

ARTICLES

High Mobility Group Box Chromosomal Protein 1 (HMGB1) Is an Antibacterial Factor Produced by the Human Adenoid

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ABSTRACT

Antibacterial factors were purified from human adenoid glands by tissue extraction and consecutive steps of reversed-phase chromatography and assayed for bactericidal activity against the airway pathogen *Moraxella catarrhalis* and also *Escherichia coli* and *Bacillus megaterium*. One of the most active components isolated from adenoids was identified by N-terminal sequence analysis and mass spectrometry as high mobility group box chromosomal protein 1 (HMGB1). This novel finding was further substantiated by Western blot analysis, demonstrating a protein of expected size reactive with HMGB1 antiserum. Local synthesis was confirmed by reverse-transcriptase PCR and *in situ* hybridization. Adenoid-derived HMGB1 and recombinant HMGB1 revealed comparable anti-

bacterial activity at high rate. More than 95% of bacteria were eradicated within 5 min by HMGB1 in the cultures. Secretion from the adenoid gland surface was also demonstrated to contain antibacterial activity, mainly mediated by α -defensins, but not by HMGB1. We conclude that HMGB1, produced and stored intracellularly in the adenoid gland, contributes to the local antibacterial barrier defense system in the upper respiratory tract. (*Pediatr Res* 52: 148–154, 2002)

Abbreviations

HMGB1, high mobility group box chromosomal protein 1
rHMGB1, recombinant HMGB1

Innate immunity refers to the part of the eukaryotic antimicrobial defense machinery that acts and kills invaders within seconds or minutes. The main effector molecules in this system are gene-derived antimicrobial peptides (peptide antibiotics),

and this applies both to plants and animals, including invertebrates and vertebrates (1, 2). More than 500 antimicrobial peptides have been described to date, of which only 30 have been identified in humans (3). Of these, some are small peptides such as defensins and some are well-characterized larger proteins such as lysozyme, phospholipase A₂, and bactericidal permeability increasing protein, contained in neutrophil granules. Another group consists of cleaved fragments of larger proteins such as lactoferrin and cathepsin G.

It was recently reported that nasal secretion contains antimicrobial activity (4) and that the oral mucosa and salivary glands produce β -defensins (5). The adenoid or pharyngeal tonsil is a part of the mucosa-associated lymphatic tissue responsible for regional immune functions in the upper respiratory tract. The adenoid has been investigated for production

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of immunoglobulins and cytokines (6) but production of antimicrobial peptides has not been studied.

HMGB1, previously known as HMGI, is a 215-amino acid residue protein that was first isolated as a nuclear factor with high electrophoretic mobility (7). The protein sequence is highly conserved, which means that, apart from two residues in the C-terminal part, the rat/mouse and human HMGB1 sequences are identical. Several studies show that HMGB1 binds with high affinity to specific DNA structures such as bent or kinked DNA (8–12) and it can also interact with Rel-containing transcription factors, e.g. nuclear factor- κ B (13). Rat/mouse HMGB1 is identical to amphoterin, a cell surface-expressed protein that has been demonstrated to enhance neurite outgrowth (14, 15) and to promote tumor growth through the receptor for advanced glycation end products (16).

HMGB1 has been shown to possess additional important extracellular proinflammatory functions. HMGB1 was discovered to act as a late mediator of endotoxin lethality (17), and increased serum levels of HMGB1 were evident in patients with sepsis (17) and hemorrhagic shock (18). In a recent study, HMGB1 was reported to cause acute lung inflammation (19). HMGB1 has also been demonstrated to act as a potent stimulus of cytokine release by human monocytes (20) and to induce chemotaxis and cytoskeleton reorganization in smooth muscle cells (21).

We here report the novel finding that the human adenoid gland can produce HMGB1, which also acts as an antibacterial factor.

METHODS

Adenoids and extraction procedure. Adenoids were surgically removed from 10 children below 6 y of age that suffered from airway obstruction. None of the patients had a history of frequent infections such as otitis, tonsillitis, or other airway infections. The frozen adenoids were homogenized and extracted overnight with 60% aqueous acetonitrile containing 1% trifluoroacetic acid (22). The extracts were centrifuged at $13,000 \times g$ for 15 min. Supernatants were pooled, lyophilized, and dissolved in water. These aliquots were tested in antibacterial assays and, if active, purified by reversed-phase HPLC.

Before surgical removal of the adenoid, secretion was collected from the surface of the gland. The secretion was diluted ($10 \times$ volume), tested for antibacterial activity, and extracted as described for the glands. All experiments were approved by the local ethical committee at Huddinge University Hospital (registration no. 155/00). Parental consent was obtained in all cases.

Reversed-phase HPLC purification. Reversed-phase chromatography of the supernatant material was performed by gradient elution with acetonitrile using a Vydac C₁₈ column (218TP54, 0.46×25 cm, Grace Vydac, Hesperia, CA, U.S.A.) with a flow rate of 0.8 mL/min. The HPLC system (Waters, Milford, MA, U.S.A.) used detection at 214 nm. Fractions were collected and lyophilized before further analysis. Solvent A was water with 0.17% trifluoroacetic acid. Solvent B was acetonitrile with 0.15% trifluoroacetic acid. All runs were started at 5% solvent B isocratic for 10 min. In the first two

purification steps, the following gradient with linear segments was used: 5–30% B during 15 min, 30–60% B during 45 min, and 60–90% B during 5 min. For the third purification step and for analytical HPLC, the gradient was 5–32% B during 10 min, 32–35% B during 45 min, and 35–90% B during 2 min.

