A molecular mimic demonstrates that phosphorylated human prolactin is a potent anti-angiogenic hormone

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Abstract

S179D prolactin (PRL) is an experimentally useful mimic of naturally phosphorylated human prolactin. S179D PRL, but not unmodified PRL, was found to be anti-angiogenic in both the chorio-allantoic membrane and corneal assays. Further investigation using human endothelial *in vitro* models showed reduced cell number, reduced tubule formation in Matrigel, and reduced migration and invasion, as a function of treatment with S179D PRL. Analysis of growth factors in human endothelial cells in response to S179D PRL showed: a decreased expression or release of endogenous PRL, heme-oxygenase-1, basic fibroblast growth factor (bFGF), angiogenin, epidermal growth factor and vascular endothelial growth factor; and an increased expression of inhibitors of matrix metalloproteases. S179D PRL also blocked signaling from bFGF in these cells. We conclude that this molecular mimic of a pituitary hormone is a potent anti-angiogenic protein, partly as a result of its ability to reduce utilization of several well-established endothelial autocrine growth loops, partly by its ability to block signaling from bFGF and partly because of its ability to decrease endothelial migration. These findings suggest that circulating levels of phosphorylated PRL may influence the progression of cancer and, furthermore, that S179D PRL may be a useful anti-angiogenic therapeutic.

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Introduction

Many tissues express prolactin (PRL) receptors (Costlow & McGuire 1977, Meister *et al.* 1992, Royster *et al.* 1995), indicating the likelihood of a response to PRL, the primary source of which is the pituitary. In addition, a substantial proportion of these tissues express PRL (reviewed in Ben-Jonathan *et al.* 1996) and for some, such as the mammary gland and prostate, PRL functions as an autocrine growth factor (Ben-Jonathan *et al.* 1996, Clevenger & Plank 1997, Schroeder *et al.* 2003). An autocrine PRL growth loop is present in cells representative of both steroid-dependent and -independent breast and prostate cancer (Clevenger & Plank 1997; reviewed in Ben-Jonathan *et al.* 2002), and is a potential target for therapeutics.

Since agents that regulate pituitary PRL synthesis and release are ineffective in extrapituitary tissues (Ben-Jonathan *et al.* 1996), several laboratories have developed PRL receptor antagonists (Fuh & Wells 1995, Lochan *et al.* 1995, Chen *et al.* 1998, 2002, Llovera *et al.* 2000, Xu *et al.* 2001, Beck *et al.* 2003). S179D PRL, a mimic of phosphorylated human PRL (Wang *et al.* 1996, Chen *et al.* 1998, Tuazon *et al.* 2002), was originally developed as a PRL receptor antagonist since we had shown that naturally phosphorylated PRL had this activity (Krown *et al.* 1992, Wang & Walker 1993). It was anticipated therefore that S179D PRL would impair the growth of tissues utilizing PRL as a growth factor by blocking the effects of both pituitary and autocrine PRL. Indeed, this molecule has been shown to impair the development of a number of fetal tissues (Coss *et al.*

2000, Yang *et al.* 2001, 2002), to inhibit the growth of mammary glands during pregnancy (Kuo *et al.* 2002, Naylor *et al.* 2005), to inhibit proliferation of human breast cancer cells in response to unmodified PRL (U-PRL) *in vitro* (Schroeder *et al.* 2003) and to inhibit the growth of human prostate cancer cells both *in vitro* and when grown as tumors in nude mice (Xu *et al.* 2001).

Further analysis of the effects of S179D PRL, however, made two points clear: first, that S179D PRL was not a pure antagonist (Bernichtein *et al.* 2001, Wu *et al.* 2003, Xu *et al.* 2003, Tan *et al.* 2005); and second, that it was more effective *in vivo* than *in vitro* (best illustrated in Xu *et al.* 2001). With regard to the first issue, we have demonstrated that in addition to its growth-inhibiting properties, S179D PRL promotes the expression of tissue-specific genes in both the mammary gland (Kuo *et al.* 2002, Wu *et al.* 2003) and the prostate (Xu *et al.* 2003). These dual antagonist/agonist activities are brought about by the inhibition of signaling to cell proliferation (Coss *et al.* 1999, Schroeder *et al.* 2003, Wu *et al.* 2003) and by production of alternative signals which result in the expression of tissue-specific genes (Wu *et al.* 2003) and cell cycle regulatory proteins (Wu *et al.* 2005). With regard to the greater efficacy *in vivo* (Coss *et al.* 2000, Xu *et al.* 2001, Kuo *et al.* 2002, Naylor *et al.* 2005), one potential explanation is that S179D PRL acts on a variety of cells and not just the mammary and prostate epithelial cells initially studied. Among these potential targets, cells of the developing vasculature are good candidates.

In addition to being a requirement for normal development, angiogenesis is also an essential element of many pathological processes, including tumor growth and metastasis, diabetic retinopathy and neurofibromatosis type 1 (reviewed in Folkman 1995, Ozerdem 2005). The development of anti-angiogenic therapies for treatment of these pathologies has therefore become an increasingly important goal of biomedical research. Endothelial cells have been shown to express PRL receptors (Corbacho *et al.* 2000, Merkle *et al.* 2000) as well as a unique receptor that binds a cleaved form of PRL (Clapp & Weiner 1992). We therefore undertook the current study to determine whether part of the *in vivo* anti-growth efficacy of S179D PRL was the result of anti-angiogenic activity.

Materials and methods

Recombinant prolactins

Recombinant U-PRL and S179D PRL were produced as previously described (Chen *et al.* 1998). Endotoxin

levels in the preparations were detected and measured with the *Limulus* amoebocyte lysate assay (E-Toxate kit; Sigma).

