encoding for amino acids 31–40 and 77–119. An entire region of 108 bp encoding for amino acids 41–76 was missing in this transcript (Fig. 1B). The sequence of this smaller transcript showed third-base substitutions at codons 95 (cytosine instead of guanine) and 103 (guanine instead of adenine) with respect to a reported pituitary

Figure 2 Metabolically labeled BBCEC culture media immunoprecipitated with NHPP bovine PRL antiserum (α bPRL) or normal rabbit serum (NRS). PRL-like proteins and the [^{35} S]-labeled bovine PRL (bPRL) are indicated (arrows), in addition to their relative molecular mass (Mr).

bovine PRL cDNA (pBPRL72) (Sasavage *et al.* 1982). However, both substitutions have been reported for another PRL cDNA clone (pBPRL4) also obtained from the bovine pituitary (Sasavage *et al.* 1982). The 430-bp PCR product was not sequenced.

Detection of PRL-like proteins in endothelial cells

Metabolic labeling and immunoprecipitation To assay for the translational products of PRL transcripts in endothelial cells, protein synthesis was metabolically labeled with [^{35} S]-cysteine and [^{35} S]-methionine in BBCEC and the [^{35} S]-labeled PRL-like proteins immunoprecipitated with bovine PRL antiserum (NHPP). PRL antiserum, but not pre-immune serum, precipitated [^{35} S]-PRL-like proteins with apparent molecular masses of 23, 21 and 14 kDa in BBCEC-conditioned media (Fig. 2). Also, a labeled, diffuse PRL-like protein band was detected within the 22-kDa region. Sizes were estimated by comigration with 23-kDa bovine PRL and molecular mass markers. No labeled PRL-like proteins were detected in BBCEC lysates (not shown).

Stimulation of Nb2 cell growth To analyze the PRL-like nature of endothelial proteins, BBCEC were seeded at different densities and co-cultured with PRL-responsive Nb2 rat lymphoma cells. BBCEC significantly stimulated the proliferation of Nb2 cells (Figs 3 and 4). The greatest level of activity in the conditioned media of BBCEC–Nb2 co-cultures was equivalent to 30 pg/ml pituitary PRL as estimated by serial dose–response effects of bovine PRL standard (Fig. 3). Stimulation of Nb2 cell growth by endothelial cells and bovine PRL standard was abolished in the presence of the NHPP bovine PRL antiserum, but not affected by preimmune serum (Fig. 3). Moreover, in experiments depicted in Fig. 4, in which greater values of [^{3} H]thymidine incorporation followed a longer

Figure 3 [^{3} H]Thymidine incorporation into Nb2 cells cultured in the absence (control) or presence of increasing concentrations of bovine PRL (bPRL) or co-cultured with BBCEC at various cellular densities in the absence (no addition) or presence of normal rabbit serum (NRS), or of NHPP bovine PRL antiserum (α bPRL). [^{3} H]Thymidine was added to all cultures for 4 h. * $P < 0.05$ compared with controls.

Figure 4 [^{3} H]Thymidine incorporation into Nb2 cells cultured in the absence (control) or presence of BBCEC at two cellular densities without further treatment (white bars) or after being cultured with locally produced antisera against bovine PRL (gray bars) or ovine PRL (hatched bars). [^{3} H]Thymidine was added to all cultures for 12 h. * $P < 0.05$ compared with controls.

incubation with the tracer (12 compared with 4 h, for experiments shown in Fig. 3), the stimulation of Nb2 proliferation induced by endothelial cells was blocked by locally raised bovine PRL and ovine PRL antisera. Both of these antisera abolished the Nb2 proliferative effect of increasing concentrations of bPRL (Fig. 5A) with this inhibition being dependent on the antisera dilution (Fig. 5B).

Figure 5 Blockage of PRL-induced stimulation of $[^3\text{H}]$ thymidine incorporation into Nb2 cells by locally produced bovine or ovine PRL antisera. (A) Nb2 cells were cultured in the absence of PRL (controls) or the presence of increasing concentrations of bovine PRL (bPRL) alone (●) or together with a 1 : 500 dilution of locally produced anti-bovine PRL (□) or anti-ovine PRL (△) antisera. (B) Nb2 cells were cultured in the absence (○) or presence of bovine PRL (100 pg) alone (●) or together with increasing concentrations of locally produced anti-PRL antisera (α PRL: anti-bovine PRL, □; anti-ovine PRL, △). $[^3\text{H}]$ Thymidine was added to all cultures for 12 h.

