Matrix metalloproteases from chondrocytes generate an antiangiogenic 16 kDa prolactin

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Summary

The 16 kDa N-terminal fragment of prolactin (16K-prolactin) is a potent antiangiogenic factor. Here, we demonstrate that matrix metalloproteases (MMPs) produced and secreted by chondrocytes generate biologically functional 16K-prolactin from full-length prolactin. When incubated with human prolactin at neutral pH, chondrocyte extracts and conditioned medium, as well as chondrocytes in culture, cleaved the Ser155-Leu156 peptide bond in prolactin, yielding – upon reduction of intramolecular disulfide bonds – a 16 kDa N-terminal fragment. This 16K-prolactin inhibited basic fibroblast growth factor (FGF)-induced endothelial cell proliferation in vitro. The Ser155-Leu156 site is highly conserved, and both human and rat prolactin were cleaved at this site by chondrocytes from either species. Conversion of prolactin to 16K-prolactin by chondrocyte lysates was completely abolished by the MMP inhibitors EDTA, GM6001 or 1,10-phenanthroline. Purified MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13 cleaved human prolactin at Gln157, one residue downstream from the chondrocyte protease cleavage site, with the following relative potency: MMP-8>MMP-13>MMP-3>MMP-1=MMP-2>MMP-9. Finally, chondrocytes expressed prolactin mRNA (as revealed by RT-PCR) and they contained and released antiangiogenic N-terminal 16 kDa prolactin (detected by western blot and endothelial cell proliferation). These results suggest that several matrix metalloproteases in cartilage generate antiangiogenic 16K-prolactin from systemically derived or locally produced prolactin.

Key words: Angiogenesis inhibitor, Matrix metalloproteinases, 16K-prolactin, Chondrocytes, Proteolytic processing

Introduction

Cartilage is an avascular tissue where blood vessel invasion is highly restricted, except during endochondral bone formation (Harper and Klagsbrun, 1999; Erlebacher et al., 1995) or in degenerative joint diseases, such as osteoarthritis and rheumatoid arthritis (Walsh, 1999). Blood vessel invasion from the underlying bone can lead to cartilage resorption by triggering apoptosis of chondrocytes and degradation of the extracellular matrix (Gerber et al., 1999; Zelzer et al., 2004). Matrix metalloproteinases (MMPs) are a family of extracellular matrix-degrading enzymes that share common functional domains and activation mechanisms (Sternlicht and Werb, 2001). MMPs produced by chondrocytes are upregulated in association with blood vessel invasion (Ortega et al., 2003), and they are required for endochondral bone formation (Stickens et al., 2004) and for cartilage destruction in osteoarthritis (Billinghurst et al., 1997) and in rheumatoid arthritis (Tetlow and Woolley, 1995). Identification of factors controlling angiogenesis in cartilage can lead to the discovery of new pathways regulating cartilage remodeling and repair in both healthy and diseased states.

Because a variety of angiogenic factors are present in cartilage (Gelb et al., 1990; Twal et al., 1994; Harada et al., 1994), its avascularity probably results from naturally occurring inhibitors that prevent new vessel growth. Antiangiogenic factors identified in cartilage include inhibitors of proteases (DiMuzio et al., 1987; Folkman and Shing, 1992; Moses and Langer, 1991), chondromodulin-I (Hiraki et al., 1997), troponin-I (Moses et al., 1999), and metastatin (Liu et al., 2001). In addition, cartilage contains a group of angiogenesis inhibitors derived by proteolysis from larger proteins that are not themselves inhibitors of angiogenesis (Cao, 2001). Two such inhibitors are endostatin, a C-terminal fragment of collagen XVIII (O'Reilly et al., 1997), and angiostatin, an internal fragment of plasminogen (O'Reilly et al., 1994). Endostatin is expressed in cartilage (Pufe et al., 2004), and both endostatin and angiostatin are generated by the proteolytic action of MMPs and cathepsins present in cartilage (Felbor et al., 2000; Lijnen et al., 1998; Patterson and Sang, 1997; O'Reilly et al., 1999; Morikawa et al., 2000).

Another antiangiogenic domain is the 16 kDa N-terminal fragment of the hormone prolactin (PRL) (hereafter referred to as 16K-PRL). The 23 kDa parent molecule PRL (also referred to as 23K-PRL, full-length PRL or PRL) lacks inhibitory activity on endothelial cells (Clapp et al., 1993), whereas 16K-PRL inhibits angiogenesis in vivo and in vitro by suppressing growth-factor-induced endothelial cell proliferation (Clapp et al., 1993, Tabruyn et al., 2005), and stimulating the expression

of the type 1 plasminogen activator inhibitor (Lee et al., 1998) and endothelial cell apoptosis (Martini et al., 2000). The acidic-aspartyl endoprotease cathepsin-D has been claimed to be the protease responsible for cleaving full-length PRL to 16K-PRL (Baldocchi et al., 1993). However, the relevance of this protease is debatable because human PRL, unlike rat PRL, is resistant (Khurana et al., 1999a) or much less susceptible (Piwnica et al., 2004) to cleavage by cathepsin-D. In search of the biologically relevant PRL-cleaving protease, we reasoned that a likely source for such an enzyme would be an avascular tissue rich in antiangiogenic factors, such as cartilage. Here, we present the first report that MMPs produced by chondrocytes cleave PRL at a highly conserved site and generate biologically active 16K-PRL. We also provide evidence that MMPs may cleave PRL derived from circulation and PRL produced by chondrocytes.

Results

Proteolysis of human PRL by chondrocyte proteases

Incubation of human PRL with lysates from rat chondrocytes resulted in its partial conversion to fragments with apparent molecular masses of 17, 16, 15 and 14 kDa, as revealed by reducing SDS-PAGE (Fig. 1). The proportion of PRL fragments varied according to the pH of the incubation buffer. At pH 5, only the 14 kDa PRL fragment was produced; as the pH increased, the amount of this fragment declined and others became apparent, including the 16 kDa fragment which predominated at pH 7 (Fig. 1A). There was no proteolysis when PRL was incubated at neutral or acid pH in the absence of chondrocyte lysates (Fig. 1A,B). Addition of pepstatin-A, an inhibitor of cathepsin-D, to the incubation mixture completely abolished proteolytic cleavage by chondrocyte lysates at pH 5 but not at pH 7 (Fig. 1B). These results indicate

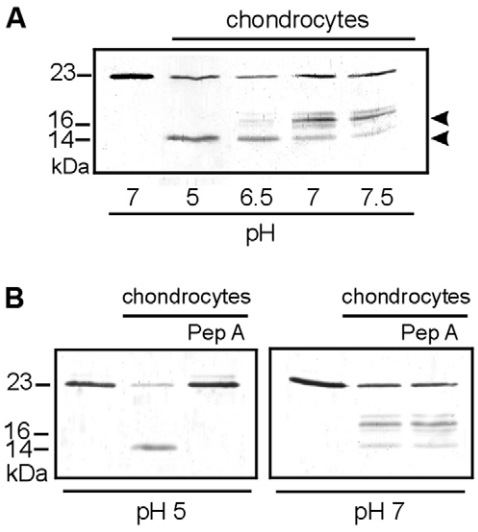

Fig. 1. Cleavage of human prolactin by chondrocyte lysates at different pHs. (A) Reducing western blot analysis of proteolytic products generated from PRL by incubation of 200 ng of human PRL with 2 μg of protein from rat chondrocyte lysates at various pH values. Arrows indicate 16 kDa and 14 kDa PRL fragments. (B) Effect of the cathepsin-D inhibitor, pepstatin-A on the acidic or neutral proteolytic activity of chondrocyte lysates. Migration of PRL isoforms of various relative molecular mass are shown on the left.

that proteases in chondrocytes can cleave PRL to generate predominantly 16 kDa and 14 kDa fragments. The acidic proteolytic activity responsible for the generation of the 14 kDa PRL fragment can be attributed to cathepsin-D, whereas a different protease(s) appears to cleave PRL to generate the 16 kDa and 14 kDa fragments at neutral pH.

