Chapter 1

Prolactin and Angiogenesis: Biological Implications of Microheterogeneity

Kambadur Muralidhar and Jaeok Lee

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1. Introduction

Prolactin (PRL) was discovered in 1928. It is found in all vertebrates including humans. The name 'prolactin' is derived from its established role, in female mammals, in mammopoiesis. That raised the first mystery regarding its role in the human male and in non-mammalian vertebrates. More than 300 effects have been produced by injecting PRL into animals of all phylogenic groups. That raised the second mystery i.e. absence of any reliable and relevant bio assay for PRL till today. Following the approaches of Reductionist Biology, prolactin has been purified and characterized from a number of vertebrate groups. That raised the third mystery i.e. extensive microheterogeneity in structure and its doubtful relevance to physiology. The mechanism of action of prolactin has been studied extensively and that gave rise to the fourth mystery as to why it does not follow the second messenger model in signaling pathways, as in the case of other membrane receptor acting hormones like epinephrine or Luteinizing hormone (LH) or FSH etc. Prolactin behaves more like a cytokine and growth factor than like a hormone! In spite of exhibiting multiple physiological effects on a variety of tissues like brain (behavior), gonadal and mammary tissues, accessory sex organs like ventral prostate, immune system of phagocytes and lymphocytes etc, no disease whose origin can be ascribed to mutations in PRL or PRL receptor genes has yet been discovered. That is the fifth mystery. There is no known clinical model of prolactin deficiency. Hyperprolactinemia due to tumors of pituitary lactotrophs is the only known pathological condition. Long term hyperprolactinemia can lead to amenorrhea in women, loss of libido in men and infertility in both.

2. Relevant biochemistry and biology of prolactin

The first observation related to prolactin was made in 1928 (Stricker and Grueter, 1928). These French scientists injected a bovine pituitary extract into pseudopregnant rabbits and

observed that such rabbits started lactating. Thereafter Riddle et al (1933) reported that the crude extract could stimulate the production of "milk" by the crop sac of pigeons. Later it was established that the same pituitary principle was responsible for both the crop "milk" production and mammary gland secretion. The same component of pituitary was purified using a pigeon crop sac development as bioassay from bovine pituitary and termed as "Prolactin" (Riddle et al 1933). Thirty years after its discovery, the first amino acid sequence of ovine Prolactin was completed in 1970 (Li et al. 1970). Prolactin from different species of fishes to mammals has since been sequenced (Sinha, 1995).

Prolactin and growth hormone from buffalo pituitaries have been purified and characterized in our laboratory (Muralidhar, et al., 1994; Khurana and Muralidhar, 1997; Maithal et al, 2001). The molecular size of buPRL monomer was 22,664 Da as determined by MALDI-TOF analysis (Panchal and Muralidhar, 2008). Enhancement of Nb2 rat lymphoma cell proliferation by the hormone has been studied (Khurana and Muralidhar, 1997). Prolactin from number species of different classes of vertebrate has been purified and characterized. Primary structure of PRL from about 45 species is known either by protein sequence directly or deduction from gene sequence or cDNA sequence (Sinha, 1995). In most of the mammalian species prolactin contains three intra-disulfide bridges between Cysteine residues 4-11, 58-174 and 191-199. Teleost PRLs, however, lack the amino terminal disulfide loop (Sinha, 1995).

The major role of prolactin in eutherians and marsupials is regulation of milk secretion and growth of mammary gland. The binding specificity of prolactin receptors does vary between species, however (Amit et al, 1997). Prolactin also plays other roles in mammals, like in immunomodulation, osmoregulation and control of parental behavior and it may be that variation in the relative importance of one or more of these provide the drive for adaptive change (Wallis, 2000).

Prolactin is biosynthesized as a larger precursor protein but processed into a ~23 kDa protein in most of the species. Many different molecular size isoforms as well as forms of PRL with post translational modifications like glycosylation, phosphorylation and sulfation have been described in literature. Dimerisation and polymerization of prolactin or aggregation with binding proteins, such as immunoglobulins, by covalent and non covalent bonds may result in high-molecular-weight forms. In general, the high-molecular weight forms have reduced biological activity (Sinha, 1995). The role of prolactin-IgG macromolecular complex in the detection and differential diagnosis of different prolactinemias is targeted primarily in clinical studies (Cavaco et al 1995).

Tyrosine sulfation is not a very common post-translational modification. Recently a review on this modification has listed 62 proteins which have been identified as tyrosine sulfated. For the majority of these, a role for sulfation in the function(s) of the proteins has not been described. In some cases sulfation is important in optimal receptor-ligand interactions (e.g.chemokine receptor binding), optimal proteolytic processing (e.g. gastrin processing), and proteolytic activation of extra cellular proteins (e.g. factor V and VIII activation) (Moore 2003).

There is only one pituitary hormone known to have sulfation of tyrosine and this is prolactin. It was found that a prolactin containing fraction had very high radioactivity when pituitary cells were incubated with radio labeled sulfate. Later it was proved that prolactin was the protein which was containing this radioactivity. Chemical analysis proved the sulfation is on the tyrosine residue/s of prolactin. Role of this tyrosine sulfated prolactin is still an unanswered question (Kohli et al 1987, Kohli et al 1988). Thus the key feature of PRL is structural microheterogeneity in various forms. The microheterogeneous isoforms of prolactin and growth hormone have been reported by our laboratory earlier. These isoforms reported included tyrosine sulphated prolactin (Kohli, et al., 1987; 1988; Chadha, et al., 1991), lower sized (19 kDa and 13 kDa) iso forms (Khurana and Muralidhar, 1997) and glycosylated isoforms (Khurana and Muralidhar, 1997) in buPRL. Similar size and charge isoforms of buffalo growth hormone have also been reported by our laboratory earlier (Maithal et al, 2001) However, the unique biological functions of the heterogeneous forms, if any, are not known yet. To serve as a frame work for investigations, we postulated two hypotheses with regard to biological significance of microheterogeneity in PRL. One was that microheterogeneous forms have different potencies in a given bioassay during ontogeny. Two, that microheterogeneous forms exhibit different biological activities in different phylogenic groups of animals during phylogeny. It was observed that the non-glycosylated isoform of buPRL was 4-5 times more potent than the glycosylated isoform of buPRL in the Nb2 rat lymphoma cell growth assay *in vitro* (Khurana and Muralidhar, 1997). Similarly it was observed that the PRL with higher isoelectric point (i.e. more basic isoform) had a higher potency than the PRL isoform with lower isoelectric point (i.e. more acidic isoform) in the same assay.