Angiogenesis assays in the chicken chorioallantoic membrane (CAM)

These assays were carried out using a modification of the method previously described (Martins-Green & Kelly 1998). Briefly, 2 ml albumin were withdrawn from 4-day fertilized eggs to drop the CAM away from the shell. Windows in the shell were made 3 h later and the eggs were incubated until day 9. Pellets were prepared by mixing 1 µg U-PRL or S179D PRL, or 500 ng basic fibroblast growth factor (bFGF), with 1% methylcellulose (1:1) in a total volume of 40 µl per pellet. Then, 40 µl drops were placed on parafilm and allowed to dry in a sterile hood for 2 h. This procedure yielded small disc-shaped pellets, 5 mm in diameter and 0.1 mm thick. These were then placed between two large blood vessels of the 9-day-old CAMs; 5 days later, the pellets and the surrounding areas were removed, fixed and the CAMs examined for the degree of angiogenesis. Angiogenesis was quantified by estimation of the avascular area. Briefly, using enlarged photographs of the pellet circles, the areas that did not contain the normal frequency of smaller vessels present in the positive controls were cut out and weighed. Weights were converted to area knowing the magnification of the images. The positive controls with normal spacing of the small vessels were considered to have no avascular area. Negative controls consisted of pellets with water and methylcellulose only, and pellets containing both S179D PRL and a 3-fold excess of rabbit anti-human PRL (provided by A Parlow, Harbor General, Torrance, CA, USA). The bFGF pellet served as a positive control. The experiment was carried out five times with ten replicates for each treatment.

Corneal angiogenesis assay

The surgical procedure for inducing corneal angiogenesis in the mouse (Kenyon *et al.* 1996) was modified to incorporate two pellets in the corneal pocket instead of just a single pellet (Ozerdem & Stallcup 2004). Slow-release polyhydroxyethyl methacrylate (hydron) (Hydro Med Sciences, Cranbury, NJ, USA) pellets (0.4×0.4×0.2 mm) were formulated to contain 45 µg sucrose aluminum sulfate (sucralfate) (Sigma) plus one of three experimental additives: 90 ng recombinant bFGF (Life Technologies, Carlsbad, CA, USA), 52.5 ng S179D PRL or 52.5 ng U-PRL. Ten mice (6 weeks old; C57BL/6) were anesthetized

with Avertin (0.015–0.017 ml/g body weight) and two pellets were implanted into the corneal stroma at a distance of 0.7 mm from the corneo-scleral limbus. Ten eyes received pairs of pellets containing bFGF and S179D PRL. Fellow eyes of each mouse received pairs of pellets containing bFGF and U-PRL. Over an 8-day period after surgery, the mice were examined to evaluate the progress of corneal angiogenesis in the operated eyes. On day 8, angiogenesis was quantified by determining the area of vascularization, as described previously (Kenyon *et al.* 1996, 1997).

Cell culture

Primary human umbilical vein endothelial cells (HUVEc) and primary human microvascular endothelial cells (HMVEc) were obtained from Clonetics (San Diego, CA, USA). The cells were usually used up to passage 5, but some repeat experiments used cells up to passage 10. HUVEc were grown on collagen-coated plates in medium M199 supplemented with 10 mM Hepes, 2.5 µg/ml thymidine, 140 USP units/ml heparin, 5 ng/ml human bFGF (Sigma), 20% fetal bovine serum (FBS; Gibco) as recommended by the supplier. HMVEc were grown on collagen-coated plates in endothelial growth medium with growth factors (EGM-2MV medium) (Clonetics).

Cell proliferation assay

HUVEc were seeded in 24-well plates at a density of 3×10^4 cells/well in 1 ml growth medium. After 24 h, cells were incubated with either U-PRL or S179D PRL in human endothelial serum-free medium containing bFGF, as before, and epidermal growth factor (EGF) at 10 ng/ml (Sigma) (SFM). Cells were further incubated for 72 h at 37°C. Cell number was determined using a colorimetric assay (Cell Titer; Promega) with the stringent modifications described previously (Huang *et al.* 2004).

Endothelial tubule formation

Matrigel with reduced growth factors (BD Biosciences, Palo Alto, CA, USA), diluted 1:1 with SFM was added (350 µl) to each well of a 24-well plate and allowed to polymerize fully at 37°C for 30 min. A suspension of 3×10^4 HUVEc/well in 400 µl SFM with reduced bFGF (2.5 ng/ml) supplemented with 5% FBS was transferred to each well. The cells were then treated with 1 µg/ml of either of the two forms of PRL. Cells were incubated for 24 h at 37°C in a humidified 5% CO₂ incubator and the tubule-like structures were observed and quantified in six random microscopic

fields towards the center of the well (at $\times 20$ magnification).

DNA content analysis

HUVEc were cultured for 3 days in SFM with reduced bFGF (2.5 ng/ml) containing 5% FBS and either U-PRL or S179D PRL (1 µg/ml). Cells were harvested by trypsinization, washed with cold Dulbecco's PBS (DPBS) and fixed with 75% ethanol in DPBS at 4°C for 30 min. Cell pellets were resuspended in 0.1% Triton X-100 (Sigma) in DPBS containing 200 µg/ml RNase (Sigma) and 10 µg/ml propidium iodide (Sigma), and incubated at room temperature for 30 min. The fluorescence of individual cells was measured with a FACScan cytofluorometer equipped with CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blotting and immunoprecipitation