Generation of 16K-PRL by neutral chondrocyte proteases is not species specific

To characterize the chondrocyte enzyme activity responsible for cleaving PRL at a neutral pH, we first determined whether, in addition to human PRL, rat PRL was susceptible to its action. Lysates from rat chondrocytes cleaved rat PRL mainly to a 16 kDa fragment and to minor fragments of 17 kDa and 15 kDa (Fig. 2A). In contrast to human PRL, no 14 kDa fragment was produced from rat PRL. Furthermore, lysates from chondrocytes from patients with osteoarthritis and from normal rat chondrocytes cleaved human PRL in an identical pattern, generating both the 16 kDa and the 14 kDa fragment (Fig. 2B). Cleavage was dose dependent, with higher protein concentrations resulting in the degradation of all PRL isoforms (Fig. 2B). Optical density values relative to that of the control PRL band incubated in the absence of chondrocyte proteins showed that, a loss of 23 kDa PRL and a similar generation and loss of 16K-PRL occurred with concentrations of human osteoarthritic chondrocyte proteins that were tenfold lower than those from normal rats (Fig. 2C). In addition, at neutral pH, lysates from human osteoarthritic chondrocytes cleaved human PRL at lower concentrations and with a different pattern than lysates from primary cultures of human skin fibroblasts, or from human U937 lymphoma cells (Fig. 2D). Lysates from human A549 lung adenocarcinoma cells were unable to cleave PRL at all protein concentrations tested (Fig. 2D).

The 16K-PRL generated by chondrocytes is the N-terminal fragment of PRL

Since there is evidence that PRL can be cleaved to yield either antiangiogenic N-terminal 16K-PRL or a 16 kDa C-terminal fragment devoid of antiangiogenic activity (Khurana et al., 1999a), we characterized the human PRL fragments generated by rat chondrocytes lysate using the monoclonal antibody INN-368 (which selectively recognizes the C-terminal end of the PRL molecule). INN-368 reacted with 23 kDa PRL and with PRL fragments of 15 kDa and 5 kDa, which gave a much weaker reaction with anti-human PRL antiserum (Fig. 3A). However, INN-368 did not recognize the 17 kDa, 16 kDa and 14 kDa fragments (Fig. 3A). Therefore, the 15 kDa and 5 kDa fragment contain a C-terminal PRL epitope that is either protected or absent in the 17 kDa, 16 kDa and 14 kDa PRL fragments.

The composition of PRL fragments was further investigated by N-terminal sequencing. In these experiments, larger amounts of human PRL digested by chondrocyte lysates at pH 7 were processed by reducing and non-reducing SDS-PAGE, and the proteins were blotted, stained with Coomassie Blue and recovered for N-terminal sequence analysis. Fig. 3B illustrates that under reducing conditions, the 16 kDa and 14 kDa fragments shared the same N-terminal sequence, LPICPGGA, as undigested human PRL, indicating that they correspond to N-terminal fragments. The 5 kDa fragment had the N-terminal sequence LQMADEE starting at residue

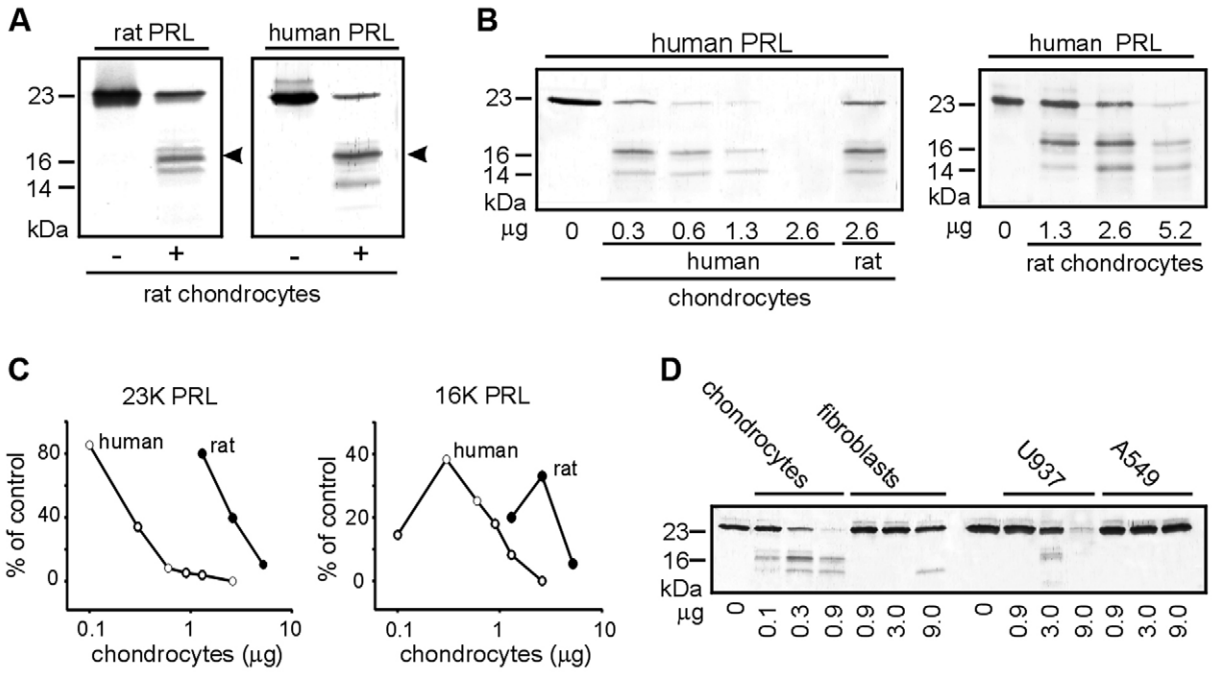

Fig. 2. Comparison of PRL-cleaving activity of lysates from rat and human chondrocytes and other human cells. (A,B) Reducing western blot analysis of the PRL proteolytic products obtained at pH 7 when 200 ng of rat or human PRL were incubated in the absence (–) or presence (+) of 2 µg of protein from lysates of rat chondrocytes (A), or when 200 ng of human PRL were incubated with the indicated protein concentrations of lysates from human or rat chondrocytes (B). Arrow indicates the 16K-PRL proteolytic product. (C) The PRL (23K-PRL) and 16K-PRL bands obtained after incubation with various protein concentrations of lysates from human or rat chondrocytes were evaluated densitometrically and the results are expressed as a percentage of the 23K-PRL band evaluated after incubation in the absence of chondrocytes. (D) Reducing western blot analysis of proteolytic cleavage products resulting from the incubation of 200 ng of human PRL with increasing concentrations of lysates from human chondrocytes, skin fibroblasts, U937 lymphoma cells, and A549 lung carcinoma cells. Positions of the PRL isoforms of the indicated relative molecular mass are shown on the left of western blots.

