

Presence of immunoreactive adrenomedullin in human and bovine milk

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Abstract

We examined by radioimmunoassay the presence of immunoreactive adrenomedullin (ir-AM) in human and bovine milk. Milk samples displaced ¹²⁵I-AM from the AM-antiserum in parallel to the standard curve. RP-HPLC revealed a main immunoreactive peak eluting as synthetic AM. Concentrations in human milk ranged between 140 and 404 pg/mL. In cow, the levels of AM were 73.5 ± 3.8 pg/mL. Bovine milk products had AM levels similar to those found in fresh bovine milk. Human milk had growth promoting activity on the human intestinal cell line Int-407 that could be partially blocked with an anti-AM antibody. © 2000 Published by Elsevier Science Inc.

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1. Introduction

Milk contains substances that have a critical role in the growth and development of the newborn. The biologically active molecules found in milk participate in multiple physiological processes including modulation of gastrointestinal functions, microbial growth control and immunoregulation. These bioactive compounds include peptide hormones and growth factors such as insulin, transferrin, lactoferrin, epidermal growth factor (EGF), transforming growth factor (TGF), nerve growth factor (NGF), and insulin-like growth factors I and II (IGF-I and IGF-II) [10,20]. The concentrations of these peptides are generally higher in colostrum than in milk and often higher than those found in blood. Due to the limited protease activity in the gastrointestinal tract of neonates, and the existence of protease inhibitors in milk [3], these peptides are likely to survive the gastrointestinal digestion, be absorbed through the gastrointestinal tract and appear intact in plasma.

In 1997, we described the expression of adrenomedullin (AM) in mouse mammary gland, showing AM immunore-

activity in the milk within the ducts from lactating glands. We also demonstrated by western blot the presence of fully processed AM peptide (Mr 6,000) in mouse milk extracts [13]. AM is a regulatory peptide which is structurally related to calcitonin-gene-related peptide (CGRP) and amylin. AM was originally isolated from a human pheochromocytoma as a peptide capable of elevating platelet cyclic AMP and inducing a hypotensive effect in rats [17]. The human mRNA is 1.6 kb long and encodes for a predicted 185 amino acid precursor from which the active full-processed 52 amino acid peptide is generated [18]. The expression of AM has been demonstrated in several tissues [29] and biologic fluids such as plasma [16], cerebro-spinal fluid [34], sweat [24], amniotic fluid [22], and urine [33]. AM has been implicated in the modulation of several physiological functions including cardiovascular tone [9], central brain activity [2,26,30], bronchodilation [15], renal function [7,14], hormone secretion [25,37], cell growth [5,27], differentiation [5], and immune response [1,36].

In view of the presence of AM in mouse milk [13] and the critical role that milk components play in the development of the neonate, we decided to examine the presence of immunoreactive AM (ir-AM) in milk from human, cow and several cow's milk products. In an attempt to further study the role of AM in the development of the gastrointestinal tract we examined the effect of milk and anti-AM on the growth of a human intestinal cell line.

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2. Materials and methods

2.1. Collection of samples

Human milk samples were obtained from three healthy women with healthy full-term infants. We also measured AM concentration in fresh bovine milk (USDA, Beltsville, MD) as well as in bovine milk-based infant formulas and commercially available bovine milk products. All the samples were stored at -20°C until processed.

2.2. Preparation of milk extracts

Frozen milk samples were thawed, centrifuged at $10,000\times g$ at 1°C to remove the fat, and passed through a microfilter ($0.2\ \mu\text{m}$ pore). Extraction of the peptides was performed using reverse-phase Sep-Pak C-18 cartridges (Waters, Milford, MA) as previously reported [21,23]. Briefly, cartridges were washed once with 80% methanol followed by 0.9% NaCl. Milk samples were mixed with an equal volume of phosphate buffer saline containing 0.1% alkali-treated casein and 0.1% Triton X-100, pH 7.4. Samples were applied to the columns and, after washing with 0.9% NaCl, AM was eluted with 80% isopropanol containing 125 mM HCl. Extracts were freeze-dried to remove the organic solvent.