Effect of anti-PRL antibodies on endothelial cell growth

To investigate a possible effect of endothelial PRLs on endothelial cell growth, we determined whether proliferation of BBCEC in culture could be affected by PRL-directed antibodies, capable of neutralizing the action of endogenous PRLs. Antiserum to bovine PRL inhibited both basal and bFGF-stimulated proliferation of BBCEC as determined directly by cell numbers (Fig. 6A), and by $[^3\text{H}]$ thymidine incorporation into DNA (Fig. 6B). Inhibition of both parameters was dose-dependent. After incubation with antiserum (1 : 1000), the number of BBCEC was similar to the number of cells seeded, which indicated that, at this antiserum dilution, there was a total blockage of endothelial cell proliferation (Fig. 6A). In addition, bFGF-stimulated $[^3\text{H}]$ thymidine incorporation

Figure 6 Effect of anti-bovine PRL antibodies on BBCEC proliferation. (A) Effect of NHPP bovine PRL-antiserum (α bPRL) on number of BBCEC. Cells were seeded (stippled bar) and cultured (Inc.) for 60 h in 5% calf serum in the absence (white bar) or presence of bFGF (1 ng/ml) alone (black bar), or together with 10^{-5} or 10^{-3} NHPP α bPRL (hatched bars). (B) Dose-dependent effect of NHPP α bPRL on $[^3\text{H}]$ thymidine incorporation into BBCEC. Cells were cultured in 0.1% calf serum in the absence (◇) or presence of bFGF (1 ng/ml) alone (▼), or together with bPRL (100 nM, △), NRS (□), NHPP α bPRL (○), or NHPP α bPRL in combination with bPRL (100 nM, ●). * $P < 0.05$ compared with bFGF-treated control; + $P < 0.05$ compared with bFGF-untreated control.

Figure 7 Dose-dependent effect of a bovine PRL monoclonal antibody (bPRL Mab) on $[^3\text{H}]$ -thymidine incorporation into BBCEC. Cells were cultured in 0.1% calf serum in the absence (◇) or presence of bFGF (1 ng/ml) alone (▼), or together with bovine PRL (100 nM, △), preimmune mouse IgG (□), bPRL Mab (○), or bPRL Mab in combination with bPRL (100 nM, ●). * $P < 0.05$ compared with bFGF-treated control.

into BBCEC was inhibited by a bovine PRL monoclonal antibody in a dose-dependent fashion (Fig. 7). The action of both polyclonal and monoclonal PRL antibodies was neutralized with exogenous PRL and no effect on BBCEC proliferation followed culture with NRS or

preimmune IgGs (Figs 6B and 7). The inhibitory effect of PRL antibodies was independent of the presence of serum in the cultures, since it was observed in cells cultured in 5% calf serum (Fig. 6A), in cells cultured with 0.1% calf serum (Figs. 6B and 7), and in cells cultured in defined medium in the absence of serum (data not shown). The bovine PRL monoclonal antibody did not modify basal or bFGF-stimulated [^{3} H]thymidine incorporation into NIH3T3 cells (data not shown). Cell viability, determined by trypan blue exclusion, was not affected by PRL antibodies at the dilutions used (data not shown) and a 1 : 100 dilution of all PRL antibodies had undetectable levels of endotoxin (<0.03 EU), arguing against a toxic action.

Discussion

The endothelium is the production site for several factors with the ability to stimulate or inhibit the formation of new blood vessels (McPherson *et al.* 1981, Schweigerer *et al.* 1987, Klagsbrun & D'Amore 1991, Auerbach & Auerbach 1994, Koyama *et al.* 1994, Nomura *et al.* 1995). Autocrine angiogenic and antiangiogenic factors provide an insight into the mechanisms by which endothelial cell growth is selectively supported or prohibited locally within a tissue microenvironment.