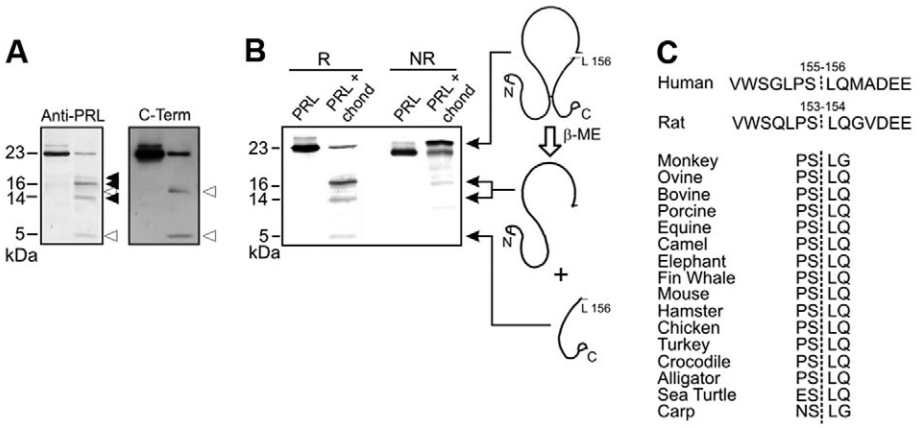

Fig. 3. Identification of PRL fragments generated by neutral proteases of chondrocytes. (A) Reducing western blot analysis of proteolytic products generated from human PRL by incubation with lysates from rat chondrocytes at pH 7 as revealed by the anti-human PRL antiserum (anti-PRL) or by monoclonal antibody INN-368 directed against the C-terminal region of PRL (C-Term). Black arrows indicate the PRL fragments detected by anti-PRL antiserum, and white arrows the PRL fragments detected by INN-368. (B) Western blot analysis under reducing (R) and non-reducing (NR) conditions illustrating the composition of proteolytic products generated from human PRL as revealed by their N-terminal sequencing. Under reducing conditions, the 16 and 14 kDa fragments gave the sequence LPICPGGA, corresponding to the N-terminus of undigested PRL, whereas the 5 kDa fragment had the N-terminal sequence LQMADEE starting at residue Leu156. Under non-reducing conditions, the 25 kDa PRL had both the N-terminal sequence of PRL and a second N-terminus starting at residue Leu156, indicating that it corresponds to a PRL cleaved between Ser155 and Leu156, which upon β-mercaptoethanol reduction (β-ME) yields the observed 16 and 5 kDa PRL products. Positions of the PRL isoforms of the indicated relative molecular mass are shown on the left of western blots. (C) Human and rat PRL amino acid sequences neighboring the chondrocyte cleavage site in the human (S155-L156) and the rat (S153-L154) hormones. The predicted chondrocyte cleavage site and its adjacent amino acids are shown for PRLs of different vertebrates.

Leu156 (Fig. 3B), suggesting that it is the peptide C-terminal to the 16 kDa human PRL. Under non-reducing conditions, most human PRL fragments remained linked by the intramolecular disulfide bond (Cys58-Cys174) because, apart from 23 kDa PRL, the major PRL variant detected was a slower migrating protein of the apparent molecular mass of 25 kDa, and only very low levels of the N-terminal 16 kDa fragment were detected (Fig. 3B). Moreover, the 25 kDa variant had both the N-terminal sequence of PRL and the N-terminus beginning at Leu156, so it corresponds to a form of PRL with a proteolytic cleavage at Leu156, resulting in two polypeptide chains, an N-terminal peptide and a C-terminal fragment starting at Leu156, that remained linked by an internal disulfide bond (Fig. 3B). Consistent with this interpretation, under reducing conditions this cleaved PRL disappears, and the 16 kDa N-terminal and 5 kDa C-terminal fragments become apparent (Fig. 3B). A fragment of PRL comprising residues 1-155 has a calculated molecular mass of 17.597 kDa, higher than the 'apparent' mass of 16 kDa estimated by reducing SDS-PAGE. This discrepancy suggests the excision of additional amino acids at the C-terminal end of the PRL fragment, before residue 155. However, the calculated mass of 5.319 kDa for PRL fragment 156-199 closely corresponds to the mass of 5 kDa estimated by reducing SDS-PAGE for the C-terminal fragment.

These findings indicate that proteases from chondrocytes cleave PRL at a neutral pH to generate N-terminal 16K-PRL. The identified cleavage site is within a highly conserved region of the PRL molecule, where both human and rat PRLs are nearly identical (Fig. 3C). Accordingly, a similar analysis with rat PRL (data not shown) indicated that chondrocyte proteases cleaved rat PRL between the Ser153-Leu154 peptide bond, a site conserved among all vertebrate PRLs (Fig. 3C).

N-terminal sequencing was also carried out on human PRL digested by chondrocyte lysates at pH 5. Non-reducing SDS-PAGE only detected PRL and a 25 kDa PRL (data not shown). The 25 kDa variant had the N-terminal sequence of human PRL and a second N-terminus IVSQVHP, starting at Ile133, which corresponds to PRL cleaved between Leu132- Ile133, a reported human PRL cleavage site for cathepsin-D (Piwnica et al., 2004). Furthermore, under reducing conditions a 14 kDa PRL was generated (Fig. 1) that had the N-terminal sequence of PRL and may correspond to residues 1-132 with a calculated mass of 15 kDa.

The 16K-PRL generated by chondrocytes inhibits endothelial cell proliferation

The 16K-PRL generated by chondrocyte lysates at pH 7 was tested for antiangiogenic activity against basic fibroblast growth factor (bFGF)-induced proliferation of endothelial cells in culture. To generate 16K-PRL, recombinant human PRL was incubated with chondrocyte lysates followed by reduction and carbamidomethylation of cysteine residues to block formation of intra- or inter-molecular disulfide bonds as reported (Ferrara et al., 1991). Because the 16K-PRL-preparation also contained intact PRL and chondrocyte proteins, a control preparation was used that lacked 16K-PRL but contained similar concentrations of PRL and chondrocyte proteins, and was subjected to reduction and carbamidomethylation. Non-reducing SDS-PAGE confirmed the presence or absence of 16K-PRL in test and control preparations, respectively, and that they did not differ in the concentration of PRL or of other significant protein contaminants (Fig. 4A). bFGF stimulated the proliferation of endothelial cells, and this effect was inhibited by the preparation containing 16K-PRL but not by the one containing only PRL (Fig. 4B).

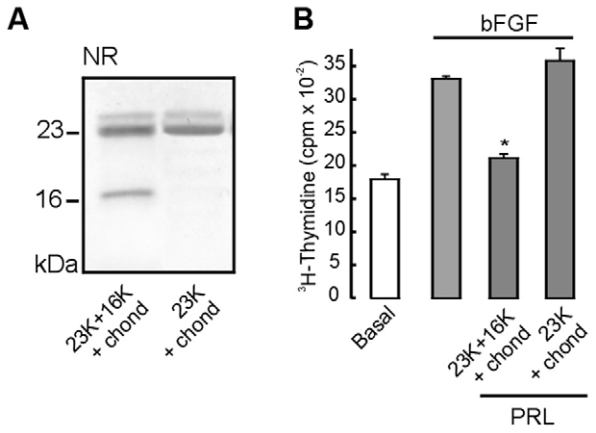

Fig. 4. The 16K-PRL generated by chondrocytes inhibits endothelial cell proliferation. (A) Coomassie-Blue-stained, non-reducing (NR) SDS-PAGE showing the PRL preparations tested for bioactivity. The preparation containing 16K-PRL was obtained by the incubation of human recombinant PRL with chondrocyte lysates, reduction, and carbamidomethylation as indicated in the methods section. In the preparation with no 16K-PRL, human PRL was added at the end of the incubation of chondrocyte lysates to avoid cleavage, and then the preparation was subjected to reduction and carbamidomethylation. The concentration of PRL in the two preparations was 200 nM and that of 16K-PRL was 100 nM as determined by non-reducing SDS-PAGE densitometry. (B) 16K-PRL inhibition of bFGF-induced proliferation of bovine umbilical vein endothelial cells. Endothelial cells were starved of serum for 24 hours and then allowed to proliferate in complete medium for an additional 24-hour period with or without 2 ng/ml bFGF and the mixture of PRL and chondrocyte proteins that did or did not contain 16K-PRL. Values are the mean ± s.e.m. of triplicate determinations *P<0.05 vs bFGF without 16K-PRL. Data are representative of three independent experiments.