2.1. Prolactin from peripheral tissues

The cells of the anterior pituitary gland which synthesize and secrete prolactin were initially described by light microscopy using conventional staining techniques (Herlant 1964). These cells designated lactotrophs or mammotrophs comprise 20-50% of the cellular population of the anterior pituitary gland depending on the sex and physiological status of the animal. The first observation that prolactin is produced in the brain was by Fuxe (Fuxe et al 1977) who found prolactin immuno reactivity in hypothalamic axon terminals. Prolactin immuno reactivity was subsequently found in the telencephalon in the cerebral cortex, hippocampus amygdala, septum (Devito 1988), caudate putamen (Emanuele et al 1992), brain stem (Devito 1988), cerebellum (Seroogy et al 1988), spinal cord (Siaud et al 1989), choroids plexi, and the circum ventricular organs (Thompson 1982).

Several approaches have been taken to prove that prolactin is found in the hypothalamus and that it is synthesized locally, independent of prolactin synthesis in the pituitary gland. Indeed, hypophysectomy has no effect on the amount of immuno reactive prolactin in the male hypothalamus and only diminishes but does not abolish the quantity of immuno reactive prolactin in the female rat hypothalamus (Devito 1988). With the use of

conventional peptide mapping and sequencing of a polymerase chain reaction (PCR) product of hypothalamic cDNA from intact and hypophysectomized rats (Wilson et al 1992), it has been established that the primary structure of prolactin of hypothalamic origin is identical to that of the prolactin of the anterior pituitary (Wilson et al 1992). The deciduas produce a prolactin-like molecule that is indistinguishable from pituitary prolactin in human (Andersen 1990, Riddick et al 1978), Finally, the non pregnant uterus has been shown to be a source of prolactin as well. Indeed a decidual-like prolactin, indistinguishable from pituitary prolactin (Gellersen et al 1989) has been identified in the myometrium of nonpregnant rats (Ben-Jonathan et.al, 2008). In addition to uptake of prolactin from the blood, the mammary epithelial cells of lactating animals are capable of synthesizing prolactin. The presence of prolactin mRNA (Kurtz et al 1993) as well as synthesis of immunoreactive prolactin by mammary epithelial cells of lactating rats has been described (Lkhider et al 1996). A great deal of evidence suggests that lymphocytes can be a source of prolactin as well (Gala et al 1994, Montgomery et al 1990).

2.2. Receptors to Prolactin (PRLR)

The PRLR was identified as a specific, high-affinity, membrane–anchored protein (Kelly et al 1974). The cDNA encoding the rat PRLR has been prepared. As is true for their respective ligands, receptors for PRL and GH (GHR) are also closely related (Boutin et al 1988, Kelly et al 1991). Both are single–pass transmembrane chains and despite a relatively low degree (~30%) of sequence identity, they share several structural and functional features (Kelly et al 1991, Goffin et al 1998). The gene encoding human PRLR is located on chromosome 5 (p13-14) and contains at least 10 exons for an overall length exceeding 100 kb (Arden et al 1990). multiple isoforms of membrane-bound PRLR resulting from alternative splicing of the primary transcript have been identified in several species (Boutin et al 1988)..

3. Prolactin and angiogenesis

Angiogenesis has been described well. A number of factors regulate this process in the body (Iyer and Acharya, 2002). We report here that naturally occurring lower size isoforms of buPRL, Cathepsin derived peptide fragments of prolactin and synthetic peptide from the internal sequence of both prolactin and growth hormone were found to exhibit anti-angiogenic activity in endothelial cell migration and CAM assays. Further using in silico methods, the three dimensional structure of buffalo prolactin was arrived at including the location of the anti angiogenic peptide sequence. A 16 K PRL as a newly generated N-terminal 16 K fragment resulting from the proteolysis of rat PRL by acidified mammary extracts was discovered and reported in 1980 (Mittra 1980a and 1980b). Since then, this PRL fragment has received considerable attention from the scientific community. The protease responsible for the cleavage of rat PRL into 16K was identified as Cathepsin D, The 16K PRL was shown to have lost PRL receptor binding ability but otherwise to have acquired the ability to specifically bind another membrane receptor (Clapp and Weiner 1992) through

which it exerts antiangiogenic activity (Clapp et al 1993). Although this receptor is still not identified, some of its downstream signaling targets have been elucidated (D'Angelo et al 1999, Corbacho et al 2000).However; many questions related to the biology of 16K PRL remain unanswered. First, although the majority of investigations have used rat 16K PRL, results are much less clear for other species, especially humans, in which PRL was recently reported to be resistant to Cathepsin D (Khurana et al 1999a and 1999b). This contrasts with the findings indicating that hPRL yields partial, but reproducible, proteolysis leading to N-terminal 16K like PRL fragments when incubated with this protease. Second, because it may be generated both centrally (Clapp et al 1994) and at the periphery, such as in pulmonary fibroblasts (Corbacho et al 2000) and endothelial cells (Corbacho et al 2000), the site/s of 16K PRL generation remains to be clearly identified. Hence, whether all sites of extra pituitary PRL synthesis can generate 16K PRL from endogenous 23K PRL or alternatively, whether circulating PRL is internalized before the proteolyzed form is exported (or both) remains open to investigation. Also, the sub-cellular compartment/s in which appropriate proteolysis conditions are found remain/s to be identified, although one can not discard the possibility that the cleavage of takes place in extra cellular milieu. In humans, although various recombinant forms of 16K hPRL were shown to be antiangiogenic, no insight into the biological relevance of hPRL *in vivo* was provided. It is relevant to ask also whether, the effects of PRL on tumors *in vivo* should be viewed from a new angle, considering a balance between the mitogenic and angiogenic (pro-tumor) activities of full-length PRL versus the antiangiogenic (anti tumor) activity of 16K-PRL (Goffin et al 1999).

