RNase 7, a Novel Innate Immune Defense Antimicrobial Protein of Healthy Human Skin*

Received for publication, July 29, 2002, and in revised form, August 27, 2002
Published, JBC Papers in Press, September 18, 2002, DOI 10.1074/jbc.M207587200

Jürgen Harder and Jens-Michael Schröder‡

From the Clinical Research Unit, Department of Dermatology, University Hospital Kiel, Schittenhelmstrasse 7, D-24105 Kiel, Germany

We analyzed healthy human skin for the presence of endogenous antimicrobial proteins that might explain the unusually high resistance of human skin against infections. A novel 14.5-kDa antimicrobial ribonuclease, termed RNase 7, was isolated from skin-derived stratum corneum. RNase 7 exhibited potent ribonuclease activity and thus may contribute to the well known ribonuclease activity of human skin. RNase 7 revealed broad spectrum antimicrobial activity against many pathogenic microorganisms and remarkably potent activity (lethal dose of 90% < 30 nM) against a vancomycin-resistant Enterococcus faecium. Molecular cloning from skin-derived primary keratinocytes and purification of RNase 7 from supernatants of cultured primary keratinocytes indicate that keratinocytes represent the major cellular source in skin and that RNase 7 is secreted. RNase 7 mRNA expression was detected in various epithelial tissues including skin, respiratory tract, genitourinary tract, and at a low level, in the gut. In addition to a constitutive expression, RNase 7 mRNA was induced in cultured primary keratinocytes by interleukin-1β, interferon-γ, and bacterial challenge. This is the first report demonstrating RNases as a novel class of epithelial inducible antimicrobial proteins, which may play an important role in the innate immune defense system of human epithelia.

The epithelia of macroorganisms represent the first barrier against invading harmful microorganisms. Therefore macroorganisms have to develop strategies to prevent microorganisms from entering the epithelia. It has been shown that the epithelia of plants, invertebrates, and vertebrates have the capacity to release antimicrobial proteins that rapidly kill invading microorganisms (1). Recent investigations have demonstrated that human epithelia also mount an innate chemical defense system by producing antimicrobial peptides, thus offering a fast response to invading microorganisms (2).

The increasing number of reports demonstrating the expression of antimicrobial peptides in human skin reflects the significance of antimicrobial proteins in a cutaneous innate chemical defense system. A member of the cathelicidin family, the serine protease inhibitor antileukoprotease, a novel anionic peptide called dermcidin, and three members of the β-defensin family have been reported to contribute to a human skin defense system.

Proteolysis of the 20-kDa precursor of the human cathelicidin, named hCAP-18, forms the antimicrobial active, α-helical 37-amino acid-containing antimicrobial peptide LL-37. This antimicrobial peptide is expressed in inflamed skin but not in normal skin (3).

The human serine protease inhibitor antileukoprotease was isolated from human stratum corneum and detected in supernatants of cultured human primary keratinocytes. In addition to its antiprotease activity, antileukoprotease exhibits high antimicrobial activity against a broad range of microorganisms, indicating that antileukoprotease contributes to the high resistance of the epidermis against infections and proteolysis (4).

Dermcidin is a novel anionic antimicrobial peptide produced exclusively by human sweat glands (5). This proteolytically processed antimicrobial peptide is secreted into the sweat, and its antimicrobial activity is not affected by the low pH value and high salt concentrations of human sweat.

One major class of human skin antimicrobial peptides comprises the β-defensins, small (4–5 kDa) cationic antimicrobial peptides. Three human β-defensins (hBD-1, hBD-2, and hBD-3) have been demonstrated to be expressed in human skin. The first human β-defensin, hBD-1, was originally purified from hemofiltrates (6), and constitutive mRNA expression has been detected in various epithelia (7–10). In human skin hBD-1 transcripts have been identified in suprabasal keratinocytes and in sweat ducts within the dermis (11). As yet, however, there is no report demonstrating that bioactive hBD-1 peptide can be extracted from human skin.