To detect PRL receptor (PRLR) isoforms, equal quantities of lysate (1 mg protein) from treated and untreated HUVEc were immunoprecipitated with 1 µg mouse anti-PRLR which recognizes the receptor extracellular domain (Zymed, San Francisco, CA, USA; catalog number, 35–9200); the immunocomplexes were captured with 100 µl protein A-agarose bead slurry (Upstate Biotechnology, Inc., Lake Placid, NY, USA) and thoroughly washed. The beads were then resuspended in 50 µl SDS-loading buffer and, after boiling for 5 min, 20 µl of the supernatant were subjected to SDS-PAGE and subsequent transfer to a polyvinylidene fluoride (PVDF) membrane. Anti-PRLR was used at 1:1000 dilution. Antigen–antibody interactions were detected using horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence (ECL; Amersham). To detect activation of extracellular-regulated kinase (ERK) 1 and 2, cells were cultured in the absence of bFGF for 16 h and then treated with bFGF (25 ng/ml) or bFGF and S179D PRL (1 µg/ml) for the times indicated. Cell lysate proteins were resolved on the gel and anti-phosphoERK was used for Western blots (dilution 1:1000; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). The blots were stripped and re-probed with anti-total ERK (dilution 1:1000; Santa Cruz Biotechnology) to control for loading.

Cloning ring migration assay

A cloning ring, used to create a defined initial area of cells, was placed in the middle of a 35 mm Petri dish and a suspension of 10^4 HMVEc in 100 µl EGM-2MV

was poured inside the ring and incubated at 37°C for 40 min for cell attachment. After this period, the cloning ring was carefully removed and the edges of the circle formed, were marked. One milliliter of medium (plus the various treatments) was then added. The cells were treated for 3 days and cell migration was assessed on the third day.

Chemotaxis assay

HMVEc (10^6 cells) in 100 µl EGM-2MV medium were plated on the underside of transwell membranes (6.5 mm Transwell filter inserts; membrane pore size, 8 µm; Corning Costar Corporation, Corning, NY, USA) by inverting the insert. The cells were allowed to adhere for 30 min, and then the wells were turned right side up and medium was added to both chambers: 100 µl in the top chamber with 5 ng/ml bFGF alone, or bFGF plus U-PRL or S179D PRL (1 µg/ml); 1 ml in the bottom chamber with EGM-2MV only. The cells were allowed to migrate towards the bFGF for 4 h. In order to evaluate migration, the cells on the undersides of the chambers were removed by wiping them away with a cotton swab, and the membranes were then fixed and stained with 2% toluidine blue in 4% formaldehyde. Migrated cells on top of the filter were photographed and counted in ten fields per filter at $\times 10$ magnification to obtain the average relative number of migrating cells per treatment (Li *et al.* 2004).

Establishment of rat aorta culture

The proximal aorta was obtained from adult Sprague–Dawley rats as described previously by Wang *et al.* (2004), and 1 mm rings were sectioned. Twenty-four-well plates were pre-coated with 200 µl Matrigel (BD Biosciences) diluted 1:1 with EGM-2MV. An aortic ring was placed on the top of this layer and covered with additional Matrigel (200 µl). After Matrigel polymerization, 500 µl EGM-2MV containing 2% FBS (medium for preferential growth of endothelial cells according to Wang *et al.* (2004)) were added to each well and the rings were incubated for 24 h. After this incubation, fresh medium was added with either U-PRL or S179D PRL (1 or 2 µg/ml), and incubation continued for 8–10 days.

Qualitative and quantitative measurement of cell out-growth in the aorta ring assay

Qualitative assessment was performed as described previously (Wang *et al.* 2004) with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-*H*-tetrazolium

bromide) staining. Quantitative assessment used an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-*H*-tetrazolium) assay to measure the relative number of viable cells growing from the aorta rings. After removal of the ring of aorta, 300 µl freshly prepared solution containing 100 µg MTS and 1 µg PMS (phenazine methosulfate) in DPBS was added to each well and incubated for 24 h at 37°C. The medium used for this study preferentially selects for endothelial out-growth, but cell number will include some fibroblasts and smooth muscle cells (Hata-Sugi *et al.* 2002).

Gene expression by semi-quantitative and real-time RT-PCR

Total RNA from non-confluent HUVEc (2×10^6 cells/100 mm dish) was isolated with Trizol (Gibco/Invitrogen). The primers used for RT-PCR are given in Table 1. Expression was normalized to GAPDH mRNA for semi-quantitative RT-PCR, and to β -actin for real-time RT-PCR.

Semi-quantitative RT-PCR

Five micrograms of total RNA from each sample were used for cDNA synthesis using Super Script First Strand Synthesis (Invitrogen). A total volume of 2 µl cDNA from the RT reaction was used with the PCR Master Mix (Invitrogen). The amplification program consisted of 95°C for 2 min, followed by 30 cycles consisting of a denaturation step (95°C for 30 s), an annealing step (55 or 56°C) and an extension step (72°C for 30 s). The amplified products were detected by 1% agarose/Tris-borate EDTA (TBE) (0.089 M Tris and borate, 0.002 M Na-EDTA) gel electrophoresis and ethidium bromide staining.

Quantitative real-time RT-PCR

Twenty five microliter reactions were prepared in 96-well optical reaction plates (ABI Prism; Applied Biosystems) using SYBR green master mix (Applied Biosystems, Foster City, CA, USA). Using the ABI Prism 7700 (Applied Biosystems), samples were heated to 50°C for 2 min, then 95°C for 10 min, before 40 cycles at 95°C for 15 s and then 55 or 56°C for 1 min. Real-time data were analyzed using the comparative C_T method (Bustin 2000).