3.1. Purification of buffalo PRL monomer from a discarded acid pellet

Buffalo pituitary prolactin in monomeric form can be prepared (Panchal and Muralidhar, 2008). The lower size isoforms of buffalo PRL can be separated from the monomer as well as the higher sized isoforms by differential alcohol fractionation (Panchal and Muralidhar, 2008) This lower size isoform containing fraction was designated as (AP) P-190-70. The APP-I, a semi-pure buPRL from buffalo pituitaries (Chaudhary, et al., 2004), has higher sized forms (>34.9 kDa, 34.9 kDa and 25.9 kDa (26 K)) and lower sized forms (18.4 kDa (18 K), 14.5 kDa (14 K) and <14.5 kDa) as well as buPRL monomer (23.4 kDa). The lower sized forms, approximately 18 K and 14 K under non-reducing conditions transform into 19 K and 13 K bands under reducing conditions (Khurana and Muralidhar, 1997). The 26 K PRL under non-reducing condition is probably the isoform nicked in the large loop of intact form but with intact disulfide bonds (Mittra I, 1980a; 1980b). Khurana and Muralidhar (1997) reported that 25 K buPRL (under non-reducing condition of SDS-PAGE) disappeared under reducing condition of the gel. The size isoform mixture, APP-I could be separated by differential alcohol precipitation. APP-I 70 (higher sized form mixture) and APP-I 90-70 (lower sized form mixture) were checked by SDS-PAGE analysis. The lower sized form mixture, APP-I 90-70, was confirmed to be free from higher sized forms by immunoblotting analysis. The reason for the successful separation is that the two different sized mixtures had different pI. Buffalo PRL monomer has pI 5.1~5.45 (Chadha, et al., 1991). The higher sized forms and

monomer are probably soluble in the alkaline ethanol. The buPRL lower sized peptides had opposite property.

3.2. Anti-angiogenic activity of (AP) P-I 90-70 fraction, lower size isoforms mixture

It has been known that N-terminal 16K PRL fragment had an inhibitory effect on angiogenesis i.e. the formation of new blood vessels (Folkman and Shing 1992), in rat (Ferrara, et al 1991) and in human (Clapp, et al 1993) both *in vitro* and *ex vivo*. Thus, it was of interest to know whether the lower size forms mixture including 18K and 14K size forms i.e. naturally occurring PRL peptides, have the anti-angiogenic activity or not. Hence (AP) P-I 90-70 fraction was tested with endothelial cell migration assay (*in vitro*) and chick egg yolk membrane assay (*ex vivo*) (Figure 1). Approximately 7 % inhibition of cell migration compared to control was observed after 4 hrs and 8 hrs, when 30 pg/ml of (AP)P-I 90-70 was added to human immortalized umbilical vein endothelial cells, EAhy926. The exact amount of the active peptide(s) in this mixture is difficult to estimate. It is, therefore, clearly proved that the physiologically cleaved buPRL fragments (i.e. naturally occurring lower size isoforms) had an anti-angiogenic function. The C-terminal 16 K hPRL does not appear to have the anti angiogenic activity (Khurana et al 1999). Extrapolating this fact to buffalo PRL, the present PRL lower size isoforms have to be mostly N-terminal fragments. If C-terminal fragments were in the mixture of isoforms, their presence is much less than that of N-terminal ones. Or those fragments may include specific anti-angiogenic active site. This (AP)P-I 90-70 fraction needs to be further characterized. Struman et al. (1999) reported that intact human PRL, GH and PL exhibited angiogenic activity. However, our results indicate unequivocally that the buPRL monomer had no significant stimulatory or inhibitory effect on blood vessel formation in CAM and endothelial cell migration assays.

3.3. Peptides derived from buffalo PRL by enzymatic digestion have anti angiogenic activity

Most of the studies about the antiangiogenic N-terminal fragment cleaved by Cathepsin D (CD) have been done in the case of rat and human PRLs (Ferrara et al. 1991). It was therefore hypothesized that buffalo pituitary PRL can be cleaved by Cathepsin D, and that the fragment could inhibit angiogenesis via endothelial interference. Here, we demonstrate that buPRL size isoform produced by CD have an inhibitory action on angiogenesis. However, it was not clear which sequence in the N-terminal fragment plays a role in antiangiogenesis. Highly pure buPRL monomer was obtained from pituitary glands with the protocol standardized in our laboratory designed with differential ethanol extractions to separate both different sized forms followed by a single Sephacryl S-200 chromatography (Panchal and Muralidhar, *2008*). The selected fractions after S-200 gel filtration showed more than 95% size homogeneity of buPRL as determined by 15% SDS-PAGE and densitometry.