Two other human β-defensins, hBD-2 and hBD-3, have been isolated from lesional psoriatic skin (12, 13). In human keratinocytes, hBD-2 and hBD-3 gene expression is highly up-regulated by bacteria and proinflammatory cytokines (12–15). This might be the reason why inflamed skin (i.e., psoriatic skin) contains high amounts of hBD-2 and hBD-3, whereas healthy skin contains only low amounts, if any, of these β-defensins (12, 13, 15).

To gain more insight into the production of antimicrobial proteins by healthy skin, we biochemically analyzed the extracts obtained from a healthy person’s stratum corneum for the presence of antimicrobial proteins. As a result, we report here the discovery of a novel human epithelial and highly antimicrobial active RNase termed RNase 7 as a major antimicrobial protein of healthy skin.

EXPERIMENTAL PROCEDURES

Culture of Epithelial Cells—Foreskin-derived keratinocytes and primary airway epithelial cells were prepared and cultured as described.
SDS-PAGE in the presence of 8M urea and Tricine (19) under non-
fication of hBD-2 (16). Electrophoretic mobility was investigated using
Escherichia coli
formed exact mass of 14,546.063 Da.

Purification and Characterization of RNase 7—Stratum corneum
derived from the heel (50 g) was extracted with acidic ethanolic citrate
buffer as described previously (17). Stratum corneum extracts or the
supernatants of cultured primary keratinocytes were dialyzed against equilibration buffer (10 mm Tris citrate, pH 7.4) using Amicon
YM3 filters and then applied to a heparin affinity column (1-ml Hi-
Trap™, Amersham Biosciences), which was previously equilibrated with
equilibration buffer. Heparin affinity column bound material was then eluted using 10 ml of 2 × NaCl in equilibration buffer at a flow rate of
1 ml/min. Eluted material was dialyzed against 0.1% trifluoroacetic acid,
acetic acid, pH 3, and applied to a preparative wide-pore RP-8-HPLC™
column (300 × 7 mm, C18, Nucleosil, 250 × 12.6 mm, Macherey and Nagel)
that was previously equilibrated with 0.1% (v/v) trifluoroacetic acid in
high performance liquid chromatography (HPLC) grade water containing
20% (v/v) acetonitrile. Proteins were eluted with a gradient of increas-
ing concentrations of acetonitrile containing 0.1% (v/v) trifluoroacetic acid
(flow rate of 2 ml/min). Aliquots (10–30 µl) of each fraction were
lyophilized, dissolved in 5 µl of 0.1% (v/v) aqueous acetic acid, and
tested for antimicrobial activity against Staphylococcus aureus and
Escherichia coli using a radial diffusion plate assay (18).

Fractions containing antimicrobial activity were further purified by
cation exchange HPLC followed by RP-18-HPLC as described for puri-
fication of HBD-2 (16). Electrophoretic mobility was investigated using
SDS-PAGE in the presence of 8 M urea and Tricine (19) under non-
fication of hBD-2 (16). Electrophoretic mobility was investigated using

Antimicrobial Assays—Antimicrobial activity of the resulting HPLC
fractions was determined using a radial diffusion agarose assay system
(18). Antimicrobial activity of purified RNase 7 was estimated using a
microlidulation assay system (13). Briefly, test organisms were incu-
bated with various concentrations of RNase 7 in 100 µl of 10 mM sodium
phosphate buffer (pH 7.4) containing 1% (v/v) trypsinoyc soy broth for
3 h at 37 °C. The antibiotic activity of RNase 7 was analyzed by plating
serial dilutions of the incubation mixtures and determining the number
of colony-forming units (CFUs) the following day. The limit of detection
(1 colony per plate) was equal to 1 × 10^5 CFU/ml.

Analysis of Cytotoxic Activity—Primary keratinocytes were seeded in a
96-well tissue culture plate (Falcon, 10^4 cells/well) and incubated with
200 µg/ml RNase 7 in bovine pituitary extract-free KGM medium for
16 h. After incubation, cell death was determined by measuring lactate
dehydrogenase activity released from the cytosol of damaged cells into
the supernatant using the cytoxicity detection kit (Roche Molecular
Biochemicals). Addition of 0.1% Triton X-100 served as a positive
control.