The neutral PRL-cleaving proteases are secreted by chondrocytes and identified as MMPs

To investigate whether PRL can be cleaved extracellularly by neutral proteases secreted by chondrocytes, human PRL was incubated with chondrocyte-conditioned medium at pH 7. Reducing western blots showed that PRL was partially cleaved, primarily to a 16 kDa fragment and much less to a 17 kDa fragment, by chondrocyte-conditioned medium but not by non-conditioned medium (Fig. 5A). N-terminal amino acid sequencing under reducing and non-reducing conditions (Table 1) indicate that the 16K-PRL is the N-terminal fragment of the molecule that results from the reduction of PRL cleaved at Ser155-Leu156. Therefore, chondrocytes secrete the neutral PRL protease(s) that generates antiangiogenic 16K-PRL. Cleavage of 16K-PRL from PRL was completely abolished by heat-inactivation of chondrocyte-conditioned medium (last lane in Fig. 5B) or by the MMP inhibitors EDTA, 1,10-phenanthroline or GM6001 (Fig. 5B,C) but not by other protease inhibitors (Fig. 5B), including the serine-protease inhibitors aprotinin or PMSF (not shown), the cysteine protease

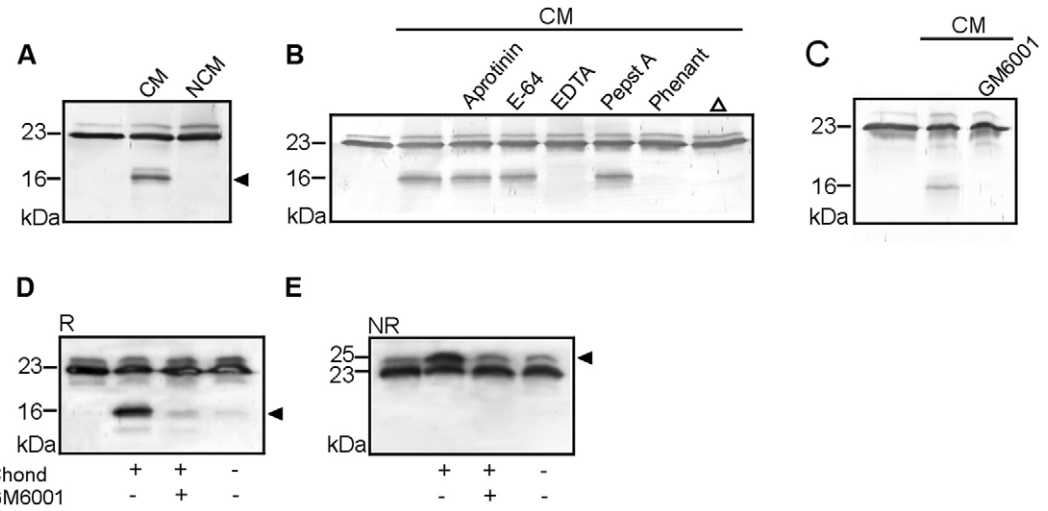

Fig. 5. The neutral PRL-cleaving proteases are secreted by chondrocytes and identified as MMPs. (A) Reducing western blot analysis of proteolytic products generated from PRL by incubation of 200 ng of human PRL with 5 μl of conditioned medium (CM) or non-conditioned medium (NCM), obtained after incubation with or without rat chondrocytes, respectively. Arrows indicate the 16 kDa fragment. (B) Reducing western blot analysis of proteolytic products generated from PRL by incubation of human PRL with non-conditioned medium (lane 1) or with chondrocyte-conditioned medium either alone (lane 2) or together with the serine protease inhibitor aprotinin (10 μg/ml), cysteine protease inhibitor E-64 (25 μg/ml), aspartyl protease inhibitor pepstatin A (Pepst A, 1.4 μM), MMP inhibitors EDTA (5 mM) and 1,10-phenanthroline (Phenant, 10 mM), or after heat inactivation (90°C for 30 min) of conditioned medium (Δ). (C) Reducing western blot analysis of proteolytic products generated from PRL by incubation of human PRL with chondrocyte conditioned medium in the absence or presence of the MMP inhibitor GM6001 (10 μg/ml). (D,E) Reducing (R) and non-reducing (NR) western blot analysis of proteolytic products generated from PRL by incubation of 8 μg of human PRL with or without 10^6 chondrocytes in the absence or presence of the MMP inhibitor GM6001 (10 μg/ml). Arrows indicate 16 and 25 kDa fragments. Positions of the PRL isoforms of the indicated relative molecular mass are shown on the left of western blots.

inhibitor E64 or the aspartyl protease inhibitor pepstatin-A. Furthermore, whole chondrocytes in culture generated 16K-PRL and the cleaved isoform from human PRL as indicated by reducing and non-reducing western blots (Fig. 5D,E), respectively, and this proteolysis was prevented by GM6001. Therefore, antiangiogenic 16K-PRL can be generated extracellularly by MMPs secreted by chondrocytes.

Purified MMPs generate N-terminal 16K-PRL

Several purified MMPs known to be present and secreted by chondrocytes were investigated to determine the nature of the putative MMPs responsible for the cleavage of PRL. Treatment of human PRL with purified MMP-8, MMP-13, MMP-1, MMP-2 and MMP-9 resulted in its partial conversion to predominant fragments of 17 kDa and 16 kDa as revealed by

Table 1. Estimated composition of human PRL cleaved products determined by N-terminal sequencing and 'apparent' molecular mass in SDS-PAGE

	Reducing		Non-reducing	
Lysate	16K-PRL	L^1 —— ?	25K-PRL	L^1 —— ? L^{156} —— C^{199}
	14K-PRL	L^1 —— ?		└ S — S ┘
	5K-PRL	L^{156} —— C^{199}	16K-PRL	L^1 —— ?
Conditioned medium	16K-PRL	L^1 —— ?	25K-PRL	L^1 —— ? L^{156} —— C^{199}
				└ S — S ┘
MMP-8, MMP-13	17K-PRL	L^1 —— L^{156}	25K-PRL	L^1 —— L^{156} Q^{157} —— C^{199}
	16K-PRL	L^1 —— ?		└ S — S ┘
MMP-3	17K-PRL	L^1 —— L^{156}	25K-PRL	L^1 —— L^{156} Q^{157} —— C^{199}
				└ S — S ┘
	12K-PRL	L^1 —— A^{111}	24K-PRL	L^1 —— A^{111} I^{112} —— C^{199}
				└ S — S ┘
MMP-1, MMP-2, MMP-9	17K-PRL	L^1 —— L^{156}		
	16K-PRL	L^1 —— ?	25K-PRL	L^1 —— L^{156} Q^{157} —— C^{199}
	16K-PRL	A^{54} —— C^{199}		└ S — S ┘
	12K-PRL	L^1 —— A^{111}		

Probable C-terminal residues are indicated in those cases where the calculated mass is similar to that estimated by SDS-PAGE.
└ S — S ┘ indicate disulfide bridges.

reducing western blots, whereas MMP-3 generated the 17 kDa fragment but not the 16 kDa fragment (Fig. 6A). In addition, smaller fragments of 12 kDa and 9 kDa were detected after incubation with MMP-3, MMP-1, MMP-2 and MMP-9. The 17 kDa, 16 kDa and 12 kDa PRL fragments had the N-terminal sequence of undigested PRL (Table 1), except for a part of the 16 kDa fragment generated by MMP-1, which had the N-terminal sequence AINSCH starting at Ala54 (Table 1). When the cleaved products were analyzed under non-reducing conditions, only 23 kDa PRL and a 25 kDa variant were detected after incubation with MMP-8 and MMP-13, whereas MMP-3, MMP-1, MMP-2 and MMP-9 generated an additional 24 kDa PRL isoform, and MMP-1 also produced a 16 kDa fragment (Fig. 6B). For all MMPs, the 25 kDa variant had the N-terminal sequence of PRL and a second N-terminal sequence QMADEE beginning at Gln157, one residue downstream from the chondrocyte protease cleavage site (Table 1). The 24 kDa PRL had the N-terminal sequence of PRL and a second N-terminus (ILSKAV) beginning at Ile112. Finally, the 16K-PRL generated by MMP-1 and evaluated under non-reducing conditions had the N-terminus starting at Ala54.