The purified buPRL monomer, intact form, was confirmed to be free from protease contamination especially from the acidic protease treatment. Intact buPRL was incubated

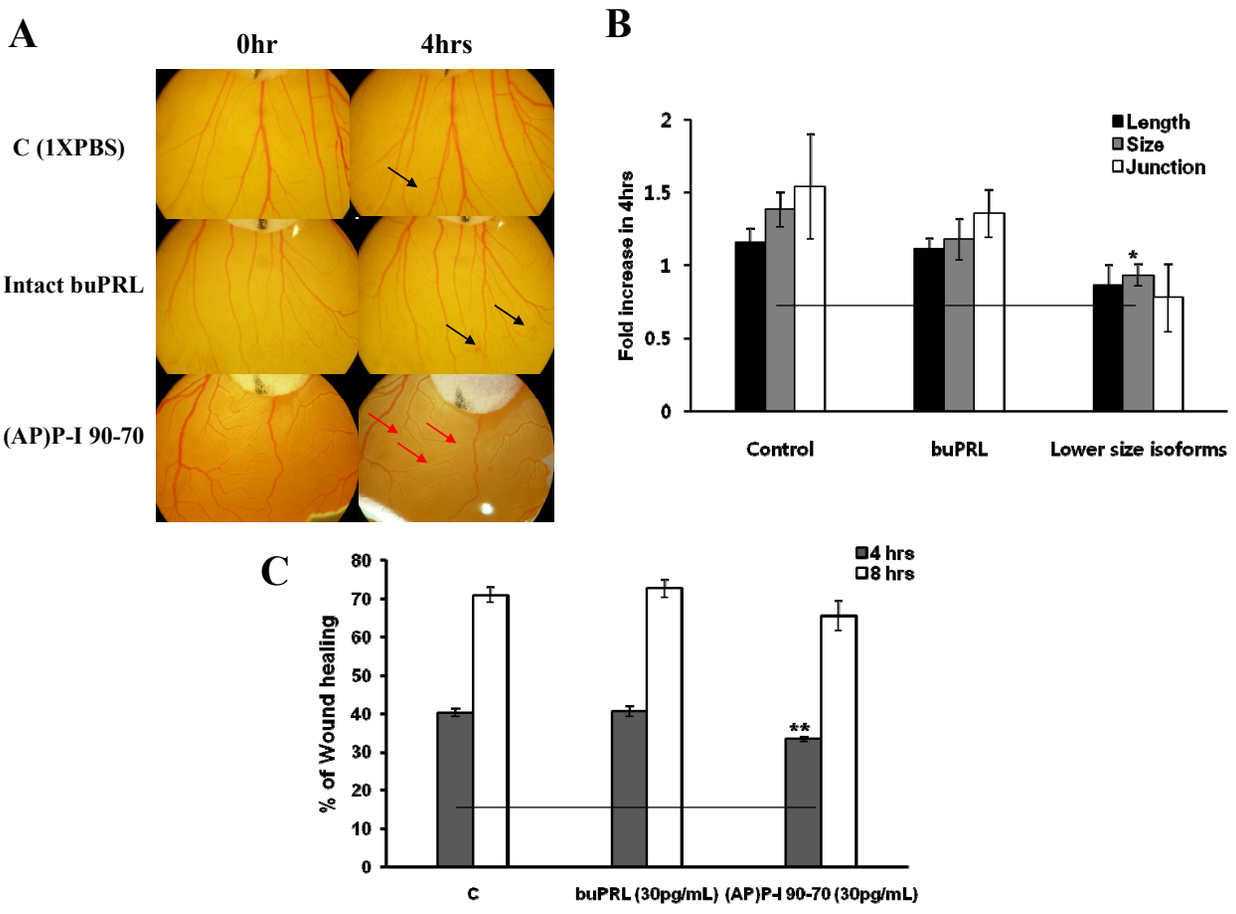

Figure 1. Antiangiogenic activity by *ex vivo* and *in vitro* assays. A, Results of CAM assay. Eggs at 6th day of incubation were used for the assay. B, Analysis of the results of A. C, Results of endothelial cell migration assay. Values are means ± SEM. $P < 0.05$ vs control. **Significantly different from control cells ($P < 0.001$).

with distilled water and 20 mM citrate/phosphate buffer including salt (i.e. pH 3.0) at 37°C, respectively, and then analyzed by electrophoresis at frequent intervals. When the intact buPRL was incubated in distilled water for 6 h, no lower-sized fragments were detected in SDS-PAGE either under non-reducing or reducing conditions. However, when the intact buPRL was incubated under the acidic conditions, a lower-sized form, approximately 21 kDa, was observed in SDS-PAGE under reducing conditions.. The lower form did not significantly increase in type and quantity during the incubation time, up to 12 h. Protease-free intact buPRL was incubated with bovine spleen CD (the ratio of substrate vs enzyme = 100:1) in acidic condition (pH 3.0) 37°C,and then the peptide mixture was separated in a 15% SDS-PAGE under reducing conditions.. Results indicated that the intact buPRL was cleaved by CD at pH 3.0 and the molecular weight of cleaved peptides were approximately 18.39 kDa (18K), 14 kDa (14K), 11.16 kDa (11K) and 7.47 kDa (7K). These sizes were identified by immunological analysis using buPRL antiserum (Figure 2B). The mixture did not show a significant contamination of CD (42.12 kDa and 29 kDa) (Fig. 2A) This enzymatic cleavage was confirmed when in the presence of pepstatin A (Marks et al. 1973), a known

acidic protease inhibitor, CD did not generate lower-sized peptides from the monomer under non-reducing conditions to just below monomer under reducing conditions (Fig.2D and 2E, L1). The amount of cleaved isoform under non-reducing condition decreased at 6 h incubation (Fig. 2D, L6).

