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Heparin affin regulatory peptide in milk: its involvement in mammary gland homeostasis

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Abstract

HARP (heparin affin regulatory peptide) is a heparin binding growth factor implicated in cellular growth and differentiation. Previously, HARP had been localized in the human mammary, in both alveolar epithelial and myoepithelial cells although HARP mRNAs were only expressed by myoepithelial cells [J. Histochem. Cytochem. 45 (1997) 1]. In the present study, we demonstrate that HARP is secreted in human mature milk with concentrations ranging from 17.68 ± 6.4 ng/ml in mature milk to 59.9 ± 11.22 ng/ml in colostrum. In vitro, HARP was found to be mitogenic on human mammary epithelial and myoepithelial cell lines and correlated with the expression of its high affinity receptor tyrosine kinase ALK (anaplastic lymphoma kinase). In vivo, ALK is expressed in both mammary epithelial and myoepithelial cells, suggesting that HARP could act in vivo as a paracrine and autocrine growth factor in the regulation of the mammary gland development and its homeostatic maintenance during pregnancy and lactation.

Keywords: HARP; Pleiotrophin; Human milk; Anaplastic lymphoma kinase; Mammary epithelial cells

Heparin affin regulatory peptide (HARP) [2], also known as pleiotrophin (PTN) [3] or heparin-binding growth associated molecule (HB-GAM) [4], is an 18 kDa secreted polypeptide that constitutes with midkine (MK) a two-member family of heparin-binding growth factor [5]. HARP plays a key role in cellular growth, differentiation and was also demonstrated to be involved in angiogenesis and tumor growth [6]. In vitro, HARP displays several biological activities. HARP promotes the neurite outgrowth of embryonic neurons [4], stimulates the proliferation of a wide range of cells including endothelial, epithelial, and fibroblastic cells [6], and prevents apoptosis [7]. HARP also induces endothelial cell migration and invasion of collagen and enhances plasminogen activator activity. The receptors involved in neurite outgrowth activity of HARP are syndecan-3 and the tyrosine phosphatase receptor RPTPβ [8,9]. Binding of HARP to the ALK (anaplastic lymphoma kinase) orphan tyrosine kinase receptor has been linked to its mitogenic, transforming [10,11], and anti-apoptotic [7] activities. HARP binds to ALK receptor through its 25 last C-terminal amino acids [10]. Indeed, a synthetic peptide P111–136 corresponding to these residues can bind to ALK receptor and inhibit the mitogenic and transforming activities of HARP, whereas a truncated mutant HΔ111–136 deleted of its 25 last amino acids and devoid of those activities is unable to bind ALK but inhibits HARP activities via a heterodimerization [10,12].

Initially isolated from neonatal brain [4], HARP was shown to be expressed in non-neuronal tissues including heart [13], uterus [14,15], cartilage [16], bone [17], and the mammary gland [1]. In this organ, HARP mRNA is expressed in alveolar myoepithelial cells and also in endothelial and smooth muscle cells of blood vessels. HARP

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protein is localized in capillaries, arterioles, and also in the region including both alveolar epithelial and myoepithelial cells, suggesting a paracrine role of HARP in the glandular epithelial cells [1]. This localization prompted us to investigate the presence of HARP in human breast milk. Indeed, maternal milk contains a variety of growth factors including epidermal growth factor (EGF) [18] and HB-EGF [19], insulin-like growth factors (IGFs) [20], transforming growth factor (TGF)-β [21], hepatocyte growth factor (HGF) [22], vascular endothelial growth factor (VEGF) [23,24] and also cytokines such as IL6 [25], and tumor necrosis factor (TNFα) [26]. These polypeptides are supposed to play various biological effects during the pre- and post-partum stages of pregnancy including development of the mammary gland, lactogenesis, stimulation of immunological signals for the host defense, and post-natal maturation of the gastrointestinal system.

The present study was undertaken to investigate the presence of HARP in human colostrum and mature milk and to determine the putative biological functions of HARP in the mother or the neonate.