Protein array

In order to detect the influence of S179D PRL on the release of angiogenesis-related factors, an angiogenesis antibody array was used (Chemicon International,

Table 1 Oligonucleotide sequences employed

Primer name	5'-Oligonucleotide sequence-3'
PRLR-LF-reverse	GATTGTGATGCTCATCTGTTGGA
PRLR-LF-forward	TCCAGGTATGTGGGTTTCAT
PRLR-deleted-LF-forward	ATCATGATGGTCAATGCCACTA
PRLR-deleted-LF-reverse	TGGGGTTCCTCACACTTTTC
PRLR-SF1A-reverse	GATAGTGAGGACCAGCATCTAATG
PRLR-SF1A-forward	TGGACTGTGGTCAATGTTGC
PRLR-SF1B-reverse	CATGAATGATACAACCGTGTGG
PRLR-SF1B-forward	CAACATCAAGGGGTACACCTC
PRL-EXON2	GCAGTTGTTGTTGTGGATGATT
PRL-EXON5	GATGCCAGGTGACCTTCGAGA
BETA ACTIN-forward	AAAGACCTGTACGCCAACAC
BETA ACTIN-reverse	GTCATACTCCTGCTTGCTGAT
CTGF-forward	GAGGAAACATTAAGAACGGCAAA
CTGF-reverse	CGGCACAGGTCTTGATGA
TIMP-1 forward	CTGCGGATACCTCCACAGGTC
TIMP-1 reverse	GCAAGAGTCCATCCTGCAGTT
TIMP-2 forward	TTGAGAGTGGACCACACTGCGC
TIMP-2 reverse	CTGGCAACCTACAACAGACCC
IL-8 forward	ATAAGCAGGCCTCCAACGC
IL-8 reverse	GAGCTGGACCAGTCGAAACC
VEGF-forward	GCCTTGCTGCTCTACCTCCA
VEGF-reverse	CAAGGCCCCACAGGGATTTT
HO-1-forward	CAGGCAGAGAATGCTGAGTTC
HO-1-reverse	GATGTTGAGCAGGAACGCAGT
TSP-1-forward	CCCTTCAAAACAAATAGGAGTTCA
TSP-1-reverse	ATCCTGTGATTCCAAATGCCAG
ANG-2-forward	TGGGATTTGGTAACCCTTCA
ANG-2-reverse	GTAAGCCTCATTCCTTCCC
ANG-1 forward	GCAACTGGAGCTGATGGACACA
ANG-1reverse	CATCTGCACAGTCTCTAAATGGT
FGF-forward	TGTGCTAACCGTTACCTGGCT
FGF-reverse	CAGTGCCACATACCAACTG
ANGIOGENIN-forward	TGGGCGTTTTGTTGTTGGTCTTC
ANGIOGENIN-reverse	CGTTTCTGAACCCCGCTGTGG
FGFR-reverse	GCCAGCAGTCCCGCATCATCAT
FGFR-forward	GACGCAACAGAGAAAGACTTGT
VEGFR-1-reverse	GATGTAGTCTTTACCATCCTG
VEGFR-1-forward	CAAGTGCCAGAGGCATGGAGTT
VEGFR-2-reverse	TGCCAGCAGTCCAGCATGGTCTG
VEGFR-2-forward	GAGGGCCTCTCATGGTGATTGT
GAPDH-reverse	GGCATGGACTGTGGTCATGAG
GAPDH-forward	TGCCACCACCACTGCTTAGC

Inc. Temecula, CA, USA; CHEMIARRAY human angiogenesis antibody array). For this assay, a control consisting of HUVEc cultured in SFM plus bFGF (2.5 ng/ml) and EGF (1 ng/ml), and a treatment group with the same medium plus S179D PRL (1 µg/ml), was used. The conditioned medium was then retrieved after a 24-h incubation. Serum-free medium was chosen to reduce the background quantities of angio-active compounds. Normalization from array to array

utilized the positive control included by the manufacturer for this purpose.

Statistical analysis

All numerical data are presented as the mean $\pm$ s.e. Except where noted, all experiments used at least triplicates and were conducted a minimum of three times. Statistical significance was calculated using Student's *t* test to compare individual means with or without Bonferroni corrections, as appropriate. A *P* value of <0.05 was considered significant. For the corneal angiogenesis assay, the two groups were compared by Mann–Whitney test.

Animal subjects

All procedures involving the use of animals received prior approval from institutional committees and were in accordance with guidelines from the American Association for Laboratory Animal Care, the United States Department of Agriculture and the National Institutes of Health (NIH).

Results

In vivo assays

The chick CAM assay allowed investigation of the effects of U-PRL and S179D PRL *in vivo* in a tissue undergoing a normal angiogenic process. U-PRL had no discernable effect and hence was chosen as the control for purposes of illustration (Fig. 1). The inhibitory effect of S179D PRL on this angiogenic process is evident: apart from the original two blood vessels between which the pellets were placed, the samples treated with S179D PRL were essentially devoid of blood vessels within the area occupied by the pellet. Even beyond the perimeter of the pellet, the vessels appeared less substantial than their counterparts in the U-PRL control. The anti-angiogenic effects were quantified to produce Fig. 1C, where the avascular area within the pellet perimeter was measured. The anti-angiogenic activity of S179D PRL was almost completely reversed by the presence of an anti-PRL antibody.

In addition to testing the anti-angiogenic activity in the chick embryo system, S179D PRL was tested in the mouse corneal angiogenesis assay. In this assay, the normally avascular cornea develops blood vessels in response to an angiogenic compound such as bFGF. The bFGF is placed in a slow-release hydron pellet and a second pellet containing the potential anti-angiogenic material is placed alongside. The great sensitivity of

Figure 1 Effects in the CAM assay. (A) U-PRL-treated CAM was indistinguishable from the untreated CAMs. (B) S179D PRL-treated CAM. The white arrows mark the edges of the pellets, which contained a total of 1 μ g in the case of each PRL and 500 ng bFGF. (C) Quantification of the avascular area and results of blockade of the effect of S179D PRL with an antibody. # $P < 0.01$ versus saline control (C) or U-PRL.

this assay also allowed us to analyze much lower concentrations of S179D PRL. Because U-PRL had no angiogenic activity in the previous assay and preliminary experiments demonstrated no angiogenic activity *in vitro*, it was used in this part of the study as a control that differed from the S179D PRL by only one amino acid. As shown in Fig. 2, as little as 52 ng S179D PRL were able to reduce angiogenesis in response to 90 ng bFGF, by 50%. On a molar basis the S179D PRL was present at half the concentration of bFGF.