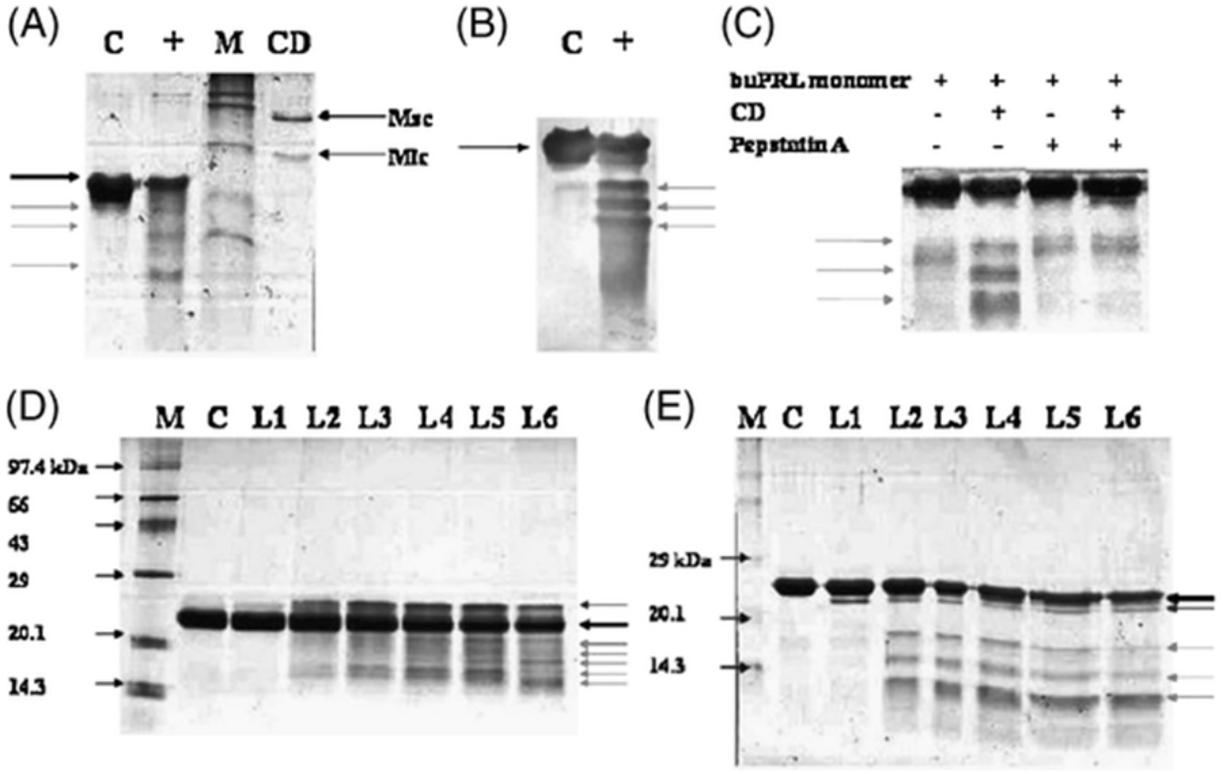

Figure 2. (**A**) Results of SDS-PAGE analysis after colloidal Coomassie blue staining. Lane C, buPRL monomer (5 μg); Lane +, buPRL monomer (5 μg) incubated with CD; Lane M, molecular weight marker; Lane CD, Cathepsin D (7.49 μ g); Msc, mature single chain (42.12 kDa); Mlc, mature large chain (29 kDa). (**B**) Immunoblot of **A** with anti-buPRL serum indicating the cleaved fragments of buPRL by CD. (**C**) Effect of pepstatin A on buPRL cleavage by CD. (**D**) Time course of action by CD on buPRL monomer. L1–L6 represent analysis of reaction mixture by SDS-PAGE under non-reducing conditions after 0 and 30 min, and 1, 2, 3 and 6 h, respectively. Lane C is the control where no enzyme was added; Lane M represents marker. (**E**) Same as **D** except that the gel was run under the reducing conditions. (Taken from Jaeok Lee et al 2011 with permission).

Cell migration analysis by wound healing assay was performed in EAhy926. The cell line expresses factor VIII-related antigen and has the same morphological distribution as primary endothelial cells with a doubling time of 12 h (Edgell et al. 1983).

A dose-dependent (3.3 pg/mL to 330 μg/mL) inhibition by buPRL peptide mixture including intact form on migration of EAhy926 cells was observed. Hence, 33 pg/mL of the peptide mixture was used for early stage (on the day 4) CAM assay, one of the *ex vivo* methods to study angiogenesis (Ribatti et al.1996*)*.The blood vessel generation reduced with the dose of the peptide mixture in a time-dependent fashion. These studies were done with the peptide mixture which was not separated from the undigested PRL, if any. This meant that in our hands the PRL monomer had neither antiangiogenic nor angiogenic activity. Further intact

PRL was not binding to the putative receptors to which the peptides were supposed to bind and exhibit their antiangiogenic activity. Prolactin is known to bind to PRL- specific receptors. Hence it can also be concluded, tentatively at least, that Cathepsin digested PRL – derived peptides do not work through PRL receptors for their anti angiogenic activity.

3.4. The peptide mixture separated from PRL retains antiangiogenic activity

The results described above were confirmed when the peptide mixture was separated from the undigested intact PRL on an FPLC column and then tested for antiangiogenic activity (Jaeok Lee et al 2011). It is interesting to note the cleavage pattern of prolactins from different species when exposed to Cathepsin D is different. For example, the sizes of fragments obtained by CD from buffalo prolactin are different from the sizes of cleaved fragments of PRL from : rat pituitary (16K and 8K) (Mittra, 1980a, b*),* mouse pituitary (16K and 8K) (Sinha and Gilligan, 1984), and human pituitary (17K, 16.5K, 15K, 11K, 8K and 5K) (Piwnica et al*.* 2006) PRL (Fig.3) These different patterns of cleavage could be due to micro-variation in the primary structure among species. Comparing the cleaved sequences between human and rat PRLs, Tyr147-Pro148-Val149-Trp150-Ser151 of human and Tyr145-Leu146-Val147-Trp148-Ser149 of rat are homologous (Piwnica et a*l*. 2006) (Fig.3B). The presence of Pro148 and Leu132 controls the cleavage pattern in hPRL (Piwnica et al 2006). Pro148 prevented high cleavage efficiency at 147–148 and 150–151 sites, while Leu132 produced the additional cleavage at 132–133 sites (Piwnica et al. 2006). Buffalo PRL has the same sequence at 147–151 site as in hPRL, but not at residue 132 (Fig. 3B). The Pro148 in buPRL also seems to link to a specific cleavage pattern. Hence, 18K and 14K of buPRL were considered homologous to 17K and 15K of hPRL, respectively. We could not detect a fragment similar to the 16.5K fragment of hPRL.