2.3. Radioimmunoassay for adrenomedullin

Concentrations of AM in the extracts were measured by radioimmunoassay as previously described [23]. Extracts were reconstituted in 0.4 ml of radioimmunoassay buffer (10 mM Phosphate, 50 mM EDTA, 135 mM NaCl, 5 mM NaHCO_3 , 0.05% Triton X-100, 0.1% Tween-20, 0.1% alkali-treated casein, pH 7.4). 0.1 ml of sample or standard human adrenomedullin (Peninsula Laboratories, Belmont, CA) were preincubated 18 h at 4°C with 0.1 ml of primary antibody (Phoenix Pharmaceuticals, Mountain View, CA). 0.1 ml of ^{125}I -AM (Phoenix Pharmaceuticals) were added (10,000 cpm) and the mixture was incubated at 4°C for 18 h. Bound tracer was separated by polyethylenglycol-facilitated precipitation with goat antirabbit serum and normal rabbit serum. The supernatant was aspirated and the radioactivity in the pellet determined in a gamma counter. The recovery of unlabeled AM added to the sample was $72.9\% \pm 1.5\%$, whereas recovery of ^{125}I -labeled AM was $82.7\% \pm 4.4\%$.

2.4. Reverse-phase HPLC

Analytical reverse-phase high-performance liquid chromatography (HPLC) was performed using a C18 column ($4.6 \times 250\ \text{mm}$, Vydac, Hesperina, CA). A sample of human milk, extracted with the Sep-Pak C-18 cartridges, was mixed with an equal volume of 10% (v/v) acetonitrile 0.2% (v/v) trifluoroacetic acid, processed through a $0.2\ \mu\text{m}$ filter and loaded onto the column. The column was eluted

with a linear gradient (5% to 60%) of acetonitrile containing 0.075% (v/v) trifluoroacetic acid, at a flow rate of 1 ml/min over 60 min. Each fraction (1 ml) was collected, freeze dried and radioimmunoassayed. The recovery of AM during the chromatographic procedure was 90%.

2.5. Proliferation assays

A human intestinal cell line, Intestine 407 (Int-407) (ATCC, Gaithersburg, MD), was used to investigate the growth effects of human milk. The cells were maintained in Minimal Essential Medium (Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (FCS) (Life Technologies). The MTT Proliferation Assay (Promega, Madison, WI) was carried out in serum-free conditions as previously reported [12]. Briefly, cells were seeded in a 96-well plate at $1-2 \times 10^4$ cells/well, and appropriate concentrations of milk and anti-AM monoclonal antibody MoAb-G6 [27] were added. After 5 days of incubation at 37°C and 5% CO_2 in a humid incubator, the MTT colorimetric assay was carried out following the instructions from the manufacturer. The plate was read at a wavelength of 540 nm. Eight independent wells per treatment were averaged.

2.6. Statistical analysis

The values obtained in the MTT assays were subjected to the Student's *t* test. A P value less than 0.05 was considered significant.

3. Results

Milk samples were extracted and assayed to measure the concentrations of ir-AM. The dilution curves obtained from human and cow milk in the radioimmunoassay were parallel to that of the synthetic human AM used in the standard curve (Fig. 1). Figs. 2A and 2B show AM concentrations measured in milk samples from three different human donors. The milk concentrations of ir-AM in the two first donors were $140.3 \pm 12.5\ \text{pg/mL}$ and $162.7 \pm 15.6\ \text{pg/mL}$ (mean \pm SEM). In the third donor, the levels were measured in samples obtained at intervals during the lactation period; the AM levels in milk during the progression of the lactation varied between 225.0 and 404.3 pg/mL without an apparent pattern of change in the AM concentrations (Fig. 2B).

The concentration of ir-AM in fresh bovine milk was $73.5 \pm 3.8\ \text{pg/mL}$. Different milk products (whole milk, 2% milk, and skim milk) gave comparable levels to those found in fresh bovine milk (Fig. 2C). In the case of the bovine milk-based infant formulas, the levels were also similar to those found in fresh bovine milk (59.5 ± 3.4 and $57.8 \pm 6.5\ \text{pg/mL}$, Fig. 2C).