Materials and methods

Materials. Culture medium, fetal calf serum, non-essential amino-acids, and trypsin/EDTA were supplied by Invitrogen (Cergy pontoise, France). BSA was obtained from Sigma (Saint Quentin Fallavier, France), Immobilon P was from Millipore (Saint Quentin en Yvelines, France), goat anti-human HARP antibodies were from R&D (Oxon, UK), horseradish peroxidase-conjugated rabbit anti-goat immunoglobulins (Jackson), TMB substrate kit (Pierce), and Superblocker solution (Pierce) were from Interchim (Montluçon, France), goat anti-ALK antibodies were from Santa-Cruz (Tebu S.A., France), and avidin–biotin blocking kit, antigen unmasking solution, biotinylated anti-goat IgG, avidin–biotin–alkaline phosphatase complex, and Vector red substrate were from Vector laboratories (Burligame, Canada).

Milk samples. Samples of human breast milk were obtained from healthy mothers of healthy full-term babies. All mothers volunteered to participate in this study. Milk samples were collected on days 1–4 after delivery (colostrum) ($n = 7$) and at least 8 days after delivery (milk) ($n = 10$).

HARP measurement. Concentrations of HARP in milk samples were measured as described previously [27]. Briefly, 0.25 μg/well of a heparin–BSA complex was coated in 50 mM Tris–HCl, pH 7.4, supplemented with 12.7 mM EDTA in 96-well plates overnight at 4 °C. After three washes with PBS containing 0.05% Tween 20 (washing buffer), wells were saturated for 1 h at room temperature with PBS containing 3% BSA. Samples (100 μl/well) diluted with PBS containing 1% BSA (incubation buffer) were incubated at 4 °C overnight. The anti-human antibody diluted at 250 ng/ml with incubation buffer was added (100 μl/well) for 2 h at room temperature. Between each step, the plate was washed three times with washing buffer. Peroxidase-labelled rabbit anti-goat antibody diluted in incubation buffer was then added and the peroxidase activity was measured using 3,3′,5,5′-tetramethyl benzidine, dihydrochloride according to the supplier. Absorbances were read at 450 nm and the concentration of HARP was determined with a titration curve from 40 to 1200 pg/ml.

Purification of human recombinant HARP. HARP was purified from conditioned media of NIH-3T3 cells expressing HARP as described previously [12]. Briefly, conditioned medium containing HARP proteins was buffered to pH 7.4 with 20 mM Hepes, ionic strength adjusted to 0.5 M NaCl, and then loaded on a 10 ml heparin–Sepharose column. Bound proteins were eluted with 20 mM Hepes, 2 M NaCl, pH 7.4, and further purified using a cation-exchange Mono-S column (FPLC system, Amersham–Pharmacia). The purification was carried out in 50 mM Tris–HCl, pH 7.4, and proteins were eluted using a 0.4–2 M NaCl gradient.

Immunoblotting of milk HARP. Milk HARP was concentrated using heparin–Sepharose beads before SDS–PAGE analysis. Purified proteins were analyzed as previously described [12].

Cells and culture. Caco-2 cells (a generous gift from Dr. Alain Servin, University of Pharmacy, Chatenay-Malabry, France) were maintained in DMEM containing 4.5 g/L glucose supplemented with 10% FCS and 0.1 nM non-essential amino acids. Hs-578BST cells (ATCC, MD, USA) were cultured in DMEM containing 4.5 g/L glucose supplemented with 10% FCS, 10 μg/ml insulin, and 30 ng/ml Epidermal Growth Factor (EGF). Hs-578T cells (ATCC, MD, USA) were cultured in DMEM containing 4.5 g/L glucose supplemented with 10% FCS and 10 μg/ml insulin. Cells were grown in a humidified atmosphere at 37 °C with 10 or 7% CO_2 for Caco-2 and Hs-578BST or Hs-578 T cells, respectively.

Cell proliferation assay. Cells were seeded and cultured in 12-well plates in complete medium for 24 h (see above) and samples were added after a 24 h starvation which corresponded to insulin depletion for Hs-578BST cells and culture without (Caco-2 cells) or with 2% SVF (Hs-578T cells). The proliferative effect was assessed by counting cells using a Malassez hematocytometer. Hs-578BST, Caco-2, and Hs-578T cells were, respectively, seeded at a density of 3×10^3, 4×10^4, and 5×10^3 cells/cm^2 and counted 8, 2, and 3 days after sample addition. For Hs-578BST cells, samples were added every 2 days.