Angiogenesis, the process of developing new blood vessels from preexisting vessels, is crucial to reproduction, growth and wound healing (Folkman and Shing 1992). In the last two decades, numerous studies reported novel angiogenic regulators (Iyer and Acharya, 2002). Among the angiogenic regulators, hPRL fragment is one of the very paradoxical molecules. Its intact form has pro-angiogenic action (Struman et al. 1999), inducing cell proliferation and motility, and relates to breast carcinogenesis (Clevenger et al. 2003). The PRL fragments as well as intact PRL were are also known to be secreted from vascular endothelial cells (Ochoa et al. 2001). The antiangiogenic and anti mitogenic effects of peptide fragments were observed in BBBE and HUVE cells, even in the presence of PRL antibody. This suggested the possibility that they act as autocrine regulators of angiogenesis, (Clapp et al. 1998; Corbacho et al. 2000).

3.5. Buffalo pituitary PRL derived peptides are active at pico gram level

In the present study, the antiangiogenic activity of buPRL-derived peptides was demonstrated *in vitro* and ex *vivo*. Endothelial cell migration is fundamental to angiogenesis (Lamalice et al. 2007), because endothelial cells, derived from meso dermal cells, form capillary blood and lymphatic vessels (Venes and Thomas 2001).

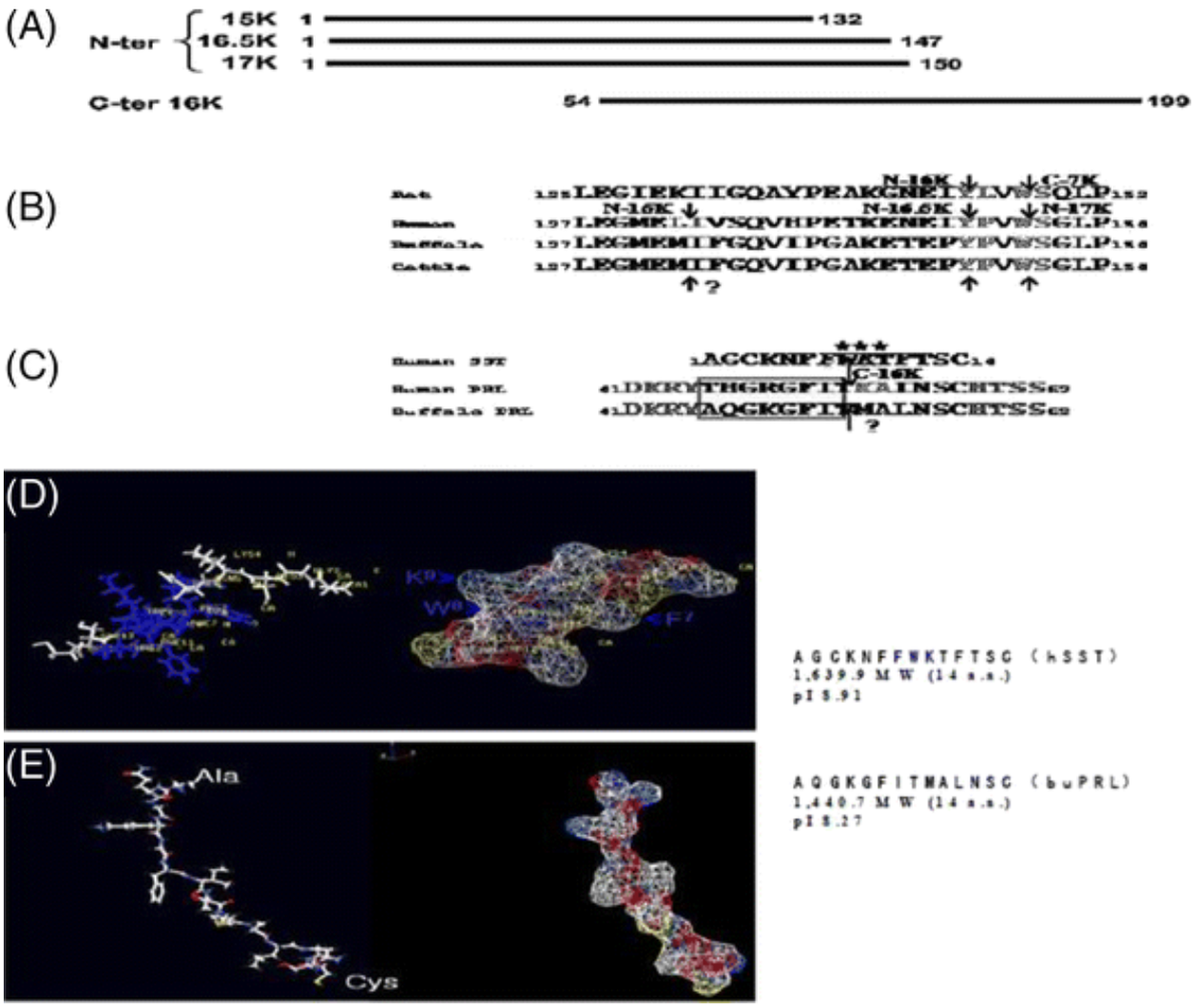

Figure 3. CD and thrombin cleavage site in the primary sequences of rat, human, buffalo and cattle PRLs. (**A**) In human PRL three N-termini by CD and C-terminus by thrombin (figure from Piwnica, et al. 2004). (**B**) CD cleavage sites in rat, human, buffalo and cattle PRLs. (**C**) The SST aligned sequences of buPRL and buGH are partially or completely involved in the sequence of N-terminus fragment known as antiangiogenic factor. Triple * in hSST is the specific binding site to SSTRs. The square window indicates the considered sequence has an antiangiogenic action. Arrows indicate the cleavage sites. (**D**) hSST in SWISS-Pdb viewer 4.0. Blue arrows and alphabets represent SST receptor binding sites. (**E**) The synthetic peptide derived from buPRL. Molecular size and pI of each peptide were gained from ExPASy Proteomics tools. (Taken from Jaeok Lee et al, 2011 with permission)

Earlier reports found antiangiogenic activity of r16K (Ferrara et al. 1991) and recombinant h16K (Clapp et.al. 1993; D'Angelo et.al. 1999) in micromolar concentrations. The present study demonstrates that buPRL-derived peptides have an antiangiogenic activity in nanomolar concentration. Even the cleaved fragments mixed with intact buPRL, considered a pro-angiogenic factor (Struman et al. 1999), are effective at the lower concentration level. The results of our study prove that buPRL peptides cleaved by CD have the antiangiogenic action, and these peptides must have come from N-terminal side. Mechanistic studies were undertaken and it was observed that the peptide mixture obtained by Cathepsin D digestion

of prolactin was capable of antagonizing the action of Bradykinin (BK) in terms of NO production.

NO, produced by NO synthase (NOS), is a second messenger in the regulation of physiological and pathophysiological process in the cardiovascular, nervous and immune systems (Moncada and Higgs 1993; Nathan and Xie 1994). NO is involved in cell migration and cell protection (Lee et al. 2005; Kolluru et al. 2008). NO-mediated pathway is a well known signaling mechanism of angiogenesis, vasopermeability and vasorelaxation in relation to VEGF (Ferreira and Henzel, 1989; Ishikawa *et al.