Enzymatic Activity of RNase 7—The ribonuclease activity of RNase 7
was determined against a standard yeast tRNA substrate as described
previously (22). Briefly, 8 pmol of RNase 7 was incubated in 0.5 ml of 40
mM sodium phosphate, pH 7.0, containing varying concentrations
(4–40 µg) of yeast tRNA (Sigma) at 37 °C. The reaction was stopped by
the addition of 0.5 ml of 20 mM lanthanum nitrate with 3% perchloric
acid, and insoluble tRNA was removed by centrifugation for 10 min at
12,000 × g. The amount of solubilized tRNA was determined by meas-
uring the absorbance at 260 nm, with an absorbance of 1.0 correspond-
ing to 40 µg of RNA. Michaelis constants (K_m (µM)) and turnover num-
bers (k_cat (s⁻¹)) were determined from the appropriate intercepts of
double reciprocal (Lineweaver-Burk) plots as described (22).

Cloning of RNase 7 cDNA from Primary Keratinocytes—Total RNA
obtained from primary human keratinocytes was reverse-transcribed
using standard reagents (Invitrogen). A 5′-RACE (rapid amplification
of cDNA ends) strategy (23) was used to amplify an RNase 7 specific
sequence from the cDNA. Two degenerate primers (5′-TGTTTTATA
(A/G)AT/CT/CAGCC/CT/ATG/3′ and 5′-CA/A/G/TGTTTTAA/G/AT-
TCCAGC/3′) were designed based on RNase 7 amino acid sequence data

1 The abbreviations used are: HPLC, high performance liquid chro-
matography; CFU, colony forming unit; GADH, glyceraldehyde-3-
phosphate dehydrogenase; RT-PCR, reverse transcriptase-PCR; ECP,
eosinophil cationic protein/RNase 7; EDN, eosinophil-derived neuro-
toxin; RP, reverse phase; Tricine, N-tris(hydroxymethyl)methylglycine;
RACE, rapid amplification of cDNA ends.
Samples were incubated for an initial denaturing at 95 °C for 10 min, followed by 45 cycles, each cycle consisting of 95 °C for 15 s, 60 °C (touchdown of 1 °C cycle from 66–60 °C) for 5 s, and 72 °C for 10 s. Cycle-to-cycle fluorescence emission readings were monitored at 72 °C at the end of each cycle and analyzed using LightCycler Software (Roche Molecular Biochemicals). Melting curves were generated after each run to confirm amplification of specific transcripts. RNase 7 intron-spanning primers were derived from the chromosome 14-derived sequence similarity of RNase 7 to members of the RNase A superfamily, we were able to isolate a healthy person’s heparin column. Bound proteins were then separated by preparative reversed-phase C8 HPLC, and HPLC fractions were analyzed for antimicrobial activity against S. aureus (Fig. 1A). One of the HPLC fractions, which was found to contain high antimicrobial activity, was further purified using microexchange HPLC (data not shown) and microreversed-phase C18 HPLC (Fig. 1B). Tricine-SDS-urea-polyacrylamide gel electrophoretic analyses revealed a single band migrating as a 17-kDa polypeptide (Fig. 1B, inset). NANOeletro spray ionization mass spectrometry revealed an exact molecular mass of 14,546.06 Da (Fig. 1D). NH2-terminal amino acid sequence analyses by Edman degradation gave the sequence shown in Fig. 1C, which indicated a new human antimicrobial protein. Using degenerated primers, the complementary DNA (cDNA) was isolated from primary keratinocytes. This cDNA (sequence data have been submitted to the GenBankTM/EBI databases (accession numbers AJ131212 and AJ306608)). Amino acid sequence similarity of RNase 7 to members of the RNase A superfamily significantly improved that RNase 7 might exhibit ribonuclease activity. To test this hypothesis, we analyzed RNase 7 for its ability to digest yeast tRNA in a standard assay. As a result we detected high enzymatic activity of RNase 7 (Michaelis constant $K_m = 2.2 \mu M$; catalytic constant $k_{cat} = 5.1 s^{-1}$), which was 50-fold more catalytically active (catalytic efficiency $k_{cat}/K_m = 2.3 \times 10^6 M^{-1} s^{-1}$) than baculovirus-derived recombinant eosiophilic cationic protein/RNase 3 (catalytic efficiency $k_{cat}/K_m = 4.9 \times 10^4 M^{-1} s^{-1}$; data obtained from Ref. 24).
RNase 7 Exhibits Broad Spectrum Antimicrobial Activity—
Analyses of the in vitro antimicrobial properties of natural RNase 7 revealed high antimicrobial activity against several potentially pathogenic Gram-positive bacteria (S. aureus, Propionibacterium acnes), Gram-negative bacteria (Pseudomonas aeruginosa, E. coli) and the yeast Candida albicans (Fig. 3). Furthermore, RNase 7 exhibited extremely high activity against vancomycin-resistant E. faecium (lethal doses that achieve a CFU reduction of 90%, LD$_{90}$ < 0.03 μM, Fig. 3).