Detection of ALK mRNA by RT-PCR. Total RNA was prepared from Caco-2, Hs-578BST, and NIH-3T3 cell lines using the RNA Instapure kit (Eurogentec, Belgium) according to manufacturer's instructions. Random primed cDNA was generated from 2 μg total RNA using the Superscript II reverse transcriptase (Invitrogen, Cergy-Pontoise, France). PCR was performed using *Taq* polymerase (Promega, Charbonnieres, France) with 10 μl of the reverse transcription products in a final volume of 50 μl. Specific primers from the region coding for the extracellular domain (ECD) of human or murine ALK were used as described by Stoica et al. [28]. The amplification reaction was performed in a GeneAmp 9600 PCR system (Perkin–Elmer) and the PCR products were electrophoresed on a 2% agarose gel.

Immunohistochemistry on paraffin-embedded sections. Paraffin-embedded tissue sections from normal human breast tissue (three different patients) were deparaffinized and rehydrated. Epitope retrieval was performed by incubating tissue sections in 10 mM sodium citrate buffer, pH 6 (unmasking solution, Vector), at 100 °C for 30 min followed by cooling down for 20 min. After three washes in PBS containing 0.1% (v/v) Tween 20 (PBS-T), non-specific binding sites were blocked by incubating slides with PBS-T containing 1% (v/v) fetal calf serum and 1% (w/v) gelatin at RT for 1 h. After a blocking step with an avidin–biotin blocking kit, sections were incubated 1 h at 37 °C with a mix of three polyclonal anti-ALK antibodies (C-19, N-20, and T-18) at 5 μg/ml each diluted in PBS-T containing 1% gelatin. After two washes in PBS-T, sections were further incubated for 30 min at RT with a biotinylated anti-goat antibody at a dilution of 1:500 in PBS-T containing 1% gelatin. After two washes in PBS-T, sections were incubated with an avidin–biotin–alkaline phosphatase complex and red coloration was obtained using the Vector red substrate. Sections were then counterstained with Harri's hematoxylin before mounting in Eukitt.

Results

HARP is present in human milk and colostrum

Many studies had described the presence of growth factors such as HGF [22] or VEGF [23] in human colostrum and milk. Since HARP was expressed and

localized in the alveolar myoepithelial cells in the human mammary gland [1], we investigated the presence of HARP in human milk of healthy mothers of full-term babies using Western blot and ELISA assay. As the consistency of breast milk varies according to the stage of lactation, we therefore determined the concentration of HARP in colostrum (collected between the first and fourth days after delivery) and in milk collected at least 8 days after delivery. HARP concentration was estimated to 59.9 ± 11.2 ng/ml ($n = 7$) in the human colostrum and significantly lower ($p < 0.0001$) in the milk 17.68 ± 6.4 ng/ml ($n = 10$) (Fig. 1A).

In order to check the presence of HARP in milk, HARP was concentrated on heparin–Sepharose beads and analyzed by Western blotting using an anti-human HARP antibody (Fig. 1B). A specific signal was detected in milk (Fig. 1B, lane 2) and the apparent molecular weight was similar to that of eucaryotic recombinant human HARP (Fig. 1B, lane 1) [29].

