Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells

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Abstract We compared the expression of human α- and β-defensins by various human tissues. mRNA for α-defensins HNP1–3, abundant in bone marrow, was detected in peripheral blood leukocytes, spleen and thymus by RT-PCR, which revealed α-defensins HD5 and HD6 only in the small intestine. In contrast, the pancreas and kidney expressed high levels of hBD-1 and lower levels of this β-defensin were found in many organs by RT-PCR (salivary gland > trachea > prostate and placenta > thymus, testis, small intestine). hBD-1 mRNA was produced constitutively by cultured normal human epithelial cells derived from the trachea, bronchi, small airways and the mammary gland. These largely non-overlapping tissue distributions of human α- and β-defensins suggest that hBD-1 may be positioned to defend epithelial cells and mucosae from infection, whereas expression of HNP1–3 in neutrophils and HD5 and HD6 in Paneth cells allows these α-defensins to participate in systemic and small intestinal host defenses, respectively.

Key words: Antimicrobial peptide; Defensin; Epithelial cell; hBD-1

1. Introduction

Mammalian defensins are 3.5–4.5 kDa antimicrobial peptides whose β-sheet structures are stabilized by three intramolecular cystine disulfide bonds [1]. Two families, called α- and β-defensins, exist in mammals and birds [2–7]. Six α-defensins and one β-defensin are known in humans (Fig. 1). α-Defensins HNP1, -2, -3 and -4 are found in neutrophils [1] and HD5 and HD6 in small intestinal Paneth cells [8–10]. The only known human β-defensin, hBD-1, was isolated from plasma and noted to be expressed by the kidney and the female reproductive tract [11]. β-defensins occur in leukocytes of cattle [2,3] and fowl [4,5] and in bovine tracheal and lingual epithelial cells [6,7]. The human *hBD-1* gene is on chromosome 8p23, within 100–150 kb of the gene for HNP1, suggesting that α- and β-defensins descended from a shared ancestral gene [12]. To gain additional insights into the respective roles of the two defensin families, we compared the expression of α- and β-defensins in various normal human tissues.

2. Materials and methods

2.1. Cell culture and total RNA purification

Primary cultures of the following normal human cells were obtained from Clonetics Corporation (San Diego, CA): first-passage bronchial/tracheal epithelial cells, second-passage small airway epithelial cells and sixth-passage mammary epithelial cells. Cells were passaged twice to 80–90% confluence in Corning 75 cm^2 tissue culture flasks. The serum-free medium (SFM) was changed every other day, and consisted of a basal medium (Clonetics) supplemented with 0.5 μg/ml hydrocortisone, 0.5 ng/ml recombinant human epidermal growth factor, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B.

Bronchial/tracheal epithelial cells were cultured in SFM with 52 μg/ml bovine pituitary extract. Mammary epithelial cells were grown in SFM with 52 μg/ml bovine pituitary extract and 10 ng/ml of epidermal growth factor, but without epinephrine, transferrin, retinoic acid or tri-iodothyronine. Small airway epithelial cells were grown in SFM containing 30 μg/ml of bovine pituitary extract and 0.5 mg/ml of fatty acid-free bovine serum albumin. The HTB-41 human submaxillary epidermoid carcinoma cell line and HTB-81 prostatic carcinoma cell lines were obtained from the American Type Culture Collection, Rockville, MD. The submaxillary cells were grown in McCoy 5A medium (Gibco, Grand Island, NY) and the prostate cells in Eagle's Minimal Essential Medium (Gibco), both supplemented with 10% heat-inactivated fetal calf serum.

In some experiments, RNA was harvested from cells cultured for 4–20 h with one of the following stimuli: 100 ng ml^{-1} *E. coli* 055:B5 lipopolysaccharide (Sigma), 100 ng ml^{-1} phorbol myristate acetate (Sigma), 80 ng ml^{-1} recombinant human interleukin 6 (Gibco, Grand Island, NY), 100 U ml^{-1} recombinant human interferon-γ (Boehringer Mannheim, Indianapolis, IN) or 20 ng ml^{-1} TNF-α (Boehringer Mannheim) and subjected to RT-PCR as described below.