Recent work has implicated PRL-related proteins as potential regulators of angiogenesis (Ferrara *et al.* 1991, Clapp & Weiner 1992, Clapp *et al.* 1993, 1994, Jackson *et al.* 1994). The present study extends and strengthens those discoveries by showing that PRLs may be produced by the endothelial cells themselves, and that they may constitute a novel group of autocrine regulators of angiogenesis. The current results are consistent with the expression of the PRL gene in endothelial cells and with PRL-related proteins acting in an autocrine manner to regulate endothelial cell proliferation.

Expression of the PRL gene in endothelial cells is indicated by RT-PCR–Southern blot and sequence analyses of total RNA from BBCEC. Specificity to endothelial cells is suggested by the lack of PRL gene PCR amplification signal in NIH/3T3 fibroblasts. A PCR product with the same size and sequence as that amplified from the pituitary gland corresponding to the full-length 23 kDa PRL mRNA was identified in BBCEC. In addition, smaller PRL transcripts of 430 and 384 bp were detected. The 384-bp transcript was found to be devoid of a 108-bp region comprising codons encoding for amino acids 41–76. This region corresponds to exon 3 in the human PRL gene and in the rat PRL gene (Gubbins *et al.* 1980, Truong *et al.* 1984). Although genomic sequence data for bovine PRL remain incomplete, the homology found among species strongly suggests that this small transcript could correspond to a novel PRL mRNA in which exon 2 is being spliced directly onto exon 4 without

affecting the reading frame. This alternative splicing predicts a protein with a molecular mass of about 20 kDa. Although the full-length PRL mRNA is responsible for 23-kDa PRL, the bulk hormone in most tissues of origin, the third-exon-deleted PRL transcript has not been found elsewhere in other PRL-producing tissues. This could imply that its expression, and perhaps the function of the protein encoded by it, may be endothelial-cell-specific.

Support for the translation of the PRL gene in endothelial cells is provided by metabolic labeling–immunoprecipitation studies. PRL-like antigens, with apparent molecular masses of 23, 21 and 14 kDa, were found to be synthesized and secreted into the culture medium of BBCEC. The sizes of the 23- and 21-kDa proteins paralleled those expected for the translational products of two of the PRL mRNAs detected in BBCEC – that is, the full-length PRL transcript and the third-exon-deleted alternative spliced mRNA. In addition, a PRL-like protein of about 22 kDa appears to have been detected that may correspond to the translational product of the 430-bp PRL transcript also found in BBCEC. Interestingly, the smaller, 14-kDa, protein has a size similar to that of a previously identified 14-kDa fragment of PRL with antiangiogenic effects (Clapp *et al.* 1994). Although the final identification of these proteins remains to be determined, these results are consistent with PRLs being produced and secreted by endothelial cells.

Further support for the secretion of PRLs by endothelial cells resulted from co-culturing BBCEC with the pre-T rat lymphoma Nb2 cells. Mitogenesis of Nb2 cells is highly dependent on PRL-like factors (Shiu *et al.* 1983). BBCEC stimulated the proliferation of Nb2 cells with a potency directly related to endothelial cell number. The PRL-like nature of the stimulatory factor(s) is indicated by the fact that PRL-directed antibodies, able to block the stimulatory action of exogenous PRL, abolished the stimulation of Nb2 cell proliferation by endothelial cells.

It is clear that the level of PRL gene expression and of the PRL-like proteins in endothelial cells is low. Highly sensitive techniques (RT-PCR and radioactive metabolic labeling) are required for their detection, and picogram amounts of biologically active PRLs were measured. The observed low level of PRL expression may be of significant value. Sensitive techniques are a common requirement for the detection of locally acting factors, particularly when little is known about regulation of their expression. The mRNA of vascular endothelial cell growth factor (VEGF) is nondetectable by Northern blots in retinal cells grown under normal oxygen conditions, but easily detected after hypoxia (Shima *et al.* 1995). Low levels of PRL expression, requiring RT-PCR and metabolic labeling, have been reported in neurons and immune cells in which PRLs are known to function as autocrine paracrine regulatory factors (Ben-Jonathan *et al.* 1996, Yu-Lee 1997).