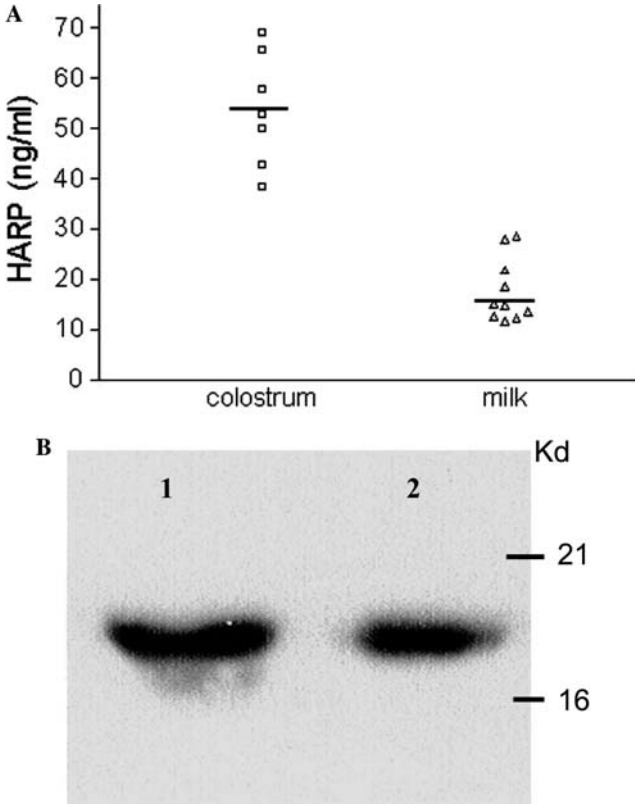

Fig. 1. HARP expression in human colostrum and milk. (A) HARP concentration was determined using an enzyme-linked immunosorbent assay (see Materials and methods) in human colostrum ($n = 7$) (milk collected 1–4 days after delivery) and in human milk collected at least 8 days after delivery ($n = 10$) of healthy mothers of full-term babies. (B) HARP present in milk was concentrated using heparin–Sepharose beads. Purified proteins (lane 2) and 10 ng of recombinant human HARP (lane 1) were immunoblotted with goat anti-human HARP antibodies after SDS–PAGE under reducing conditions.

The mitogenic activity of HARP on human mammary epithelial and myoepithelial cells in vitro is correlated with the expression of the ALK receptor

To assess whether HARP present in milk was involved in mammary gland homeostasis or in the maturation of the development of the neonate intestine, the mitogenic activity of human recombinant HARP was tested on (i) the Hs-578BST alveolar myoepithelial cell line, (ii) the HS-578T epithelial cell line, and (iii) the Caco-2 intestinal epithelial cell line as described under "Materials and methods." A dose–response curve was performed on these three cell lines using human recombinant HARP purified from culture medium of HARP-expressing NIH-3T3 cells [12] (Fig. 2A). For Hs-578BST, the proliferative effect was determined after 8 days of stimulation and the stimulation was, respectively, limited to 2 and 3 days for Hs-578T cells and Caco-2 cells in order to prevent cell death of control cells. Human recombinant HARP was shown to induce Hs-578BST and Hs-578T cell proliferation in a dose-dependent manner (Fig. 2A). In contrast, when the mitogenic activity of HARP was tested on Caco-2 cells, no proliferation was observed for concentrations up to 9 nM HARP whereas 10% FCS induced a twofold stimulation of proliferation under these conditions (Fig. 2A).

As the ALK tyrosine kinase receptor had been recently reported to bind HARP with a high affinity ($K_d = 32$ pM) and to transduce HARP-mediated signals, the expression profile of this molecule in the three cell lines was investigated. No ALK proteins were detected using Western blot experiments but this could be explained by a low level of ALK. Hence, RT-PCR analysis was then performed to test its expression and NIH-3T3 cells, used as a cellular model to test HARP mitogenic activity [12], were used as a positive control. Using the primers described in Materials and methods, 236 and 239-bp products corresponding, respectively, to human and murine ALK were observed with Hs-578BST (Fig. 2B, lane 3) and NIH-3T3 (Fig. 2B, lane 4) cells. In contrast, no signal was detected with Caco-2 cells (Fig. 2B, lane 2). Furthermore, the presence of ALK receptor on Hs-578T cells was previously described [10], the expression of ALK mRNA was then correlated to the mitogenic activity of HARP in those cells.

ALK protein is localized in normal human mammary epithelial and myoepithelial cells in vivo

Since mammary myoepithelial and epithelial cell lines were responsive to HARP mitogenic activity, certainly through ALK receptors, the in vivo expression of this molecule in such cells was questioned. By immunostaining with a mix of three specific anti-ALK antibodies, ALK receptor was localized in both epithelial