To further characterize the ir-AM in milk, a human milk sample was fractionated by reverse-phase HPLC and ir-AM

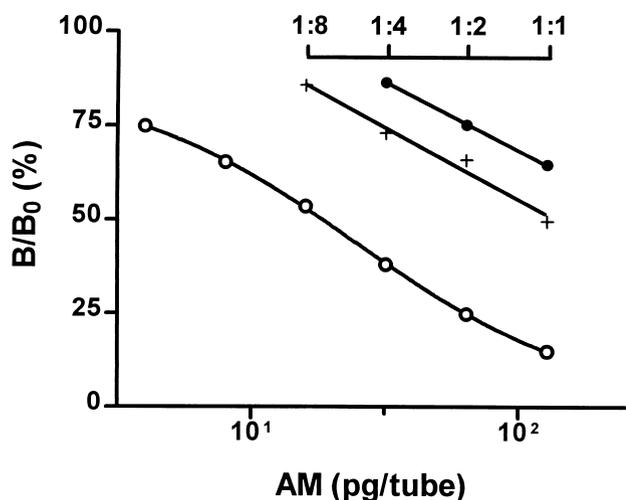


Fig. 1. Competitive binding curves generated by human and bovine milk in the AM radioimmunoassay. The dilution curves of human milk (+) and bovine milk (●) were compared with the standard curve of synthetic AM (○). B/B_0 represents the ratio of radioactivity bound to that bound in the absence of added standard. The scale bar over the milk curves represents the different milk dilutions.

was quantified by radioimmunoassay in each fraction (Fig. 3). The major peak of AM immunoreactivity eluted at the same fraction as synthetic human AM, confirming that the ir-AM corresponded to the fully processed active peptide. A second peak was obtained later (Fig. 3).

We used the MTT assay to examine the growth promoting activity of human milk on the intestinal human cell line Int-407. Milk produced an increase in the proliferation of these cells in a dose-dependent manner (Fig. 4A). The addition of MoAb-G6, a monoclonal antibody that blocks the biologic activity of AM [27], caused a dose-dependent suppression of growth (Fig. 4B).

4. Discussion

The present work shows that ir-AM is present in human and bovine milk. Ir-AM was quantified by radioimmunoassay. In the assay, the milk samples displaced ^{125}I -AM from the specific AM-antiserum in parallel to the standard curve. Reverse-phase HPLC of human milk revealed a main immunoreactive peak eluting at the same position as synthetic AM. A second peak more hydrophobic than AM was detected. The identity of this peak is unknown, however, in a previous report we demonstrated that after fractionation by C18-HPLC of conditioned medium from a human tumor cell line (H720) several fractions showed immunoreactivity with AM antibodies. One of these peaks corresponded to the elution position of synthetic AM. Another peak that eluted later in the chromatography was analyzed by western blot and corresponded to the unprocessed AM precursor and an intermediate form [27]. This fact suggests that the immunoreactivity detected here could also correspond to an AM