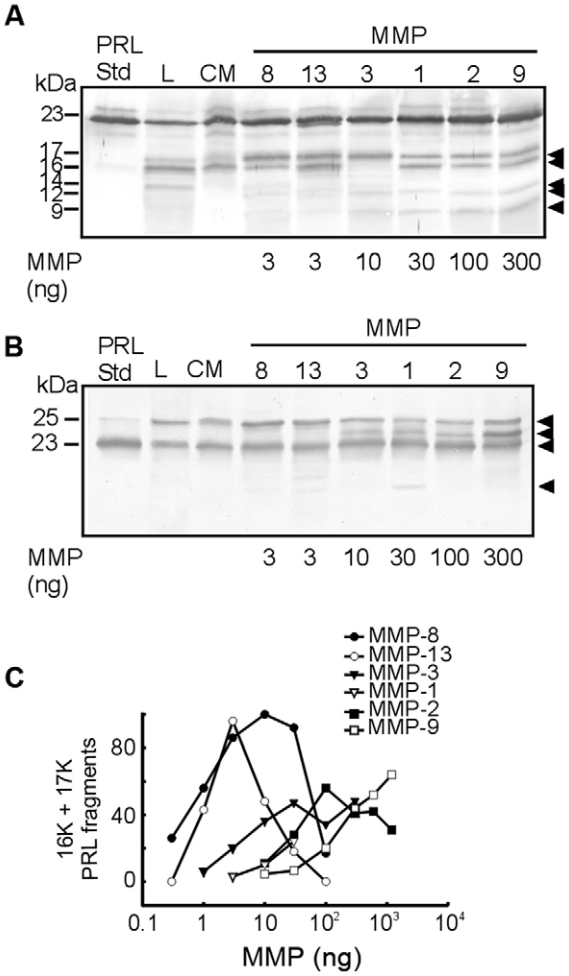

Fig. 6. Purified MMPs cleave PRL. Reducing (A) and non-reducing (B) western blot analysis of proteolytic products generated from PRL by incubation of 200 ng of human PRL with 2 μg of protein of rat chondrocyte lysate (L), rat chondrocyte-conditioned medium (CM), or with the indicated amount (ng of protein) of purified MMP-8, MMP-13, MMP-3, MMP-1, MMP-2 or MMP-9. The non-incubated human PRL standard (PRL std) is shown. Arrows indicate the various PRL proteolytic products and their relative molecular mass is indicated on the left of western blots. (C) The densitometric values of the 16-kDa-PRL and 17-kDa-PRL fragments generated by incubation with various concentrations of the purified MMPs were expressed as percentage of that of the 23-kDa-PRL band in the non-incubated PRL standard.

Cleavage of PRL at the Leu156-Gln157 peptide bond would yield, upon reduction, a fragment comprising residues 1-156 and a calculated mass of 17.710 kDa, which may correspond to the 17 kDa PRL observed under reducing conditions. Likewise, cleavage of PRL at the Ala111-Ile112 peptide bond would yield, upon reduction, fragments comprising residues 1-111 (12.593 kDa) and residues 112-199 (10.322 kDa), which may correspond to the observed 12 and 9 kDa PRLs, respectively. Finally, cleavage at Ala54 would yield a C-terminal fragment comprising residues 54-199 (16.861 kDa). This cleavage site precedes the intramolecular disulfide bond Cys58-Cys174 of PRL, so that the resulting C-terminal 16K-PRL can be detected in both reducing and non-reducing conditions.

The ability of the various MMPs to generate 17 kDa and 16 kDa N-terminal PRLs was compared by plotting the amounts (determined by densitometry after reducing SDS-PAGE) of the two PRL fragments generated by increasing MMP concentrations (Fig. 6C). MMP-8 and MMP-13 were the most potent, because they produced both PRL fragments at lower concentrations than the other MMPs (Fig. 6C). Also, MMP-8 and MMP-13 degraded both PRL fragments, whereas – at the concentrations tested – none of the other MMPs degraded any of the PRL isoforms (Fig. 6C). MMP-8 generated and degraded both PRL fragments at lower concentrations than MMP-13 (Fig. 6C). The other MMPs cleaved PRL with the apparent relative potency of MMP-3>MMP-1= MMP-2>MMP-9. Thus, the relative potency was MMP-8>MMP-13>MMP-3>MMP-1= MMP-2>MMP-9.

PRL mRNA, PRL and 16K-PRL are expressed in chondrocytes

The expression of PRL mRNA was demonstrated in rat chondrocytes by reverse transcriptase (RT)-PCR (Fig. 7A). Amplification yielded a single product with the expected length of 220 bp, similar to the control PCR band from rat PRL cDNA. No signal was detected in the negative control without reverse transcriptase. The presence of PRL proteins was investigated by western blotting, using INN-368 or INN-1 monoclonal antibodies that selectively recognize the C-terminal or the N-terminal end of PRL, respectively (Aranda et al., 2005). Selectivity was confirmed by comparing antibody binding to a mixture of purified rat standards of 23K-PRL, N-terminal 16K-PRL (residues 1-145), and C-terminal 6 kDa PRL (6K-PRL, residues 149-198) (Fig. 7B). The anti-rat PRL antiserum recognized all three rat PRL isoforms. The two monoclonal antibodies reacted with full-length PRL, but only INN-368 detected the C-terminal 6K-PRL standard and only INN-1 bound to the purified N-terminal 16K-PRL standard (Fig. 7B), confirming the location of the epitopes for each antibody. The anti-rat PRL antiserum reacted with chondrocyte proteins co-migrating with 23K-PRL and with 16K-PRL (Fig. 7B). These proteins were also detected by the N-Term antibody INN-1. However, the C-Term INN-368 antibody reacted only

with the 23K-PRL and not with the 16K-PRL-like protein, indicating that the C-terminal epitope is either protected or absent in this 16 kDa protein. The preceding experiments were carried out with chondrocyte lysate containing 200 μg of protein, so lysate used in the PRL-cleaving experiments (containing 2 μg of protein) is not enough to be detected by western blot analysis. Therefore, PRL-immunoreactive proteins in the cleaving experiments exclusively derive from the exogenous hormone.