* 1989), BK (Regoli and Barabe 1980; Ferreira et al. 1992) and Acetyl choline (Ach) actions (Palmer et al. 1989). Gonzalez et al. (2004) reported that human and rat 16K PRL fragments inhibited NOS activation induced not only by VEGF and BK but also by Ach, in endothelial cells. Buffalo PRL fragments seem not to be correlated to the pathway stimulated by Ach in EAhy926 (Jaeok Lee et al, 2011). Moreover, the buPRL peptides were more effective in inhibiting the BK-mediated NO mechanism than that by VEGF. It is not known, however, how this peptide blocks the NO pathway. We envisage two possible mechanisms. One is that the peptides act as receptor antagonists of angiogenic factors, and the other is that the peptides induce another pathway to inhibit NO production driven by these angiogenic factors. Besides, the role of individual amino acid residues in the antiangiogenic action is not yet clear.

It has been observed that these proteolytically derived peptides from buffalo PRL are more potent than Somatostatin (Jaeok Lee et al, 2011). Somatostatin, a small peptide (14 amino acids) secreted from hypothalamus, is a negative regulator of growth hormone (GH) release (Norman 1997). Widely distributed throughout the body, SST binds to five different subtypes of its cognate receptors (SSTR1-5), one of them being a G-protein-coupled receptor present on the cell membrane (Patel *et al.* 1990; Lahlou *et al.* 2004). While the five SSTRs bind the natural peptide, SSTR2, SSTR3, and SSTR5 can bind its synthetic analogues (Lahlou et al. 2004). SST and its analogues inhibit angiogenesis and also the production and secretion of angiogenic factors including VEGF (Barrie *et al.* 1993). It was reported that SST blocked Kaposi sarcoma (KS) cells (KS-Imm), isolated from a kidney-transplanted, immuno suppressed patient and also highly angiogenic, xenografts into nude (nu/nu) mice through angiostasis (Albini et al. 1999). SST also induced cell death of human somatotrph tumor cells (SSTR2) (Ferrante *et al.* 2006). In the study with vascular endothelial cell (dominated by SSTR3) by Reisine and Bell 1995), SST inhibited cell proliferation through blocking of both endothelial NOS (eNOS) and MAPK activations. SST is a powerful anti-tumour angiostatic agent. The results of our work confirm that SST and 14K buPRL are significant antiangiogenic factors, and 14K is more effective than SST in the antiangiogenesis assays. The antiangiogenesis brought about by buPRL fragment seems to be related to the inhibition of vasopermeability of endothelial cells.

It is interesting to note that a synthetic peptide corresponding to the Somatostatin sequence within the amino acid sequence of buffalo pituitary Growth hormone is capable of antagonizing Bradykinin induced angiogenesis in terms of nitric oxide production (Syamantak Majumder et al, 2009). To what receptors do these lower size isoforms of buPRL bind in their antiangiogenic activity? In our hands, intact buffalo PRL had neither pro- nor

anti- angiogenic activity. The peptides may act through PRL receptors or through other receptors like those to Bradykinin or VEGF. They may also have their own distinct receptors. As they are capable of inhibiting VEGF or Bradykinin induced angiogenesis, it can be safely concluded that the peptides might work through VEGF or Bradykinin receptors rather than PRL- specific receptors.

3.6. Antiangiogenic activity of the Synthetic peptides

Generally, the conformation of the cleaved fragment is either new and unstable or native-like which is less stable than that of the original (Creighton, 1993). No experimental details are available on the conformation of the cleaved fragment. Nevertheless, a significant difference in the N-terminal 14K PRL structure from the intact PRL was that the loop between the first and the second α-helices were loosened and more opened to the environment. In the intact form, the loop was close to the fourth α-helix.