In support of the assumption that the PRL level of expression in endothelial cells is of significant value, polyclonal and monoclonal PRL antibodies inhibited the proliferation of BBCEC in culture. The action of PRL antibodies was found to be specific because their effect was neutralized with exogenous PRL, and no action followed culture of the cells with preimmune antibodies. Furthermore, in support of endothelial cells as the source of these PRL-like proteins, the action of PRL antibodies was not dependent on the presence of serum in the culture medium. The fact that PRL antibodies inhibited the growth of endothelial cells is consistent with the possibility of endothelial-derived PRLs acting in an autocrine manner to stimulate cell proliferation.

The conclusion of PRLs being angiogenic is surprising, given that the proteolytic fragments of PRL inhibit (rather than stimulate) endothelial cell proliferation, and full-length PRL neither binds nor affects endothelial cells (Ferrara *et al.* 1991, Clapp & Weiner 1992, Clapp *et al.* 1993, 1994). In contrast, PRLs with opposite effects on angiogenesis may not be unlikely. Two PRL-related proteins, proliferin and proliferin-related protein, have been shown to stimulate and inhibit respectively endothelial cell migration (Jackson *et al.* 1994). The conclusive identification of angiogenic PRL-like molecules awaits their purification and subsequent characterization. Nevertheless, alternative explanations to a PRL stimulatory action may also be feasible. For example, the inhibitory action of PRL antibodies could have been the result of an anti-proliferative signal, rather than of a block of a proliferative signal; addition of the antibodies to the cell cultures could have more effectively crosslinked an anti-angiogenic PRL fragment bound at the cell surface, thereby triggering an anti-proliferative signal. Comparisons of the effects given by bivalent and monovalent antibodies should provide valuable insights on this latter possibility.

No conclusions can be drawn on which one of the endothelial-derived PRLs (23-, 21- and 14-kDa isoforms) could be responsible for the proposed autocrine effects on endothelial cell proliferation. It is likely, however, that the active PRL(s) may differ structurally from pituitary 23-kDa PRL. Full-length 23-kDa pituitary prolactin was found to be inactive on endothelial cells (Ferrara *et al.* 1991, Clapp *et al.* 1993, 1994), a result confirmed recently using a wide range of doses of 23-kDa PRL alone or in combination with bFGF (C Clapp, unpublished observations). Furthermore, the receptors for pituitary 23-kDa PRL appear to be absent from endothelial cells. Full-length pituitary PRL shows no binding affinity for endothelial cell membranes (Clapp & Weiner 1992) and RT-PCR experiments with PRL-receptor-specific primers have failed to detect the cloned PRL receptors in endothelial cells (C Clapp and P A Kelly, unpublished observations). In contrast, endothelial cells do express a novel receptor for PRL molecules that does not bind pituitary PRL. Ligand binding and chemical crosslinking

studies showed that 16-kDa PRL, but not pituitary 23-kDa PRL, binds to high-affinity, saturable sites in endothelial cell membranes distinct in size from the cloned PRL receptors (Clapp & Weiner 1992). A plausible hypothesis is that endothelial-derived PRLs, structurally different from pituitary 23-kDa PRL, may exert their autocrine effects through the activation of the new set of endothelial-specific receptors.

On the basis of this hypothesis, likely candidates for the active endothelial-derived PRLs are the 14-kDa PRL-like protein, as it has a size similar to that of PRL fragments that bind to endothelial cells and have effects on angiogenesis (Clapp & Weiner 1992, Clapp *et al.* 1994), and the 21-kDa protein, presumed to be the translational product of the third-exon-deleted PRL transcript. The amino acid sequence encoded by exon 3 is an important component of one of the two known binding domains of PRL to the cloned receptors, and its deletion is expected to generate a protein with reduced capacity to activate them (De Vos *et al.* 1992, Fuh *et al.* 1993, Goffin *et al.* 1995). Therefore, it is conceivable that the third-exon-spliced variant, if active, would operate through a new set of PRL receptors.

Identification of the molecular forms of PRLs produced by endothelial cells is of major importance for understanding the specific role and receptors involved in the regulation of endothelial cell function by this family of hormones and cytokines. Cloning and purification techniques involving specific antibodies are currently being undertaken.

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