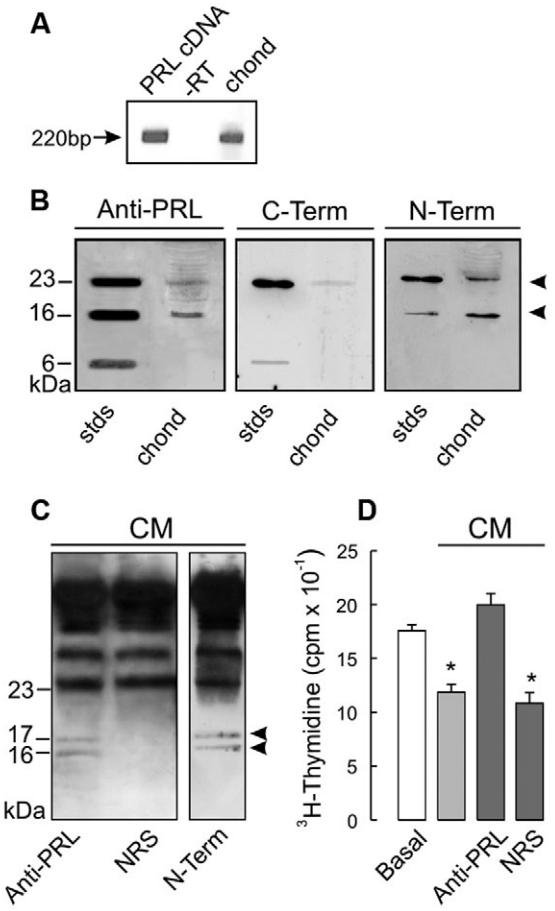

Fig. 7. PRL mRNA, PRL, and N-terminal 16K-PRL are detected in chondrocytes and in chondrocyte-conditioned medium. (A) RT-PCR product obtained from rat chondrocytes using primers specific for PRL (lane 3). PRL cDNA was used as a positive control (lane 1), and omission of reverse transcriptase served as a negative control (lane 2). (B) Reducing western blot analysis of immunoreactive PRL-like proteins in chondrocytes. Western blots with a mixture of purified standards (stds) of rat PRL, N-terminal 16K-PRL, and C-terminal 6K-PRL were run in parallel with 200 μg of chondrocyte lysate and were first probed with anti-rat PRL antiserum (anti-PRL), then stripped and reprobed with monoclonal antibody INN-368 directed against the C-terminal end of PRL (C-Term), and subsequently stripped and reprobed with monclonal antibody INN-1 directed against PRL N-terminal region (N-Term). (C) Western blot analysis of chondrocyte-conditioned medium immunoprecipitated with anti-PRL or normal rabbit serum (NRS), and blotted with either anti-PRL or INN-1. Arrows indicate immunoreactive proteins of 17 and 16 kDa. The relative molecular masses of PRL standards are indicated. (D) Proliferation of bovine umbilical vein endothelial cells incubated in the absence (basal) or presence of 10 μl of chondrocyte-conditioned medium alone or together with a 1:400 dilution of anti-PRL or NRS. Values are the mean ± s.e.m. of three independent experiments. *P<0.05 vs basal.

Antiangiogenic N-terminal PRL fragments are present in chondrocyte-conditioned medium

Immunoprecipitation-western blot analysis revealed the presence of PRL immunoreactive proteins of 17 kDa and 16 kDa in chondrocyte-conditioned medium (Fig. 7C). These proteins reacted with anti-rat PRL antiserum and with the N-Term antibody INN-1. The PRL nature of these proteins was confirmed by their absence when, instead of the anti-rat PRL antiserum, normal rabbit serum was used for immunoprecipitation. By contrast, proteins of higher molecular mass (≥23 kDa) were non-specific, because they were immunoprecipitated by both anti-PRL antiserum and normal serum. Based on their affinity for the N-terminal specific monoclonal antibody, we conclude that the 17 kDa and 16 kDa proteins correspond to N-terminal fragments of PRL. To investigate whether these PRL fragments have antiangiogenic properties, the effect of chondrocyte-conditioned medium was evaluated on the proliferation of endothelial cells in culture in the absence or presence of anti-PRL antiserum or normal serum (Fig. 7D). Chondrocyte-conditioned medium inhibited the proliferation of endothelial cells in culture, and this inhibition was reversed by anti-rat PRL antiserum but not by normal serum (Fig. 7D). This finding indicates that the antiangiogenic effect of chondrocyte-conditioned medium depends on PRL-like antigens that might correspond to the N-terminal 17 kDa and 16 kDa PRL fragments.

Discussion

These studies demonstrate for the first time that MMPs cleave PRL to generate antiangiogenic 16K-PRL. 16K-PRL blocks a variety of endothelial cell functions (Corbacho et al., 2002; Gonzalez et al., 2004; Lee et al., 2005) and inhibits angiogenesis in ocular tissues (Dueñas et al., 1999; Pan et al., 2004; Aranda et al., 2005) and in tumors (Bentzien et al., 2001; Kim et al., 2003). The specific enzymes responsible for generating 16K-PRL are largely unknown. MMPs cleave various proteins to release their bioactive forms, including the antiangiogenic fragments angiostatin (Patterson and Sang, 1997; Lijnen et al., 1998; O'Reilly et al., 1999) and endostatin (Lin et al., 2001). Considering that the release of antiangiogenic fragments occurs in avascular tissues such as cartilage (Pufe et al., 2004), we focused on whether chondrocyte MMPs could process PRL to 16K-PRL.

We demonstrate that MMPs produced and secreted by chondrocytes cleave PRL to generate biologically active 16K-PRL. Cleavage occurs at Ser155-Leu156, a highly conserved site in PRL, indicating that this processing can occur in all vertebrate species. Consistent with this suggestion, both rat and human PRL were cleaved at this site by either human or rat chondrocytes. However, the MMPs in chondrocytes that are responsible for cleaving PRL remain unclear. MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13 are produced in chondrocytes (Barrett et al., 1998), but the purified enzymes cleaved PRL between Leu156-Gln157, one residue downstream from the chondrocyte cleavage site. Whereas this difference may imply that none of these MMPs is the active chondrocyte enzyme, it is also possible that some of them are

indeed the endogenous PRL proteinases, but that their site of cleavage is altered by the complex chondrocyte setting where many MMPs are present, compartmentalized and differentially activated. Indeed, cleavage by MMPs is known to vary, depending on enzyme affinity and the presence of other substrates, such as other MMPs and proteinases (Parks et al., 2004). MMP-8 and MMP-13 could be the enzymes cleaving PRL in chondrocytes, because they proved to be the most efficient in producing 16 kDa and 17 kDa N-terminal PRL fragments. Also, PRL-cleavage was associated exclusively with human chondrocytes and not with other human cells and cell lines tested; MMP-8 and MMP-13 are restricted to few cell types including chondrocytes (Cole et al., 1996; Barrett et al., 1998), and their specificity is directed towards type II-collagen (Barret et al., 1998; Sternlicht and Werb, 2001). Finally, both MMP-8 and MMP-13 are upregulated in osteoarthritis (Billinghurst et al., 1997; Tetlow et al., 2001), and we show that chondrocytes from patients with osteoarthritis were more potent in cleaving PRL than normal rat chondrocytes.

The fact that MMPs generate antiangiogenic PRL fragments could imply that these enzymes produce PRL peptides that contribute to the prevention of vessel growth under normal conditions. However, MMP-8 and MMP-13 are upregulated in osteoarthritis, where angiogenesis occurs (Billinghurst et al., 1997; Tetlow et al., 2001), and mice deficient for both MMP-13 and MMP-9 exhibit reduced vascularization in the area of the growing bones (Stickens et al., 2004). Whereas these observations indicate that proangiogenic actions of these MMPs predominate, it is possible that such predominance reflects the degradation of antiangiogenic factors by elevated levels of MMPs associated with disease. Accordingly, osteoarthritic chondrocytes degraded PRL more efficiently than normal chondrocytes, and higher levels of MMP-8 and MMP-13 potently degraded PRL. Likewise, in MMP-13- and MMP-9-null mice, reduced vascularization could involve the increased production of angiogenesis inhibitors by redundant mechanisms compensating for the loss of the respective enzymes. Future studies addressing the relative contributions of MMPs to PRL cleavage in normal and diseased states, their relative expression levels, degree of activation and relative access towards exogenous and endogenous PRL should help clarify these issues.

Our discovery that MMPs generate antiangiogenic PRL peptides helps to resolve the open question of how PRL fragments are formed under physiological conditions. This is particularly relevant, because the only enzyme reported to convert PRL to antiangiogenic 16K-PRL was cathepsin-D, which has been challenged as the enzyme performing this function in vivo due to its reduced ability to cleave human PRL (Khurana et al., 1999a; Piwnica et al., 2004) and to its controversial activity level at the neutral pH of physiological fluids (Khurana et al., 1999a; Lkhider et al., 2004). Here, we found that cathepsin-D had no activity at neutral pH, but reported that at pH 5 chondrocyte cathepsin-D cleaved a large part of human PRL at a single site to generate a 14 kDa N-terminal fragment. These results might imply that interactions with endogenous activators in chondrocytes can augment the efficiency and increase the specificity of cathepsin-D to cleave human PRL. However, PRL cleavage by cathepsin-D would be limited to an acid pH compartment and may not occur in the extracellular space of normal chondrocytes.