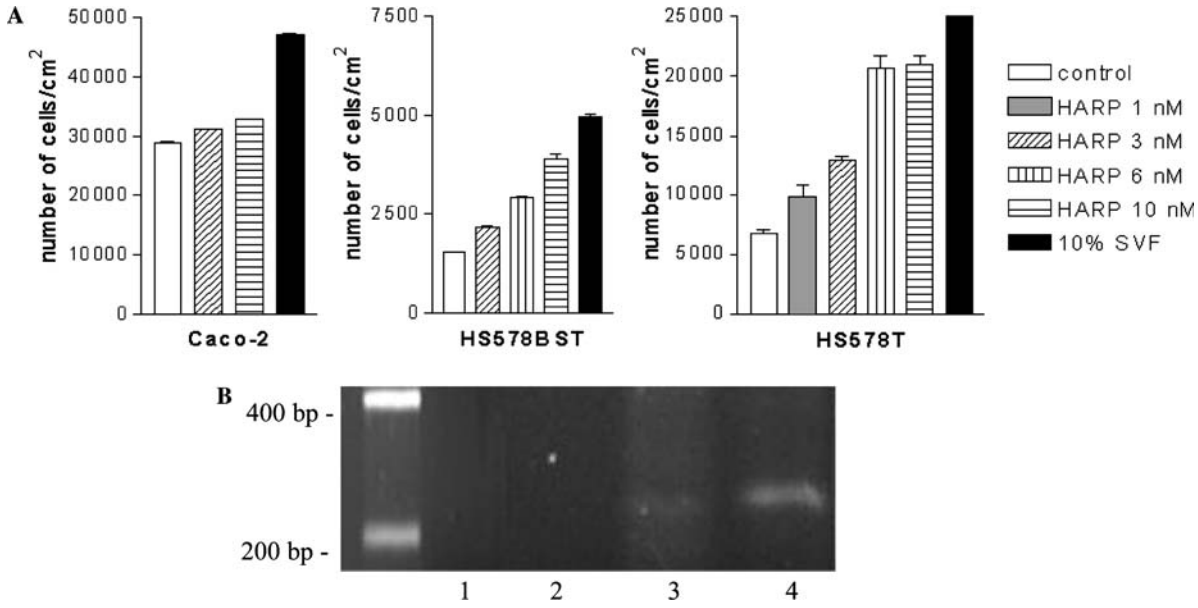

Fig. 2. The mitogenic activity of human recombinant HARP on Hs-578BST, Hs-578T, and Caco-2 cells is correlated with the expression of ALK mRNA. (A) Hs-578BST, Hs-578T, and Caco-2 cells were starved for 24 h and stimulated with different concentrations of human recombinant HARP purified from the culture medium of NIH-3T3 cells HARP. Proteins were added every 2 days and cells were numbered after 2, 3 or 8 days using a Malassez hematocymeter for Hs-578T, Caco-2 or Hs-578BST cells, respectively. For each cell line, the results are means of two separate experiments carried out in duplicate and the standard errors are indicated. (B) The transcript of ALK receptor was examined by RT-PCR using specific primers. Two micrograms of total RNA extracted from NIH-3T3 cells (lane 4), from Hs-578BST cells (lane 3), and from Caco-2 cells (lane 2) was submitted to reverse transcription and PCR amplification as described under "Materials and methods." As negative control, similar experiment was performed with water (lane 1). The size of the amplified fragment of ALK was 236- or 239-bp (respectively, human and murine ALK).

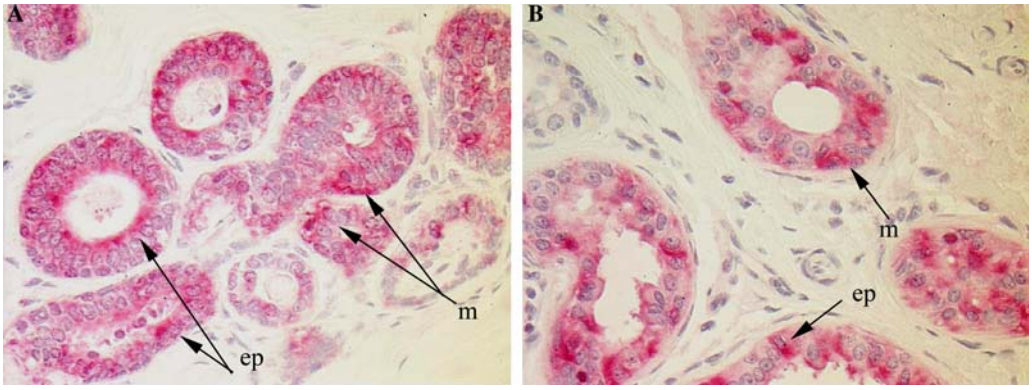

Fig. 3. Localization of ALK receptor in human normal breast. (A) and (B) represent two different sections of human normal breast lobules immunostained with a mix of three specific polyclonal anti-ALK antibodies showing immunostaining of both epithelial (ep) and myoepithelial cells (m). Magnification 200× (A), 400× (B).

and myoepithelial cells (Figs. 3A and B) with approximately 40% staining of the alveolar cells. In the stroma, the matrix compartment was clearly negative and in control, no staining was obtained with non-specific Ig (data not shown).