No cytotoxic activity toward keratinocytes was observed, not even at high RNase 7 concentrations up to 200 μg/ml (data not shown).

RNase 7 Is Expressed in Various Epithelial Tissues—To investigate the tissue distribution of RNase 7 mRNA expression, we analyzed mRNA obtained from various body sites by real-time RT-PCR. Gene expression was detected in most of the analyzed tissues including skin, respiratory tract, and genitourinary tract (Fig. 4A). Weak expression was seen in tissues of the gastrointestinal tract including stomach, small intestine, and colon.

To investigate the cellular origin of RNase 7 in human skin and in the respiratory tract, we first analyzed cultured primary keratinocytes as well as respiratory epithelial cells for RNase 7 mRNA expression using conventional RT-PCR. We found that cultured primary keratinocytes as well as primary nasal, bronchial, and nasal epithelial cells, but not skin-derived fibroblasts, expressed RNase 7 mRNA (not shown).

We then investigated whether keratinocytes release RNase 7 protein. HPLC analyses of cationic proteins secreted by cultured primary keratinocytes led to the isolation of an antimicrobial protein showing identical biochemical properties, including the exact mass of RNase 7 (14,546 Da) as revealed by nanoelectrospray mass spectrometry. We were able to purify ~10 μg of RNase 7 from the culture supernatants of ~5 × 10$^8$ unstimulated primary keratinocytes, indicating that skin keratinocytes release RNase 7.

RNase 7 Gene Expression Is Induced in Primary Keratinocytes by Proinflammatory Cytokines and Infectious Stimuli—We next investigated the effect of various proinflammatory cytokines and bacteria on the RNase 7 gene expression in cultured primary keratinocytes by real-time PCR. The low basal gene expression of RNase 7 increased 7-fold upon treatment with 10 ng/ml interferon γ and 8.5-fold by 10 ng/ml interleukin 1β. Application of 10 ng/ml tumor necrosis factor α led only to a 2.5-fold induction of RNase 7 gene expression (Fig. 4B). The contact of keratinocytes with 10$^7$ colony-forming units/ml of heat-inactivated P. aeruginosa for 16 h induced the RNase 7 gene expression 9-fold, whereas S. aureus, E. coli, and Streplococcus pyogenes gave rise to 3-fold, 2-fold and 2.1-fold increases, respectively (Fig. 4C).

DISCUSSION

With the recent discovery of two inducible epithelial antimicrobial peptides, human β-defensin-2 and -3, from lesional scales of psoriatic patients’ inflamed skin, we hypothesized that healthy skin might also constitutively produce antimicrobial peptides and proteins that may participate in a cutaneous defense system. To address this question specifically, we analyzed extracts obtained from a healthy person’s stratum corneum for the presence of antimicrobial factors.