Conversely, in cartilage, most PRL cleavage by MMPs probably occurs extracellularly. PRL is a component of human synovial fluid (Ogueta et al., 2002) that possibly derives from plasma, since most proteins with a molecular mass of less than 100 kDa readily transfer from one fluid space to the other (Perman, 1980). MMPs are secreted or anchored to the cell surface, so that PRL that diffuses through cartilage from the synovial fluid could be converted to 16K-PRL in the extracellular space and/or at the target-cell membrane, locations compatible with the physiological site of action of this peptide. Also, MMPs are known to release growth factors embedded in the extracellular matrix (Mott and Werb, 2004). PRL can bind to heparin (Khurana et al., 1999b) and MMPs could act on PRL bound to heparin sulfate proteoglycans to increase 16K-PRL bioavailability. In support of the extracellular processing of PRL in cartilage, we showed that MMPs that convert PRL to 16K-PRL are present in condrocyte-conditioned medium, and that MMPs cleave PRL added to whole chondrocytes in culture.

On the other hand, 16K-PRL can be generated by the cleavage of PRL produced by chondrocytes themselves. Our study shows for the first time that chondrocytes express PRL mRNA, PRL and N-terminal 16K-PRL. This finding is consistent with the reported expression of PRL mRNA in mesenchymal progenitor cells during chondrogenic differentiation (Ogueta et al., 2002), and it substantiates the generation of 16K-PRL by normal cartilage. The fact that the 16K-PRL fragment is found in chondrocyte lysates suggests that the cleavage of PRL can also occur intracellularly. Intracellular cleavage by MMPs has been reported for other substrates (Wang et al., 2002; Sawicki et al., 2005) and angiostatin has been found to accumulate in the cytoplasm of prostate carcinoma cells (Migita et al., 2001). Furthermore, we show that chondrocytes can secrete antiangiogenic PRL fragments in sufficient amounts to inhibit angiogenesis. Chondrocyte-conditioned medium inhibited endothelial cell proliferation, and this effect was dependent on N-terminal PRL fragments of 17 kDa and 16 kDa.

Inhibition of angiogenesis is important to maintain the vitality and function of cartilage (Gerber et al., 1999; Walsh, 1999). We hypothesize that formation of antiangiogenic 16K-PRL is one of the mechanisms contributing to cartilage avascularity. However, this in vivo role needs to be demonstrated. Targeted disruption of either PRL or the PRL receptor has not been particularly useful for dissecting the in vivo role of these peptides in angiogenesis. The PRL gene was mutated by a targeted insertion that truncated the protein at residue 117, leaving an intact N-terminal PRL fragment of 11 kDa that might retain antiangiogenic properties and therefore exhibit no change of phenotype (Horseman et al., 1997). Also, these PRL fragments do not appear to signal through the PRL receptor but rather through a distinct, endothelial cell surface receptor (Clapp and Weiner, 1992), whose activity would be unaltered by targeted deletion of the PRL receptor. However, by using RNAi silencing and antibody-neutralization experiments in the eye, endogenous PRL fragments were shown to suppress angiogenesis in healthy ocular tissue (Aranda et al., 2005). We are currently using similar approaches to investigate the antiangiogenic actions of PRL fragments in the joint.

Other important aspects of our work that warrant further

investigation are the molecular and cellular mechanisms leading to reduction of cleaved PRL. Whereas MMPs and cathepsin-D from chondrocytes efficiently cleaved PRL, and 16K-PRL was detected under reducing conditions, only minor levels – if any – were seen in non-reducing gels. Disulfide-bond reductases that require reduced glutathione or cysteine as cofactors are known to be involved in angiostatin formation (Stathakis et al., 1999). Also, thioltransferases requiring reduced glutathione and NADPH are present in the pituitary gland and can reduce intra- and inter-molecular disulfide bonds within PRL and its oligomeric forms (Lorenson et al., 1984). Because 16K-PRL has been detected in pituitary extracts under non-reducing conditions (Torner et al., 1995), the study of pituitary gland reductases should help clarify the mechanisms involved in the reduction of cleaved PRL.

Finally, the N-terminal fragments of 17 kDa, 16 kDa, 14 kDa or 12 kDa generated by the various MMPs are similar to antiangiogenic N-terminal PRL fragments of 16 kDa and 14 kDa produced from recombinant DNA (Clapp et al., 1993; Struman et al., 1999), and to antiangiogenic N-terminal fragments of 17 kDa, 16 kDa and 11 kDa generated by the proteolysis of PRL with cathepsin-D (Ferrara et al., 1991; Baldocchi et al., 1993; Piwnica et al., 2004). Therefore, an important conclusion of these and previous studies is that antiangiogenic molecules derived from PRL are not a single entity, but rather a family of peptides with different molecular masses, all containing the N-terminal region of PRL. We introduce the name ‘vasoinhibin’ to define the collection of N-terminal fragments of PRL having inhibitory actions on vascular endothelial cells. We propose that the endogenous production of vasoinhibin in the various tissues involves the action of MMPs, rather than the previously proposed cathepsin-D, and that such cleavage is of major significance in avascular tissues such as cartilage. Future work is required to demonstrate the role of these peptides in the control of cartilage avascularity and its alteration by disease.

Materials and Methods

Reagents

Rat and human PRL were from the National Hormone and Pituitary Program (NHPP, Torrance, CA), and human recombinant PRL generated in *Escherichia coli* was provided by Michael E. Hodsdon (Yale University, New Haven, CT). The N-terminal 16K-PRL and the C-terminal 6K-PRL fragments were generated by enzymatic proteolysis of rat PRL with a particulate fraction from rat mammary glands followed by gel filtration and carbamidomethylation as described (Clapp, 1987). Collagenase type-2 was from Worthington Biochemical Corporation (Lakewood, NJ), and bFGF, trypsin and culture medium-reagents from Gibco-Invitrogen Corporation, (Carlsbad, CA). MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13 were from R&D Systems (Minneapolis, MN), and MMP-1 was from Sigma Chemical Co. (St Louis, MO). All protease inhibitors were from Sigma except the MMP inhibitor N-[2(R)-2-(hydroxamido carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (referred to as GM-6001), which was from Calbiochem (San Diego, CA). Anti-rat PRL and anti-human PRL antisera were obtained and characterized as reported (Dueñas et al., 1999; Dueñas et al., 2004). Anti-rat PRL (INN-1; N-Term) and anti-human PRL (INN-368; C-Term) monoclonal antibodies that react with the N-terminal or the C-terminal end of PRL, respectively (Aranda et al., 2005), were provided by Peter Berger (Austrian Academy of Science, Innsbruck, Austria).

Chondrocyte cultures

Articular chondrocytes were isolated from human osteoarthritic femoral condylar cartilage obtained through arthroplasty of the knee joints from patients with osteoarthritis, diagnosed using the criteria of the American College of Rheumatology (Altman et al., 1986), or from femoral epiphyseal cartilage of male Wistar rats (130-150 g body weight) as described (Shakibaei et al., 1997) with modifications. Briefly, cartilage was cut into small slices, rinsed in solution A (10 mM glucose, 3 mM KCl, 130 mM NaCl, 5.25 mM Na_2HPO_4) and digested with 0.25% (w/v) trypsin in solution A for 10 minutes (rat) or 30 minutes (human) at room temperature, and then with 0.2% collagenase type-2 in the same solution for 2 hours (rat) or 24 hours (human) at 37°C. Single-cell suspensions were obtained by rinsing the cells in 0.9% NaCl, 1 mM EGTA, repeated pipetting and separation from undissolved tissue fragments using a nylon mesh with a pore-width of 50 μm. Cells were sedimented by centrifugation and either resuspended in lysis buffer [0.5% Nonidet P-40, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 1 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), pH 7], or seeded at 2×10^5 cells/cm^2 and incubated in growth medium [Dulbecco’s modified Eagle’s medium (DMEM), 5% fetal bovine serum (FBS), 50 mg/ml gentamycin] for 24 hours at 37°C. After incubation, chondrocyte-conditioned medium was collected, clarified by centrifugation (1200 ***g*** for 10 minutes), concentrated fivefold to tenfold (Centricon 3, Amicon Millipore, Billerica, MA), and stored at –70°C. Lysates from non-cultured chondrocytes were clarified by centrifugation and stored at –70°C until assayed for PRL-cleaving proteases or for the presence of the endogenous PRL fragments. For comparison, human skin fibroblasts isolated as described previously (Zhou et al., 2004), the human A549 lung adenocarcinoma cell line and the human U937 lymphoma cell line were used.