2.2. RNA purification and reverse transcriptase (RT-PCR)

Total RNA was purified from cultured cells and peripheral blood leukocytes with the RNeasy Total RNA kit (QIAGEN Inc., Chatsworth, CA). Total RNA from thymus, prostate, testis, salivary gland, skeletal muscle, placenta, small intestine, kidney, trachea, spleen, bone marrow (all human) was also purchased from Clontech (Palo Alto, CA). cDNA (20 μl) was synthesized from 1 μg of each total RNA with a Clontech cDNA kit. A 1/5 dilution of cDNA (10 μl) was added to a mixture (50 μl final volume) that contained 1 μl of dNTP mix (10 mM each), 1 μl 5′ and 3′ primers (25 μM each), and 2.5 U of cloned pfu DNA polymerase (Stratagene, La Jolla, CA), The PCR primers and the expected product sizes (Table 1) are based on published sequences [8,9,15]. Primer A-1 (sense) corresponds to nucleotides 110–136 of the hBD-1 cDNA sequence and Primer A-2 (antisense) is complementary to the 3′ end of hBD-1, immediately before its poly(A) tail [11,12]. The human β-actin control amplimer set (Clontech) generated a product of 838 bp.

PCR was performed as follows with an automated DNA thermal cycler for 25, 30, 35 cycles: denaturation, 1 min at 94°C; annealing, 1 min at 60°C; extension, 2.5 min at 72°C. We used a master reagent mix to ensure tube to tube consistency in cDNA synthesis and PCR reactions and included a negative control reaction in each experiment. Reaction products (10 μl) were visualized after electrophoresis in 1.3% agarose gels containing 1 μg ml^{-1} ethidium bromide.

2.3. Subcloning, DNA sequencing, Northern blots

PCR products of hBD-1 around 250 bp in size were cloned into pCR-Script SK vector (Stratagene, La Jolla, CA). DNA sequencing was performed with a kit, according to the manufacturer's instructions (US Biochemical Corp., Cleveland, OH). Commercial multiple tissue blots of human poly A^+ RNA were obtained from Clontech and represented the following tissues: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes. The membranes were hybridized with a radiolabeled synthetic oligonucleo-

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tide, 5′-CTTGCAGCACTTGGCCTTCCCTCTGTAACAGGTGCC-TTGAATTTTGGTAAAGATCGGGCA-3′, complementary to the hBD-1 cDNA sequence before the stop codon [11,12]. Fifteen micrograms of total RNA was hybridized to a full-length hBD-1 cDNA probe labeled by the random oligonucleotide method. The RNA bands were separated on 1% agarose formaldehyde gels and transferred to Gene Screen Plus nylon membranes (DuPont, Boston, MA) by capillary transfer.

2.4. Quantitative imaging

PCR products (30 cycles) of hBD-1 were separated on a 1.3% agarose gels, transferred to nylon membranes and hybridized with [α-^{32}P]dCTP labelled hBD-1 cDNA. Quantitative analysis was performed with a Phosphor Imager 445 SI (Molecular Dynamics, Sunnyvale, CA). To compare mRNA expression in different tissues, data from each tissue were initially normalized by the formula: relative amplified product=(P_{tissue}/P_{max})×100, wherein P_{tissue} was the amount of amplified product obtained from each tissue, and P_{max} was an amplified product obtained from kidney. The resulting data were then renormalized by setting the next highest sample (submaxillary gland) to 100%.

3. Results and discussion

Although synthesis of hBD-1 by human kidney and female productive tract [11,16] and of human α-defensins HNP1–3 and HD5 and HD6 by myeloid cells [15] and Paneth cells [8,9] respectively has been reported, the expression of these human defensins has not been systematically compared, tissue by tissue. Our Northern blots confirmed abundant production of hBD-1 by kidney, but also revealed substantial expression by the pancreas (Fig. 2). The production of hBD-1 by various sites within the female genitourinary tract [16] will be described elsewhere. The prominent expression of hBD-1 in the pancreas might be related to protecting pancreatic ductal and acinar epithelial cells from retrograde infection via the pancreatic duct, or might contribute to the antimicrobial properties of pancreatic secretions [17], thereby compensating for the low density of α-defensin producing Paneth cells in the duodenum [18].

After 25 cycles of RT-PCR, hBD-1 transcripts were evident only in the kidney and submaxillary salivary gland. In contrast, HNP1–3 transcripts were prominent after 25 RT-PCR cycles in the bone marrow, peripheral blood leukocytes, spleen and thymus (Fig. 3). After 30 cycles of PCR, hBD-1 transcripts were also seen in prostate, testis, placenta, small intestine and trachea, whereas HNP1–3 transcripts were evident in the prostate, submaxillary salivary gland, placenta and trachea.