Effects on endothelial cell number

Since S179D PRL proved to be anti-angiogenic *in vivo*, the next set of studies was designed to examine the mechanism. First, we asked what forms of the PRLR were expressed by human endothelial cells. Figure 3A illustrates PCR products designed to be specific for the long form and for both short forms

of the human PRLR by recognition of the alternately spliced intracellular regions. The form-specific amplicons were sequenced in order to verify their identity. Figure 3B illustrates that the mRNA for both short forms is translated into protein recognizable by antibodies directed against the extracellular domain of the receptor. No band at the expected molecular mass for the long form was observed even though 1 mg protein was subjected to immunoprecipitation. Subsequent experiments determined that a significant proportion of what we had identified as the long form by PCR was missing part of the extracellular domain of the receptor, and hence was unlikely to be detected with the antibody used. Figure 3C shows the result of PCRs that indicate the presence of full-length long receptor (FLF) and a version of the long receptor with part of the extracellular domain deleted (DLF). Careful analysis of the structure and signaling capabilities of this form of the receptor is the subject of continued

Figure 2 S179D PRL is anti-angiogenic in the dual hydon pellet mouse cornea assay. (A) Scarcity of bFGF-induced (90 ng/pellet) angiogenic vessels in a S179D PRL-treated (52.5 ng per pellet) cornea. (B) Abundance of bFGF-induced angiogenic vessels in a U-PRL-treated (52.5 ng per pellet) cornea (control). Arrows indicate vessels penetrating the cornea from limbus. P denotes the location of the implanted hydon pellets. Scale bar, 250 μ m in A and B. (C) The magnitude of angiogenesis induced by bFGF was significantly smaller (0.2827 mm²) in corneas implanted with S179D PRL-containing hydon pellets compared with those treated with hydon pellets containing U-PRL (0.5717 mm²) (Mann–Whitney test, $P=0.0147$).

investigation, but a delta S1 version of the long receptor has been previously reported in a wide variety of tissues (Kline *et al.* 2002).

To determine the effect of S179D PRL on endothelial cell number, an assay was conducted that measured viable cell number after a 3-day incubation. In order to survive and proliferate in culture, human endothelial cells require the presence of bFGF, sufficient heparin for the bFGF to be biologically active and either serum or EGF. The effect of S179D PRL was therefore over and above the effect of these angiogenic medium components. As shown in Fig. 4A, high concentrations (500–1000 ng/ml) of S179D PRL

caused a decrease in viable cell number. In most assays, the response was modest at 25% (as illustrated), but was as large as 40% on occasion. Lower concentrations of S179D PRL were without significant effect in this 3-day incubation. High concentrations of U-PRL were also without effect. This served as one measure of the specificity of the response since U-PRL and S179D PRL only differ by one amino acid and each is made by recombinant technology in *Escherichia coli*. Since both molecules are expressed at very similar levels during production, they are therefore likely to contain very similar levels of contaminating substances. Because of the sensitivity of endothelial cells

Figure 3 Expression of PRLR in HUVEc. RT-PCR (A) and immunoprecipitation and Western blotting (B) for the PRLR isoforms. (A) Lane 1 contains a base pair ladder; lane 2, the long PRLR product (200 bp); lane 3, the short PRLR 1a product (174 bp); lane 4, the short PRLR 1b product (179 bp). (B) Lanes 1 and 2 show lysates of HEK cells transfected with equal amounts of vector coding for the short 1b form and short 1a form of the PRLR respectively to provide positive controls; lane 3 shows the untreated HUVEc immunoprecipitated extract. H, heavy chain; L, light chain; SF1a, short form 1a; SF1b, short form 1b. The numbers to the right indicate the molecular masses calculated from co-run molecular mass markers. (C) PCR amplicons from HMVEc which result from the presence of full-length and extracellularly deleted versions of the long form of the receptor. FLF, amplicon from the full-length long form of the receptor; DLF, amplicon from the extracellularly deleted long form of the receptor. Human prostate cancer cell line (PC3), PC3 cell positive control for the expression of the full-length long form; Ec, endothelial cell.

to bacterial endotoxins and the high concentrations of S179D PRL required to see an effect in the 3-day incubation, we measured the levels of endotoxin in each PRL and performed an endotoxin control. The level of endotoxin in the two recombinant PRLs was similar at less than 0.00166 EU/ μ g protein. An endotoxin control performed at more than 10-fold this concentration was without effect on viable cell number (labeled 'L' in Fig. 4A).

To be sure that the activity of S179D PRL was not due to the presence of misfolded protein (this occurs

more frequently with S179D PRL than U-PRL), conditioned medium from eukaryotic cells over-expressing each PRL was also analyzed (shaded bars in Fig. 4A). Eukaryotic cells have a number of quality control steps that eliminate misfolded proteins from the secretory pathway and a variety of chaperone proteins aid in the proper folding of secreted proteins (Krishna *et al.* 2004). The PRLs expressed in eukaryotic cells had indistinguishable activity from those expressed in *E. coli*. A final control conducted was to pretreat the *E. coli* proteins with 10 μ g/ml polymyxin-B. Polymyxin B binds and inactivates endotoxin. This also did not change the activity of the S179D PRL (Fig. 4B). We conclude therefore that although the effect of S179D PRL on human endothelial cells requires high concentrations under the conditions used, it is a specific response.