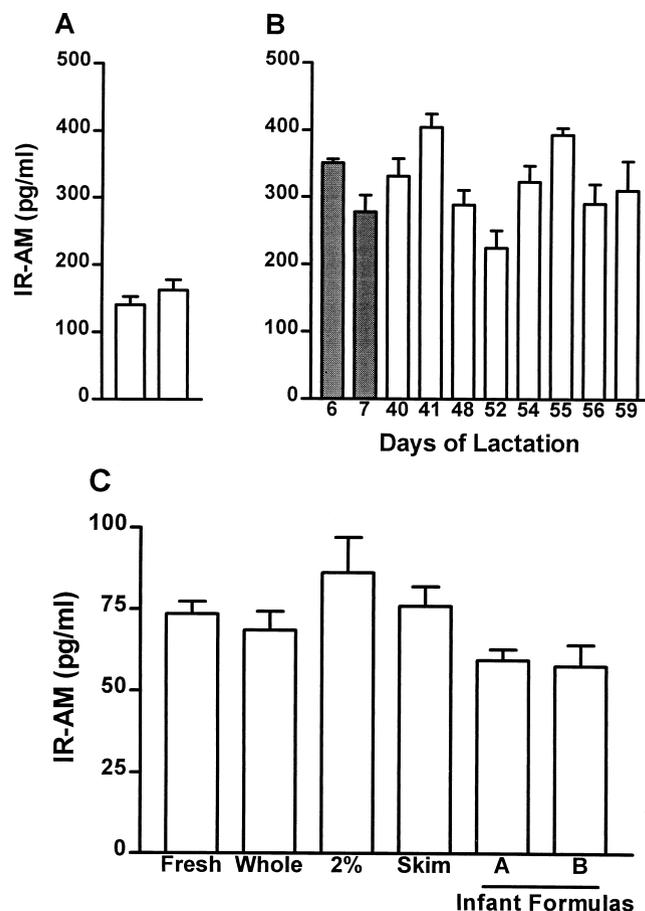


Fig. 2. (A) Ir-AM concentrations in milk samples obtained from two different women. (B) Evolution of ir-AM during the lactation period in a third human donor. The shaded bars correspond to colostrum samples. (C) Ir-AM concentrations in fresh bovine milk, different commercially available milk products, and bovine milk-based infant formulas.

intermediate form. The ir-AM concentrations found in milk ranged from 140 to 404 pg/mL. The normal plasma concentration of AM is in the range of 6 to 60 pg/ml [11], about 35 pg/mL in the case of women post-partum [28], therefore,

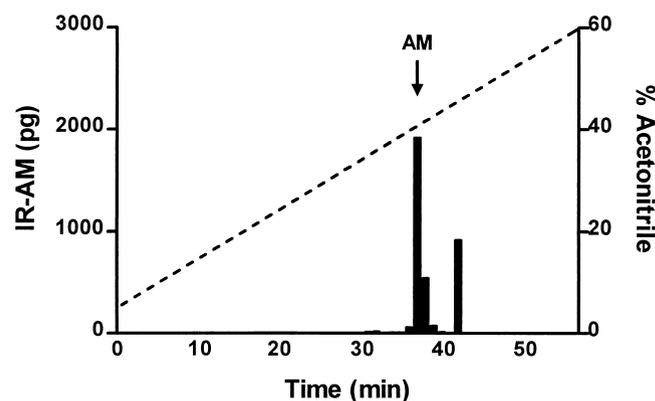


Fig. 3. Ir-AM profile generated by reverse-phase HPLC of human milk. The arrow indicates the elution position of synthetic human AM. The dotted line indicates the acetonitrile gradient.