As a result, we report here the isolation of a novel antimicrobial protein with a broad spectrum of high antimicrobial activity. Amino acid sequence analysis and cloning of the corresponding cDNA revealed that this novel protein has a high similarity to the members of the RNase A superfamily. We termed this novel protein RNase 7, because it was the seventh discovered member of the human RNase A superfamily. Nanoelectrospray mass spectrometry revealed an exact molecular mass of 14,546 Da for RNase 7, which is 8 Da less than the theoretical mass calculated from the deduced amino acid sequence (14,553.9 kDa), suggesting that the 8 cysteine residues of RNase 7 are connected through 4 disulfide bridges.

After we had submitted the cDNA sequence for RNase 7 (GenBank$^\text{TM}$/EBI accession numbers AJ131212 and AJ306608), a human genome data bank search revealed that RNase 7 is located on a bacterial artificial chromosome clone derived from chromosome 14 (GenBank$^\text{TM}$/EBI accession number NT_019583), a region where all known RNase A superfamily members are located. Very recently the eighth member of the RNase A superfamily, RNase 8, has been discovered by screening the human genome sequence (25). RNase 7 and RNase 8 share an amino acid sequence similarity of 78% and a genomic distance of only 15,000 bp, suggesting that they may have evolved from a common ancestor gene by a duplication event. However, although these proteins show a very high similarity in their gene and protein sequence, their physiolog-
RNase 7 is the second member of the human RNase A superfamily that is known to contain antibacterial activity. It has been demonstrated that the eosinophil-derived RNase ECP (eosinophil cationic protein/RNase 3) exhibits antimicrobial activity against S. aureus and E. coli with activity (LD_{90} = 0.5–1 μM) (26) similar to that observed for RNase 7. Interestingly, RNase 7 is a highly cationic protein (theoretical pI = 10.1). In the case of ECP it has been discussed that its high positive charge (pI > 11) may be relevant for its antimicrobial activity. It remains to be determined whether the cationic properties of RNase 7 are of importance for its antimicrobial activity.

It might be of particular importance that RNase 7 is highly active in killing vancomycin-resistant E. faecium (LD_{90} < 0.03 μM). RNase 7 is on a molar basis more active against vancomycin-resistant E. faecium than human β-defensin-3 (LD_{90} = 0.5 μM) (13), indicating that RNase 7 may kill these bacteria in a different manner. The very potent antimicrobial activity of RNase 7 against multiresistant bacteria supports the idea that RNase 7 might be a useful agent to treat infections caused by antibiotic resistant bacteria.

Detection of RNase 7 gene and protein expression in primary keratinocytes together with its high abundance in stratum corneum as well as its broad spectrum of high antimicrobial activity strengthens the hypothesis that RNase 7 may participate in cutaneous innate immunity and may help to keep human skin healthy.

This is the first report demonstrating that a member of the RNase A superfamily is expressed in human skin. The finding that RNase 7 is enzymatically active links it with the well known phenomenon that human skin contains ribonuclease activities, which make it necessary to take special precautions when performing experiments with RNA (i.e. by wearing gloves). Interestingly, the molecular structures of these ribonuclease activities have not yet been reported. Our data support the hypothesis that RNase 7 may be at least in part responsible for the high ribonuclease activities associated with human skin.

We could demonstrate that in addition to human skin RNase 7 mRNA is expressed in primary nasal, tracheal, and bronchial epithelial cells, implicating a potential role of RNase 7 also for the innate immunity of the human respiratory tract. Furthermore RNase 7 mRNA is expressed in various epithelial tissues including those of the gastrointestinal tract and genitourinary tract.

The hypothesis that RNase 7 may play an important role in a cutaneous antimicrobial defense system is strengthened by the observation that contact of keratinocytes with bacteria induced RNase 7 gene expression, a finding that is known for other epithelial antimicrobial proteins like the human β-defensins, hBD-2 (12), hBD-3 (13), and hBD-4 (27). RNase 7 is the first member of the human RNase A superfamily that is known to be induced upon microbial stimulation.