PRL cleavage analyses

The activity of the enzymes that cleave PRL to 16K-PRL was assessed by incubation of 200 ng of rat or human PRL in 5 μl of incubation buffer (0.05 M Tris-HCl, 0.15 M NaCl, and 0.01 M $CaCl_2$, pH 6.5, 7, or 7.5) with either 2 μg of protein from chondrocyte lysate or 5 μl of chondrocyte-conditioned medium in a final volume of 20 μl for 24 hours at 37°C. For incubations at pH 5, 200 ng of human PRL in 5 μl of 0.1 M Tris (pH 7.4) were mixed with 2 μg of chondrocyte lysate protein in a final volume of 20 μl of 0.1 M citrate-phosphate buffer pH 5, containing 0.15 M NaCl. Purified MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13 were activated with p-aminophenylmercuric acetate (APMA) at a final concentration of 1 mM according to the manufacturer’s instructions, and were tested at the indicated concentrations under the pH 7 incubation conditions. In separate experiments, the aspartyl protease inhibitor pepstatin-A (final concentration, 1.4 μM), cysteine protease inhibitor E64 (25 μg/ml), serine protease inhibitors aprotinin (10 μg/ml) and phenylmethylsulfonyl fluoride (5 mM), or MMP inhibitors 1,10-phenanthroline (10 mM), EDTA (5 mM) and GM6001 (10 μM), were preincubated for 10-30 minutes at room temperature with 5 μl of chondrocyte-conditioned medium before adding 200 ng of human PRL and adjusting to a final volume of 20 μl with incubation buffer. In all cases, the reaction was stopped by the addition of reducing or non-reducing Laemmli buffer followed by boiling the samples for 5 minutes and fractionation on 15% SDS-PAGE. Cleavage of PRL by chondrocytes in culture, was determined by the incubation of 8 μg human PRL with 10^6 chondrocytes in 600 μl of growth medium for 24 hours in the absence or presence of 10 μM GM6001. Cleaved PRL products (in 20 μl of culture medium) were investigated on reducing or non-reducing SDS-PAGE.

Western blotting

To analyze cleaved products of PRL, the incubation samples resolved on SDS-PAGE were transferred to nitrocellulose membranes and blots were probed with a 1:2000 dilution of anti-rat PRL or anti-human PRL antisera. The antigen-antibody complex was detected by using the alkaline phosphatase second antibody kit (Bio-Rad Laboratories, Hercules, CA) or horseradish peroxidase (HRP)-coupled secondary antibodies and enhanced chemiluminescence (ECL, superSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL). To assay for endogenous PRL fragments, lysates from 2×10^6 chondrocytes (200 μg of protein) were subjected to SDS-PAGE on a 15% acrylamide gel under reducing conditions, blotted and probed with the anti-rat PRL antiserum (1:1000). Membranes were then stripped and reprobed with INN-368, the C-Term anti-PRL monoclonal antibody (1:500), and subsequently stripped and reprobed with INN-1, the N-Term anti-PRL monoclonal antibody (1:1000). Detection was by ECL using HPR-conjugated secondary antibodies. Western blot analysis was also performed in chondrocyte-conditioned medium (500 μl) immunoprecipitated with anti-rat PRL antiserum or normal rabbit serum, as previously described (Corbacho et al., 2000).

Protein sequencing

Amino acid sequence analysis was performed on the major protein bands produced by cleavage of 10 μg of rat or human PRL with chondrocyte lysate (5 μg of protein) or with 10 μl of chondrocyte-conditioned medium in a final volume of 40 μl adjusted with incubation buffer pH 7. Similarly, sequencing analysis was performed on the major cleavage products obtained after incubation of 10 μg of human PRL with 50 ng of purified MMP-1, 1.5 μg of MMP-2, 100 ng of MMP-3, 30 ng of MMP-8, 1.5 μg MMP-9, or 50 ng of MMP-13. After incubation, samples were resolved by reducing or non-reducing 15% SDS-PAGE, and the proteins were transferred to Sequi-Blot PVDF membranes (Bio-Rad, Hercules CA), visualized with 0.1% Coomassie blue R-250, excised, and sequenced by automated Edman degradation using the Applied Biosystems Procise 491 Sequencer (Foster City, CA).

Endothelial-cell-proliferation assay

Bovine umbilical vein endothelial cells (BUVECs) obtained as previously described

(Cajero-Juárez et al., 2002), were cultured in F12K medium with 10% FBS and 50 U/ml penicillin-streptomycin. Proliferation assays were carried out as reported (Gonzalez et al., 2004). Briefly, BUVECs were seeded at 5000 cells/cm^2 in complete culture medium and allowed to attach 3-4 hours. Cells were then serum-starved for 24 hours with 0.5% FBS-culture medium, and the medium was replaced with complete 10% FBS-culture medium containing 2 ng/ml of bFGF with or without PRL or a mixture of PRL and 16K-PRL generated by incubation with chondrocyte lysate. In separate experiments, BUVECs were maintained in the 0.5% FBS-culture medium and treated with 10 μl of chondrocyte-conditioned medium in the absence or presence of a 1:400 dilution anti-rat PRL antiserum or normal rabbit serum. After treatment, BUVECs were allowed to proliferate for 24 hours and pulsed for the last 12 hours with 0.6 μCi [^{3}H]thymidine per 15-mm-well as described (Ferrara et al., 1991). The preparation containing PRL and 16K-PRL was obtained by incubating ten tubes, each containing 50 μg of recombinant human PRL mixed with 7 μg of protein from chondrocyte lysate in a final volume of 60 μl incubation buffer pH 7, for 24 hours at 37°C, followed by reduction and carbamidomethylation of cysteine residues to stabilize the resulting 16K-PRL fragment. Carbamidomethylation is known to increase the potency of 16K-PRL and was performed as reported (Ferrara et al., 1991). The control preparation consisted of chondrocyte lysate to which PRL was added at the end of the 24 hours incubation period (to avoid cleavage) followed by reduction and carbamidomethylation. The concentration of PRL in the two preparations was the same (200 nM), and that of 16K-PRL was 100 nM as evaluated by non-reducing SDS-PAGE and densitometry.

RT-PCR

Immediately after isolation, chondrocytes were frozen at –70°C. Total RNA was extracted and quantified, and 5 μg was reverse transcribed in a 25 μl reaction using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Two μl aliquots were used for cDNA amplification by PCR (30 seconds at 94°C, 45 seconds at 65°C, and 45 seconds at 72°C for 40 cycles). We employed a nested PCR protocol in which the first 20 μl reaction incorporated two oligonucleotides specific for rat PRL (sense primer A, 5′-TGT TCT GGT GGC GAC TGC CAG ACA CCT-3′, and antisense primer D, 5′-GCA GTT GTT TTT ATG GAC AAT TTG GCA-3′). Two μl of the first-round reaction was then amplified in a second reaction with two nested oligonucleotides (sense primer B, 5′-ACT TCT TCC CTA GCT ACT CCT GAA GAC-3′, and antisense primer C, 5′-TAT CTT TTC GAT CCC TTC GAG AAG CCG-3′). The product resulting from the second reaction was 222 bp long and spanned exons 3 and 4, ensuring that amplification of cDNA could be distinguished from that of genomic DNA.

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