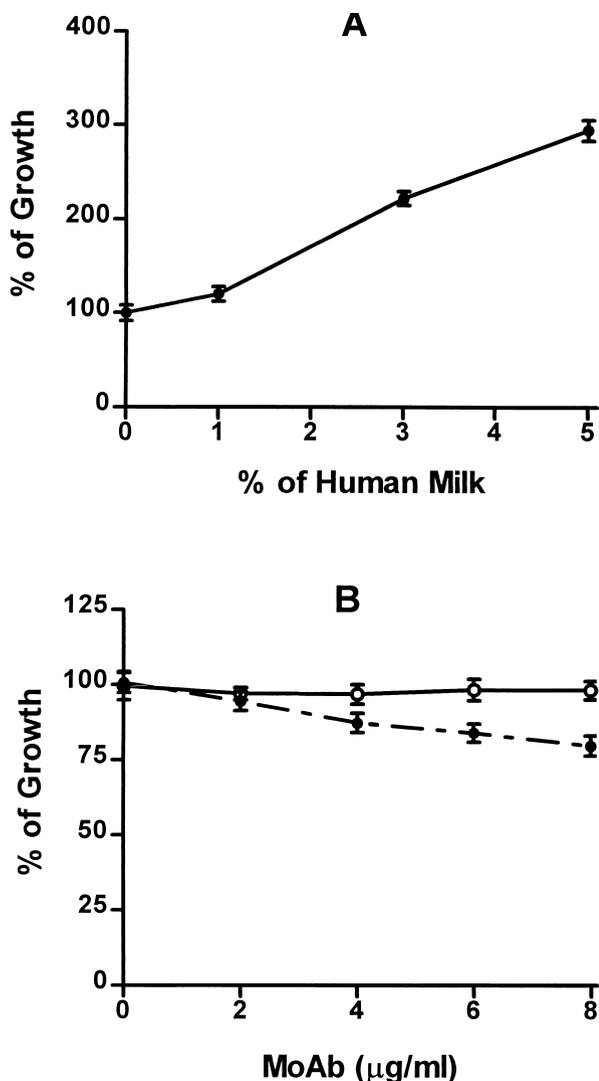


Fig. 4. Growth effects of human milk and MoAb-G6. (A) A small intestinal cell line, Int-407, was incubated in the presence of increasing concentrations of human milk, showing a dose-dependent increase of growth. All treatments showed a significant induction of growth ($P < 0.001$). (B) Int-407 was incubated in medium supplemented with 5% human milk and increasing concentrations of the monoclonal antibody MoAb-G6 (●) or its mouse myeloma isotypic control, IgAκ (○). Values are expressed as percentage of the growth as compared to the control group. The mean and standard deviation of eight values are represented. All treatments higher than $2 \mu\text{g/ml}$ of MoAb-G6 showed a significant inhibition of growth ($P < 0.001$).

we can conclude that the levels of AM in human milk are higher than those reported in plasma. This finding is in agreement with the observation that many of the human hormones and growth factors present in milk exceed their concentrations found in plasma [10]. On the other hand, the concentrations of hormones and growth factors are usually higher in colostrum than in milk, this is at least the case for EGF [4], substance P and CGRP [6]. However in the case of IGF-I the milk levels have been reported to decrease after the transition between colostrum and milk to increase after-

wards between the first and sixth week after parturition [4]. Although further investigation is required, in our study we could not find any evident progression in the AM levels during the lactation period of one of the donors. Because the presence of AM could be relevant for human health, we also analyzed the existence of AM in fresh bovine milk, commercial bovine milk and more importantly, bovine milk-based infant formulas. In cow, the detected levels of AM were found to be lower than those found in humans. Similarly, all the commercial milk products presented comparable levels of AM. The radioimmunoassay for the bovine peptide has previously been validated in bovine plasma samples [8]. In the present work, diluted samples of bovine milk displaced the tracer in parallel to human milk and synthetic AM. This fact suggests that the antibody against human AM recognizes the bovine peptide with similar affinity. However, we cannot rule out the possibility that slight differences in immunoreactivity could exist between human and bovine AM and therefore we have to be cautious in comparing concentrations between both species.

Human milk contains growth factors such as EGF, TGF- α , TGF- β , NGF, insulin, and IGF-I [20]. Milk also contains neuropeptides such as gastrin, gastrin-releasing peptide (GRP), neurotensin, peptide YY, calcitonin, CGRP, and substance P [6,20]. The presence of a substance in milk does not necessarily imply a physiologic role for that entity. The real biologic significance is difficult to establish and requires the demonstration of an effect in the offspring in response to the exposure to the substance in milk as well as the effect in response to the removal of the substance from milk [32]. However, at this juncture, it is possible to speculate that the presence of the active peptide AM in milk could have some direct impact in the development of the neonate due to the several physiological activities that have been associated to it. In relation with the gastrointestinal tract, immunoreactive AM has been detected in human stomach, duodenum, jejunum, ileum and colon [19], and specific binding sites have been detected in rat stomach [31]. This distribution suggests a role for AM in the regulation of secretor and motor functions in the gastrointestinal tract, as well as in its development during the embryogenesis and the period immediately following birth. Since the developing intestine in the neonate is considered to be one of the main target organs for the growth factors present in human milk, we have analyzed the effect of human milk in the growth of a human small intestinal epithelial cell line (Int-407). We demonstrate that milk has a growth promoting activity on this intestinal cell line. Since MoAb-G6 partially blocks the milk induced growth, AM may be one of the growth factors present in milk. AM has also been described as an agent with antimicrobial activity against gastrointestinal microorganisms [1,36]. This activity could be important in the protection of the neonate against gastroenteritis produced by intestinal pathogens. Finally, since some peptides are absorbed from the neonatal gastrointestinal tract and appear intact in plasma [35], AM could also exert

actions in the modulation of tissue growth as well as in the regulation of the immune system.

In conclusion, this report shows for the first time that human milk contains high levels of the peptide AM and that AM has a direct growth effect on intestinal cells.

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