The finding that RNase 7 exhibited both antimicrobial and ribonuclease activity gave rise to the speculation that the enzymatic activity is required for antimicrobial activity. For ECP it has been shown that its ribonuclease activity is not essential for antibacterial activity (28). Further investigations will show whether ribonuclease-inactive RNase 7 retains its antimicrobial activity.

It has been reported that the ribonuclease activity of the antiviral eosinophil-derived RNase 2/EDN and RNase 3/ECP is essential for their antiviral activity against respiratory syncytial virus (24, 29). Therefore one could speculate that the ribonuclease activity of RNase 7 may also be necessary for activity against viruses. However, in initial experiments we did not observe antiviral activity of RNase 7 against herpes simplex

**Fig. 4. Tissue distribution and regulation of RNase 7 mRNA expression.** A, RNA from various tissues was reverse-transcribed, and RNase 7 gene expression was analyzed by real-time PCR. Bars represent the relative RNase 7 transcript levels normalized to GAPDH transcript levels. B, cultured human primary keratinocytes were stimulated with the indicated cytokines (10 ng/ml) for 16 h, and RNase 7 mRNA expression was analyzed by real-time PCR. Bars represent the relative RNase 7 transcript levels normalized to GAPDH transcript levels. Data present means ± S.E. of triplicate samples. The transcript levels between unstimulated keratinocytes and the cytokine-stimulated keratinocytes were significantly different (p < 0.05, Student’s t test). C, cultured human primary keratinocytes were stimulated with the indicated bacteria (10^7/ml, heat-inactivated) for 16 h, and gene expression was analyzed by real-time PCR. Bars represent the relative RNase 7 transcript levels normalized to GAPDH transcript levels. Data present means ± S.E. of triplicate samples. The transcript levels between unstimulated keratinocytes and the bacteria-stimulated keratinocytes were significantly different (p < 0.05, Student’s t test). TNF, transforming growth factor; IFN, interferon; IL, interleukin.
virus-1.² Further experiments need to be performed with other viruses to elucidate whether RNase 7 is important as an innate antiviral effector molecule.

The role and physiological function of members of the human RNase A superfamily is not fully understood. The antiviral activity of EDN and ECP and the antibacterial and helminthotoxic activity of ECP suggests that these eosinophil-derived RNases may contribute to an eosinophil-mediated antimicrobial defense system (30). Furthermore, it has been shown that RNase 5 exhibits angiogenic activity (31). Therefore, it remains to be determined whether RNase 7 exhibits other physiological functions than to be a major epithelial antimicrobial component.

In conclusion, the isolation of a novel epithelial-derived antimicrobial RNase identifies RNases as a novel class of endogenous epithelial antimicrobial proteins that may play an important role in the innate immunity of human epithelia and offer an immediate host response against infectious agents. It is interesting to speculate that in patients suffering from recurrent epithelial infections the production of epithelial-derived antimicrobial proteins like RNase 7 might be disturbed.

Finally, the discovery of human epithelial antimicrobial proteins like RNase 7 may further inspire the development of new strategies for the treatment of infectious diseases in which conventional antibiotics fail because of the emergence of resistant bacteria.

Acknowledgments—We thank J. Quitzau, M. Brandt, and C. Gerbrecht-Glissmann for excellent technical assistance. We thank Dr. J. Bartels for assistance with the amino acid sequence analysis. We also thank Dr. S. Schubert and S. Voss (Dept. of Medical Microbiology, University of Kiel) for help with antimicrobial assays.

REFERENCES


² J. Harder, unpublished observation.
RNase 7, a Novel Innate Immune Defense Antimicrobial Protein of Healthy Human Skin

Jürgen Harder and Jens-Michael Schröder

J. Biol. Chem. 2002, 277:46779-46784. doi: 10.1074/jbc.M207587200 originally published online September 18, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207587200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 31 references, 11 of which can be accessed free at http://www.jbc.org/content/277/48/46779.full.html#ref-list-1