We found little or no evidence of HNP1–3 production by the kidney, which had large amounts of hBD-1 mRNA, the small intestine (which expressed HD5 and HD6) (Fig. 4) or the testis (which was reported to express large amounts of a

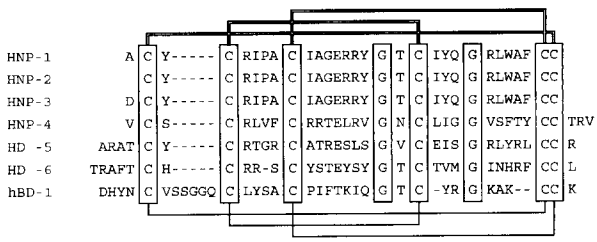

Fig. 1. Amino acid sequences of seven human defensins. hBD-1 is a β-defensin, the others are α-defensins. The typical cystine disulfide pairing of α-defensins is shown by double lines above and that typical of β-defensins is shown by the lines below the sequences. Gaps (–) were introduced to maximize the alignment of α- and β-defensins, and 8 residues that are highly conserved in α-defensins (6 cysteines and 2 glycines) are boxed.

cathelin-associated antimicrobial peptide [19,20]). Because all tissues contain multiple cellular elements that contribute to their total mRNA pool, we purified RNA from normal epithelial cells that were derived from the upper (trachea and bronchi) and lower airways or from mammary gland, and also recovered RNA from human prostate and submaxillary gland cell lines. We identified mRNA encoding hBD-1 in all of those cells by RT-PCR, but not by Northern blotting (data not shown). With the phosphor-imaging system, we performed a more quantitative evaluation of hBD-1 expression by several of the RT-PCR positive/Northern blot-negative tissues. Relative to submaxillary gland (100%), their levels of hBD-1 expression were: trachea, 76.9%; prostate, 62.7%; placenta, 54.5%; thymus, 37.8%; small intestine, 29.4%; and testis 24.8%. Considerably lower levels of product were found in the bone marrow (9.0%), skeletal muscle (8.9%), spleen (6.5%) and peripheral blood leukocytes (5.4%).

As mRNAs for bovine mucosal epithelial β-defensins, tracheal antimicrobial peptide ('TAP') and lingual antimicrobial peptide ('LAP') are induced after challenge with bacterial lipopolysaccharide, tumor necrosis factor alpha (TNF-α), and traumatic injury [7,13,14], we decided to examine hBD-1 expression for evidence of similar responsivity. To ensure our ability to detect increased numbers of hBD-1 transcripts, we mixed various proportions of RNA from cultured tracheal/bronchial epithelial cells, which contained relatively high hBD-1 transcript numbers, with RNA from cultured small airway epithelial cells, which contained relatively low transcript numbers, to a final weight of 1 μg and performed RT-PCR on the mixtures. As we saw the expected relationship between the hBD-1 RNA input and the final amount of amplified product, we were confident of our ability to detect increased relative levels of hBD-1 had they occurred.

Primary cultures of normal human tracheal/bronchial, small

Table 1
PCR primers used in this study

Primer	Sequence	Peptide	Product bp
A-1	5′-TTGTCTGAGATGGCCTCAGGTGGTAAC-3′	hBD-1	253
A-2	5′-ATACTTCAAAAGCAATTTTCCTTTAT-3′	hBD-1	253
B-1	5′-CACTCCAGGCAAGAGCTGATGAGGTTG-3′	HNP1-3	215
B-2	5′-AATGCCCAGAGTCTTCCCTGGTAGATG-3′	HNP1-3	215
C-1	5′-TGGTGGCCCTGCAGGCCCAGGCTGAG-3′	HD5	238
C-2	5′-AGTCTGTAGAGGCGGCCACTGATTTC-3′	HD5	238
D-1	5′-TCACCATCCTCACTGCTGTTCTCCTC-3′	HD6	281
D-2	5′-GAATCTGTGGTTAATACCCATGACAG-3′	HD6	281