Analysis of DNA content at the end of the 3-day incubation period in S179D PRL indicated an 8-fold increase in the percentage of cells with sub G₀/G₁ quantities of DNA (Fig. 4C). Thus, cell death contributed to the decreased cell number. Once again, the endotoxin control showed no effect.

Effects on cell migration

In order to form blood vessels, endothelial cells have to be capable of migrating. We therefore tested the ability of S179D PRL to affect cell migration with and without a chemoattractant. New capillaries are most often formed from microvessels and so HMVEc were initially used for this part of the study. When HMVEcs were plated inside a cloning ring and subsequently allowed to migrate, S179D PRL reduced the distance migrated by about 40% (Fig. 5A). Since the distance migrated in this 3-day assay would have been affected by the number of cells and we know that S179D PRL reduces the number of HMVEc (data not shown) as well as HUVEc, we also performed a shorter-term, 4 h chemotaxis assay. In this assay, S179D PRL reduced the migration of HMVEc in response to bFGF by more than 60% (Fig. 5B).

Another measure of migration is the ability to form tubules when plated in Matrigel. Randomly plated cells must digest their way through the Matrigel and arrange themselves into tubular structures. S179D PRL decreased the ability of HUVEc to form tubule structures in Matrigel (Fig. 5C).

Although HUVEc and HMVEc are considered primary cells and were mostly used at passage numbers up to 5, we wanted to test the effect of S179D PRL on the migration of endothelial cells that had never been cultured. To do this, we analyzed the outgrowth of

Figure 4 Effects on HUVEc number and DNA content. (A) The response to 500 ng/ml (S) and 1 µg/ml (S') S179D PRL, U-PRL at 1 µg/ml (U) or endotoxin at 0.02 EU/ml (10 times the concentration in the recombinant protein preparations) (L) were without effect. The open bars show the recombinant protein expressed in *E. coli* and the shaded bars, the same concentrations of proteins expressed in eukaryotic cells. (B) Result with recombinant proteins from *E. coli* with (filled bars) and without (open bars) prior incubation with polymyxin B. (C) Results of flow cytometric analysis following propidium iodide staining of control cells (C), cells treated with 0.02 EU/ml endotoxin (LPS) and cells treated with S179D PRL (S) for 3 days. On the graphs, 1 is sub G₀/G₁ amounts of DNA, most often interpreted as resulting from apoptosis; 2 is G₀/G₁; 3 is G₂/M.

endothelial cells from rings of aorta placed in primary culture. Endothelial and associated cells migrate from the intima to form sprouts of capillary-like

structures. Observation of sprout formation after 8 days showed a marked reduction in the samples treated with S179D PRL (Fig. 5D). Upon removal of

Figure 5 Effects on endothelial cell migration and tubule formation. (A) The distance migrated in a 3-day incubation period; (B) The relative number of cells that have migrated in response to a gradient of bFGF during a 4-h period. (C) The ability of endothelial cells to form themselves into tubule-like structures in the absence and presence of either U-PRL or S179D PRL. Each form of PRL was used at 1 μg/ml for panels A to C. (D) The effect of each form of PRL (2 μg/ml) on vessel sprout formation from rat aortas. (E) Quantification of the relative number of cells in the sprouts. * $P < 0.05$ versus bFGF control in both panels A and B. Abbreviations as in previous figures.

the original ring of aorta, the cells that have grown out can be quantified by formazan dye conversion assays. The results in Fig. 5E indicate that a 50% decrease in relative cell number occurred with S179D PRL. Thus

it seems clear that S179D PRL affects both the number of viable endothelial cells and the migratory capacity of endothelial cells from large vessels and microvessels alike.

Figure 6 Effects of each form of PRL (1 μ g/ml) on mRNA expression in HUVEc. (A) Results of semi-quantitative PCR for 17 genes of interest and 2 control genes for normalization purposes (GAPDH and actin). TSP, thrombospondin; CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor; HO-1, heme-oxygenase-1; TIMP-1 and -2, tissue inhibitors of matrix metalloproteases 1 and 2. (B) Results of real-time PCR for the 8 genes most affected by treatment. * $P < 0.05$ versus control (C).

Effects on gene expression (mRNA)

To determine the mechanism whereby U-PRL and S179D PRL had such different effects on endothelial cells, we analyzed expression of PRLR and a variety of angiogenesis-related molecules. As illustrated in Fig. 6A, which shows the results of semi-quantitative PCR, U-PRL and S179D PRL had different effects on the expression of a number of genes. In this battery of tests, U-PRL increased expression of endogenous PRL and heme-oxygenase-1, but otherwise was without significant effect. This is illustrated more quantitatively in Fig. 6B where the results of real-time PCR are presented for the 8 of the 17 mRNAs most affected by treatment. U-PRL increased the expression of endogenous PRL by about 4-fold and doubled the

expression of heme-oxygenase-1. Production of PRL by HUVEc has been previously reported (Clapp *et al.* 1998, Corbacho *et al.* 2000). In contrast to U-PRL, S179D PRL down-regulated the expression of endogenous PRL (about one-half) and heme-oxygenase-1 (about one-fourth), had no effect on the expression of what we labeled the long PRL receptor (which would include both the full-length and the deleted version) and up-regulated (doubled) both short forms of the PRLR. S179D PRL also down-regulated expression of bFGF (about one-fourth) and vascular endothelial growth factor (VEGF; about one-half), without significant effect on the expression of their receptors. Thrombospondin, angiogenin, angiopoietins 1 and 2, and connective tissue growth factor were unaffected at the mRNA level. Thrombospondin and connective

Figure 7 Effects of S179D PRL (1 μ g/ml) on 24-h release of angio-active molecules in HUVEc. Quantification is from two arrays, each one containing two spots for each protein. NEG, negative control on the array; ANG, angiogenin. * P < 0.05 versus control; # P < 0.01 versus control.

tissue growth factor were included because expression of these two genes has been shown to be affected in the mammary gland of mice treated with S179D PRL (Naylor *et al.* 2005).