Discussion

The localization of HARP protein in the human mammary epithelial and myoepithelial alveolar cells [1] prompted us to investigate its presence in milk and also to understand its biological functions in the mammary gland and the neonate intestinal tractus. We have characterized HARP in the colostrums (collected between 1 and 4 days after delivery) and in milk collected at least 8 days after delivery. As reported for other growth factors [20,22,23], HARP concentration was significantly greater ($p < 0.0001$) in colostrum (59.9 ± 11.2 ng/ml) than in mature milk (17.6 ± 6.4 ng/ml). Similarly, HARP concentration in mature milk was 2 orders of magnitude greater than in the serum from

normal adults (0.1 ng/ml) [27] and is in the range of the ED_{50} of HARP measured for its mitogenic activity on the different cell lines tested in vitro (reviewed in [6]). The difference between HARP concentrations in colostrum and in mature milk could be due to hormonal changes during the end of pregnancy and lactation since levels of progesterone and estrogen decreased after birth. Indeed, we have previously demonstrated that HARP mRNAs were upregulated by progesterone in rat uterus [14]. In this study, we also measured the concentration of MK which forms with HARP a family of heparin-binding growth factors and, in this case, MK concentration was similar in colostrum and milk (15.6 ng/ml, ($n = 7$) and 10.8 ng/ml ($n = 10$), respectively) (data not shown), a result in agreement with the absence of MK regulation by progesterone [14].

Polypeptides present in human milk could have various biological effects during the pre- and post-partum stages of pregnancy including development of the mammary gland, lactogenesis, stimulation of immunological signals for the host defense, and post-natal maturation of the gastrointestinal system. For example, HGF has been demonstrated to stimulate the proliferation of the intestine epithelial IEC-6 cells in vitro [22] and VEGF to bind to VEGFR-1 in Caco-2 cells without stimulating their proliferation. In this study, as a first approach, we questioned whether HARP was able to induce cellular proliferation of the neonate intestine cells or of the mother mammary gland cells. Our results are more in favor of HARP activity on human breast homeostasis during lactation. Therefore, two breast cell lines, the myoepithelial cell line Hs-578BST and the epithelial cell line Hs-578T, were stimulated by recombinant HARP in a similar range of concentrations as compared to NIH-3T3 and BEL cells, usually used to test HARP mitogenic activity [12,30]. This stimulation seems to be transduced by the recently identified HARP high affinity receptor ALK, for HARP mitogenic and transforming activities [9], whose mRNA expression was shown by RT-PCR (this study and [11]). However, HARP proteins were only secreted by Hs-578BST (3.6 ng/ml) but not by Hs-578T cells (data not shown), suggesting that HARP could present a paracrine and an autocrine mechanism of action on these epithelial cells and myoepithelial cells, respectively. These results parallel a previous study demonstrating the in vivo localization of HARP protein in myoepithelial and epithelial cells whereas HARP mRNA was restricted to myoepithelial cells [1]. Immunohistochemistry experiments performed on normal human breast also supported this hypothesis since the ALK receptor was detected in both epithelial and myoepithelial cells. However, we cannot exclude that HARP expression in the mammary gland differs from that in normal tissue during lactation. Taken together, our results suggest that in vivo HARP could act as an autocrine and a paracrine growth factor regulating mammary gland development and its homeostatic maintenance during pregnancy and lactation.

The role of HARP in the gastrointestinal tractus of the neonate was also investigated and our results suggest that HARP is not involved in the proliferation of neonate intestine cells since Caco-2 cell proliferation was not stimulated by the recombinant protein. They are in good agreement with the absence of ALK transcripts, even using RT-PCR experiment. However, even if HARP does not affect intestinal cell proliferation, we cannot exclude its role in cell metabolism (e.g., glucose uptake, Ca^{2+} entry, and permeability of mucosa to macromolecules) as it was suggested for VEGF [23] and HGF [22]. Another possible physiological role of HARP present in breast milk concerns the immunomodulation of the newborn gastrointestinal tract. Indeed, mononuclear cells present in milk have been shown to be competent for the production of many different cytokines (IL-1α, IL-1β, and IL-6) when stimulated in vitro [31] and recently HARP was demonstrated to stimulate the in vitro proliferation of PBMC [32]. So, it is tempting to speculate that HARP could stimulate the milk mononuclear cells causing an increasing production of mediator of specific and non-specific immunity [33]. This potential effect of HARP remains to be evaluated.

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