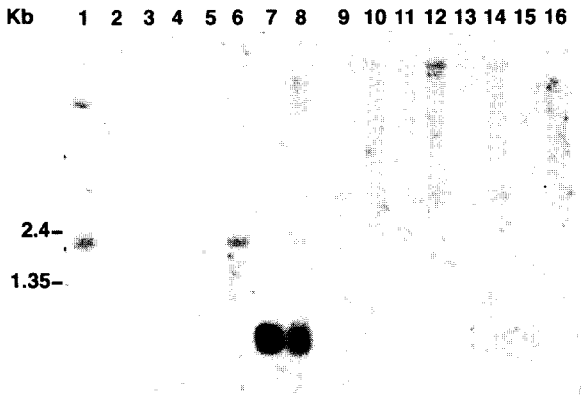

Fig. 2. Northern blot analysis of hBD-1 expression in various human tissues. The filters were loaded with human mRNA from (1) heart, (2) brain, (3) placenta, (4) lung, (5) liver, (6) skeletal muscle, (7) kidney, (8) pancreas, (9) spleen, (10) thymus, (11) prostate, (12) testis, (13) ovary, (14) small intestine, (15) colon, (16) peripheral blood leukocyte. Intense hybridization signals can be found in lanes 7 and 8 (kidney and pancreas).

airway, and mammary epithelial cells were incubated with LPS, IL-6 or phorbol myristate acetate for 4 h and 20 h, then their RNA was purified and RT-PCR was performed. We also challenged the submaxillary epithelioid carcinoma cells with different concentration of LPS (from 50 to 800 ng/ml), IL-6, PMA, IFN-γ, TNF-α, LPS+IL-6, LPS+PMA, LPS+IFN-γ, LPS+TNF-α, without inducing hBD-1 message. Under none of these experimental conditions did we observe any enhanced levels of hBD-1. We concluded, with some disappointment, that either expression hBD-1 was constitutive in these systems or that it was regulated by stimuli other than those reported for bovine TAP.

Because cattle possess at least 15 different β-defensins (13 in neutrophils [2,3] and one each (TAP and LAP) in tracheal and lingual epithelial cells) [6,7], and multiple β-defensins exist in the leukocytes of chickens and turkeys [4,5] we subcloned and sequenced 32 different 250 bp RT-PCR products derived from human submaxillary gland (8 clones), prostate (8 clones) and small intestinal RNA (8 clones) or from the cultured human tracheal cells (8 clones). All but two (93.8%) yielded sequences identical to hBD-1. One salivary gland clone had a single nucleotide difference that would change Val^6 to Ile^6 in the mature hBD-1 peptide. A single small intestinal clone also had a base pair change that would have changed Gly^{25}, a seventh cysteine residue. We concluded that one or both were likely PCR artifacts.

Overall, these data indicate that hBD-1 is expressed by epithelial cells in many human organs. In some tissues, exemplified by the kidney, pancreas, and female reproductive tract, hBD-1 mRNA is sufficiently abundant to allow its facile identification by Northern blots. Other tissues express hBD-1 in relatively smaller amounts, so that more sensitive detection techniques such as RT-PCR are required.

The current demonstration that the various defensins exhibit highly tissue-specific expression offers a potential explanation for why so many different defensin species exist within an individual species. Just as the diversification of organisms is favored by geographic isolation and environmental selection, the differential tissue expression (and consequent spatial separation) of defensins suggests that some of their molecular diversity is a result of selection pressures exerted by microbial pathogens with corresponding tissue tropisms.