Effects on protein release

The 24-h conditioned medium was analyzed by protein array to test whether effects on gene expression were translated into effects on normally secreted proteins. The 24-h time point was chosen so as not to complicate analysis with the effects of non-specific release caused by apoptosis. Figure 7 illustrates results for all of the proteins on the array that corresponded to the gene expression analyses. S179D PRL decreased release of angiogenin, EGF and bFGF. For the latter two, the effect may have been even more pronounced than is indicated in the figure since both bFGF and EGF were present at low levels as part of the culture medium. S179D PRL therefore affects the amount of several angiogenic proteins secreted, thereby decreasing the probability that they will interact with their cognate receptors, forming an autocrine growth loop.

Of the other angio-active compounds examined at the protein level, the release of three of them was elevated by treatment with S179D PRL. These were the tissue inhibitors of metalloprotease 1 and 2, and surprisingly, VEGF.

Effects of S179D PRL on signaling from bFGF

After 16 h in the absence of bFGF, re-addition of bFGF initiates phosphorylation of ERKs 1 and 2 in HUVEc (Fig. 8A). The levels of phosphorylated ERKs were high from 5 to 30 min of incubation, declined

to basal levels by 60 min and remained low through 300 min. When S179D PRL was present, bFGF showed no ability to activate ERK between 0 and 30 min, but the combination showed strong activation of ERK at 60 min and activation above control levels through 240 min (Fig. 8B). U-PRL and endotoxin were without effect on bFGF activation of ERK (data not shown). Since the gels and blots for Fig. 8A and B were run at different times in order to have a long time course, the ability of S179D PRL to inhibit ERK phosphorylation in response to bFGF at 30 min was confirmed using the same gel and blot (Fig. 8C).

Discussion

In this study we have shown for the first time that S179D PRL can be a potent anti-angiogenic compound, with its potency best illustrated in the mouse corneal angiogenesis assay. S179D PRL was effective when tested on human macro- and microvessel endothelial cells *in vitro*, and on chicken and rodent blood vessel development *in vivo*. *In vitro* assays demonstrated an effect on viable endothelial cell number and an effect on the ability of endothelial cells to migrate and form tubes reminiscent of capillaries. S179D PRL also interfered with the ability of endothelial cells to migrate in response to a chemoattractant. The chemotaxis assay was conducted in a 4-h time frame and so results were largely separate from effects on cell viability. Thus it seems that S179D PRL has two, separate, direct effects on endothelial cells: a reduction in viable cell number and an inhibition of migratory capacity. The reduction in viable cell number after a 3-day incubation correlated well with the number of cells containing sub G_0/G_1 amounts of DNA. By far the largest part of the effect on viable cell number, therefore, seems to be by the promotion of apoptosis.

Promotion of apoptosis can be brought about by removal of growth factors, and S179D PRL reduced expression or release of four very important endothelial growth factors: angiogenin, EGF, bFGF and VEGF. At the time points used, VEGF was reduced only at the mRNA level and angiogenin was reduced only at the protein level. Some of the effects on VEGF were unexpected. First, although VEGF mRNA was reduced at 3 days, the amount of protein in the medium was elevated at 24 h. The angiogenin result suggests that this was not non-specific release. Instead, it may be an initial response to pre-apoptotic cell stress. One can reasonably expect, however, that with decreased mRNA there would later be decreased protein. Second, others have found that mixed PRL

Figure 8 Effect of S179D PRL on signaling from bFGF. HUVEc were incubated without bFGF for 16 h and then bFGF (25 ng/ml) was re-supplied to the cells. (A) Time course between 0 and 300 min for bFGF activation of ERKs 1 and 2. (B) Results of co-incubation of bFGF with S179D PRL (1 µg/ml). Since the gels and blots shown in panels A and B were run at different times, panel C shows a repeat of the result of co-incubation of S179D PRL and bFGF at the 30-min time point. The 0- to 60-min lanes on panels A and B were run on the same gel and the 120- to 300-min lanes on panels A and B were run on the same gel.

preparations elevate mRNA for VEGF in other cell types (Hovey *et al.* 2001, Goldhar *et al.* 2005), whereas we observed either no effect of U-PRL or a decrease with S179D PRL. This difference may be related to the type of cell and therefore to the types of PRLR present: most cells primarily express the long form of the receptor; in our study we found that a substantial proportion of what we initially thought was long receptor was in fact missing part of the extracellular domain and hence would probably have a decreased ability to bind ligand. The predominant forms that could be recognized with an antibody against the extracellular domain of the receptor were the two short forms of the receptor. Since we saw no effect on expression of the long form receptor in response to either form of PRL and a substantial proportion of this is unlikely to bind PRL very well,

it is likely that the signaling of relevance to the actions of both PRLs in endothelial cells originates at the short receptors.

Even though S179D PRL reduced VEGF, angiogenin, bFGF and EGF — thereby reducing the use of these autocrine growth loops — effects on cell number occurred in the presence of added bFGF and EGF because both are components of the culture medium. In addition, the anti-angiogenic effect seen in the corneal assay showed that S179D PRL could counteract the effect of bFGF. S179D PRL must therefore be able to block the effects of bFGF and not just its production. This was evidently not via an effect on the expression of the FGF receptor since no change in response to S179D PRL was observed, at least at the mRNA level. Since human PRL has been shown to bind to heparin (Khurana *et al.* 1999), one hypothesis we considered was that S179D PRL competed

effectively with bFGF for heparin, while U-PRL did not. When we pre-incubated S179D PRL with heparin, however, it did not change its anti-angiogenic activity (data not shown) and hence we have excluded this as a mechanism. Next, we considered the possibility that S179D PRL interfered with signal transduction from bFGF. Our results show that S179D PRL did indeed block normal ERK activation by bFGF and yet also showed a delayed and prolonged activation of ERK. Prolonged activation of ERK has been associated in other cell systems with signaling to apoptosis (Liu *et al.* 1996, Lessor *et al.* 1998) and is consistent therefore with the effects of S179D PRL in these cells.