The disulfide bond between the loop and the fourth α-helix, between Cys58 and Cys174, in the centre of the loop might be giving more compact configuration.

We have cloned and expressed cDNA for buffalo prolactin in E.coli and the bacterially expressed buffalo prolactin was biologically active in stimulating Nb2 rat lymphoma cells in vitro to divide (Manoj Panchal and Muralidhar, 2010). From the nucleotide sequence of the cDNA, the aminoacid sequence was deduced and from the available 3- dimensional structure of human prolactin, the 3-dimensional structure of buffalo prolactin was obtained by homology modelling. Somatostatin is a known inhibitor of angiogenesis. Using bioinformatics tool of BLAST search, we could locate a similar peptide in both prolactin and growth hormone. The peptides were synthesized through commercial sources. It was observed that the synthetic peptides, both the 13-mer and the 14-mer were active in pictogram range in all the *in vitro* and *ex vivo* bioassays.

Small ligands tend to bind, relatively speaking, the interior of globular proteins, while linear ligands tend to bind in clefts on the surface of proteins (Creighton *1993*). The small and linear structure of the synthesized peptide (Figure 3E) enables it to bind to both the interior of putative receptors and to the clefts on the surface of receptors. The synthetic peptide, Ala45-Gly47-Lys48-Gly49-Phe50-Ile51-Thr52-Met53-Ala54-Leu55-Asn56-Ser57-Cys58, also showed more active than SST in the antiangiogenic action. This SST-matching sequence peptide had a structure (linear) different from SST, which had α-helix structure (Figure 3D). This active motif for antiangiogenesis is not involved in binding to the PRLR sites, especially the second half of loop 1 (His59, Pro66 and Lys69) of site 1 (Goffin et al. 1992). This result also demonstrates that Ala45 to Met53 can be the active sites for angiostatic function. The C-terminal 16K peptide (54–199 residues) of hPRL does not appear to have the function of antiangiogenesis (Khurana et al. 1999b). In the C-terminal 16K fragment of hPRL, the fourth helix is still close to the partial second loop, which becomes exposed to environment in the N-terminal 16K fragment of hPRL. Because of this, the C-terminal 16K fragment of hPRL may not have antiangiogenic action. Three residues, Phe7-Trp8-Lys9, in SST sequence

appear to have the crucial role in binding with high affinity to all SST receptors, SSTR1 to SSTR5 (Poitout et al. *2001*). However, in the synthetic sequence derived from buPRL, Ala1-Gln2-Gly3-Lys4-Gly5-Phe6-Ile7-Thr8-Met9-Ala10-Leu11-Asn12-Ser13-Cys14, 'Ile7-Thr8-Met9' is found instead of 'Phe7-Trp8-Lys9'. In the SST-matching sequence of hPRL, 'Ile51-Thr52-Lys53' replaces the SST tri-peptide sequence ('Phe7-Trp8-Lys9') positions (Figure 3C). This leads to the question of whether buPRL fragments bind to SSTR3 or not, and if the fragments bind to SSTR3, which residues in the sequence of the fragments bind to the receptor and mediate the antiangiogenic action? In the PRL fragments- and SST-mediated inhibition of the angiogenesis, there are similar mechanistic patterns. Both factors blocked VEGF-induced cell proliferation, which is known to be through the MAPK pathway in vascular endothelial cells (D'Angelo *et* al. 1999).

The hPRL and hGH tilted peptides (14-amino-acid sequence) consisting of 9 hydrophobic amino acids induce endothelial cell apoptosis and inhibit endothelial cell proliferation and capillary formation (Nguyen et al. 2006). The tilted peptide has been known to destabilize membrane and lipid core and is characterized by an asymmetric distribution of hydrophobic residues along the axis when helical. However, the synthetic tilted peptides derived from hPRL and hGH show 4 times and 32 times less activity, respectively, than the 16K hPRL *in vitro* (Nguyen et al. 2006). The synthetic peptide related to buPRL represents differences in structure and functional sensitivity from those of the tilted peptides. This peptide has similar sensitivity with 14K buPRL in the inhibition of angiogenesis *in vitro* and *ex vivo*. Although the peptide includes 9 hydrophobic amino acids, it does not form a helix (Figure 3E). These differences imply that the synthetic peptide related to buPRL has a receptor-binding mediated antiangiogenic function, rather than protein-membrane-interaction-mediated function.

4. Summary and speculation

The present study suggests that naturally occurring size iso forms of buPRL have antiangiogenic activity. Further, buPRL gives upon Cathepsin digestion a 16K-like fragment, but of 14K size and more cleaved fragments which have an antiangiogenic action. The antiangiogenic action of the fragments is, at least, related to the initial part of the sequence of the second loop between first and second α-helices. Furthermore, the synthetic peptide (derived by hSST matching area of buPRL) can be a potential anticancer therapeutic agent as well as for treatment of vascular, rheumatoid and other diseases whose etiology necessarily involves angiogenesis (Folkman 1995). Confirming the relevance of this idea requires demonstrating whether the buPRL fragments have N-terminal structure, and whether there are other sequences between Thr1 and Tyr44 having anti angiogenic action. Further work is required to explore whether SSTR3 is the specific receptor for 14K and other cleaved peptides, and if not, what is the specific receptor to the peptides, and what sequence part plays a crucial role in this antiangiogenic action? The biology of PRL is fascinating but very intriguing. More than 300 biological activities have been ascribed to PRL from various species. We have proposed two hypotheses to guide research on this hormone with regard

to the significance of microheterogeneous isoforms. Our work has provided experimental evidence in support of these two hypotheses. Much more work needs to be done to understand and demystify prolactin actions.

Author details

Kambadur Muralidhar and Jaeok Lee
Hormone Research Laboratory, Department of Zoology, University of Delhi, Delhi, India

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