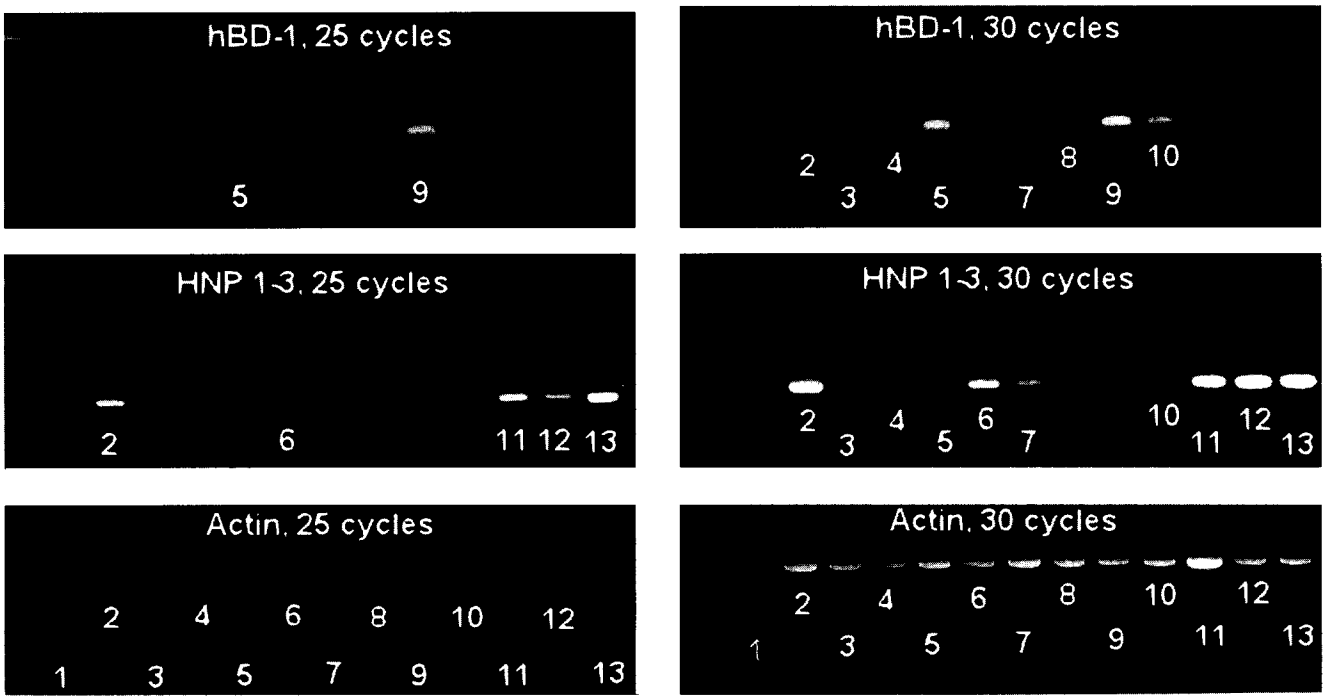

Fig. 3. Analysis of hBD-1 and HNP1–3 expression in various normal human tissues by RT-PCR. Lane 1, RNA derived from no template; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, salivary gland; lane 6, skeletal muscle; lane 7, placenta; lane 8, small intestine; lane 9, kidney; lane 10, trachea; lane 11, spleen; lane 12, peripheral blood leukocytes; lane 13, bone marrow. Top panels: hBD-1 expression, detected as a 253 bp fragment with 25 or 30 cycles of amplification. Middle panels: HNP1–3 expression, detected as a 215 bp fragment, with 25 and 30 cycles of amplification. Bottom panels: β-actin expression, detected as a 838 bp fragment, with 25 and 30 cycles of amplification.

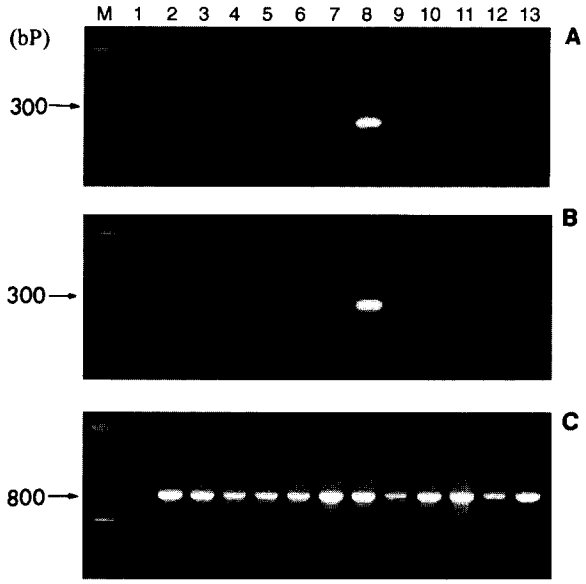

Fig. 4. RT-PCR analysis of HD5 and HD6 expression. The lane number are as defined in Fig. 3. A: HD5 expression was detected as a 238 bp fragment. B: HD6 expression was detected as a 281 bp fragment. C: β-actin expression expression was detected as a 838 bp fragment. All products were amplified for 35 cycles.

Overall, the pattern of expression of human β-defensin hBD-1 suggests that its primary role may be to defend epithelial cells and certain mucosal surfaces from microbial invasion. In contrast, the presence of α-defensins HNP1–3 in neutrophils gives these peptides access to all vascularized tissues, which permits their participation in systemic host defenses.

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