S179D PRL also decreased expression of endogenous U-PRL, but our data at this time are inconclusive about an autocrine growth-promoting role for PRL in endothelial cells, since we were unable to demonstrate any effect of additional U-PRL on cell number *in vitro* or angiogenesis *in vivo*. These results are in accord with those of Clapp *et al.* (1993) who saw no effect on HUVEc at the same dose and no effect in the CAM assay at 5-fold the dose we used. Using bovine endothelial cells, Struman *et al.* (1999) were also unable to see an angiogenic effect of PRL *in vitro*. However, they did observe an effect in the CAM assay at 20-fold the concentration used in our study, as long as the CAMs were mature and normal blood vessel development was complete (i.e. they were older than the CAMs used in our study). The only piece of data supporting a relevant function for U-PRL in the present study is the effect on heme-oxygenase-1. Heme-oxygenase-1 has been shown to be protective against apoptosis (Ohta & Yachie 2004) and so, even though U-PRL did not increase the number of cells over those in the control, it may protect against apoptosis and, in that sense, be angiogenic under some circumstances. Consistent with this idea are the reports of anti-apoptotic effects of PRL in other cells (e.g. Kochendoerfer *et al.* 2003, Perks *et al.* 2004) and, conversely, the promotion of apoptosis by S179D PRL in other cells (Yang *et al.* 2002).

Others have demonstrated a long PRLR in bovine endothelial cells (Merckle *et al.* 2000), but the primers used would not have distinguished between the intact form and any forms missing part or all of the extracellular domain. It is possible therefore that bovine and other endothelial cells also express deleted versions of the long receptor. Up-regulation of the short forms by S179D PRL suggests that these varieties play a more important role in the anti-angiogenic response. These short receptor isoforms are produced by differential splicing of the same initial transcript as the long form (Hu *et al.* 2001,

Trott *et al.* 2003) and signaling through them by S179D PRL has previously been demonstrated in other cell types (Wu *et al.* 2003). As in the present study, this signaling resulted in long-term activation of ERK (Wu *et al.* 2003). At the same time, short-term activation of ERK in response to bFGF was inhibited. This result underscores the complex nature of intracellular signaling and the importance of long time-course analyses in response to ligands.

In vitro tubule formation, sprout formation and *in vivo* angiogenesis would each be decreased by the increased expression of the inhibitors of metalloproteases, as observed upon treatment with S179D PRL, since metalloproteases are required for migration through Matrigel and the outgrowth of sprouting vessels *in vitro* and *in vivo* (Chun *et al.* 2004).

In vivo blood vessel development involves the initial migration of endothelial cells and pericytes, in response to an angiogenic stimulus, followed by remodeling of the microvascular networks. During the process of neovascular sprouting, extensive cross-talk occurs between pericytes and endothelial cells (Folkman & D'Amore 1996, Lindahl *et al.* 1997, Hellstrom *et al.* 2001, Enge *et al.* 2002, Bergers *et al.* 2003, Pietras & Hanahan 2004, Baluk *et al.* 2005, Ozerdem 2005). S179D PRL therefore may also have direct or indirect effects on pericytes and other cells that contribute to the vessel wall. In this regard, the CAM assay results show that S179D PRL not only blocked initial development of vessels, but also appeared to limit wall development of pre-existing vessels. Although S179D PRL was very potent *in vivo*, the *in vitro* activities on HUVEc required much higher concentrations. This in part may reflect the need for a large and rapid effect in order for it to be seen in 3 days *in vitro*. It may also reflect important differences in the *in vitro* versus *in vivo* microenvironments. Additionally, it may suggest indirect mechanisms of action on cell types not present in the endothelial cell cultures, such as pericytes.

A cleaved and reduced form of PRL, so-called 16K PRL, has previously been reported to be anti-angiogenic (Clapp & Weiner 1992) and also seems to block ERK signaling from bFGF (Struman *et al.* 1999). S179D PRL is a more potent anti-angiogenic compound than 16K PRL *in vivo*. It is efficacious at half the molar concentration of bFGF used in the corneal assay, while 16K PRL requires an 8-fold excess (Duenas *et al.* 1999). By contrast, 16K PRL is more effective *in vitro* (Clapp & Weiner 1992, Clapp *et al.* 1993), perhaps suggesting a greater focus of 16K PRL on endothelial cells themselves. Other members of the PRL superfamily have also been reported to have

effects on angiogenesis that have been related to initiation and termination of placental neovascularization (Jackson *et al.* 1994), or to such pathological processes as diabetic retinopathy (Frystyk 2005).

In conclusion, we have demonstrated that S179D PRL is a potent anti-angiogenic factor *in vivo*. Part of the mechanism of action in endothelial cells is through decreased expression or release of a number of autocrine angiogenic factors, part is by interference with signaling from bFGF, and part is by disruption of cell migration, the latter most likely due to increased expression and release of inhibitors of matrix metalloproteases. S179D PRL is a molecular mimic of phosphorylated human PRL. The results therefore suggest that assessment is warranted of circulating phosphorylated PRL in conditions and disorders regulated by angiogenesis. Moreover, the results suggest that S179D PRL may be a useful anti-angiogenic therapeutic.

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