Bacterial strains and assays for antibacterial activity. The bacterial strains used were *Bacillus megaterium* (Bm11), *Escherichia coli* (D21), and *Moraxella catarrhalis* (Mc) ATCC # 25238. Antibacterial activity was recorded using both an inhibition zone assay in thin agar plates (23) and as killing rates in liquid Luria-Bertani broth medium (for *B. megaterium*) (24). The plates (1-mm thickness) contained Luria-Bertani broth medium, 1% agarose, and about 10^4 – 10^5 log phase bacteria. Small wells (diameter 3 mm) were punched in the assay plates and loaded with samples of 3 μ L. After overnight incubation at 30°C, the diameters of the growth inhibition zones were measured to estimate the ability of the tested factors to kill bacteria. Lethal concentrations (LC in micromolar \pm SE), representing the lowest concentration of a substance that will inhibit bacterial growth, were calculated (23). As a method to compare diameters of inhibition zones, we also used cecropin A units. Fixed amounts of cecropin A were applied to assay plates and the inhibition zone diameters were measured. The relation between the zone diameter and the $^{10}\log$ of amount cecropin A applied was linear. One nanogram of applied cecropin A corresponds to one cecropin A unit. This relation curve was used to compare antibacterial activity between our isolated HPLC fractions. For killing rate experiments, bacteria were diluted to a concentration of 1000 cells per 5 μ L and were incubated at 37°C in the presence of HMGB1 purified from adenoid tissue or recombinant rat HMGB1. Aliquots of 10 μ L were removed at defined time points, diluted to 100 μ L, and immediately spread on Luria-Bertani plates. The plates were incubated at 37°C overnight and bacterial colonies were counted.

Structural analysis of HPLC-purified peptides and proteins. Lyophilized chromatographic fractions were dissolved in water. Purity was checked by analytical reversed-phase HPLC. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was conducted using a Finnigan MAT Lasermat 2000 instrument. Edman degradation was performed with a Procise cLC or Procise HT instrument (Applied Biosystems, Foster City, CA, U.S.A.). Electrospray mass spectra were recorded using a Q-TOF tandem mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray ion source.

Recombinant HMGB1 protein. rHMGB1 was purified from *E. coli* BL21(DE3) pLysS (Novagen, Madison, WI, U.S.A.) transfected with a pCAL-n vector (Stratagene, La Jolla, CA, U.S.A.) containing the rat HMGB1 gene (a kind gift from Dr. K.J. Tracey, North Shore University Hospital, New York, NY, U.S.A.). For exact data on cloning conditions see Ref. 17. Protein expression was induced with isopropyl-D-thiogalactopyranoside, and rHMGB1 was purified using a calmodulin column (Amersham Pharmacia Biotech, Uppsala, Sweden). Purified rHMGB1 was subsequently passed over a polymyxin B column (Pierce Chemical, Rockford, IL, U.S.A.) to reduce the lipopolysaccharide content. The purity of rHMGB1 was verified by SDS-PAGE and Western blot analysis. Protein

concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturers' protocol.

Western blot analysis and silver staining. HPLC-purified HMGB1 from human adenoids and rHMGB1 were electrophoresed in Tris-Tricine 10–20% gels (Novex, San Diego, CA, U.S.A.) and transferred to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were blocked with 1% BSA in Tris-buffered saline containing 0.1% Tween before incubation with primary rabbit anti-rat HMGB1 antibodies. Peptide antibodies (affinity purified) were generated by Innovagen AB, Lund, Sweden. The peptide used for immunizing rabbits corresponds to amino acid residues 165–183 of the HMGB1 molecule. Protein-antibody complexes were visualized using a horseradish peroxidase-conjugated donkey-anti-rabbit antibody (1:5000) (Amersham Pharmacia Biotech) and developed using the ECL-system (Amersham Pharmacia Biotech), according to the manufacturer's recommendations. Silver-staining of the gel was performed using Electran Insta-view Silver Staining Kit (BDH Laboratory Supplies, Poole, Dorset, U.K.) according to the manufacturer's protocol.

RNA extraction and reverse-transcriptase PCR analysis. Total RNA was extracted from snap-frozen adenoid tissue using an Ultraspec II Kit (Biotecx Laboratories, Houston, TX, U.S.A.) according to the manufacturer's protocol. Total RNA prepared from human monocytes isolated from leukocyte concentrates by density gradient centrifugation were used as a positive control. Primer pairs specific for HMGB1 (forward 5' GGAGGAGCATAAGAAGAAGC, reverse 5' CTGCTTGCATCTGCAGCAG, annealing temp 52°C) and the housekeeping gene β -actin (forward 5' CACACTGTGCCATCTACGA, reverse 5' GTTTCATGGATGCCACAGGA, annealing temp 58°C), the latter used as internal standard, were designed from published cDNA sequences. Superscript cDNA kit (Invitrogen, Carlsbad, CA, U.S.A.) and RNase Inhibitor (Roche Diagnostics Corp., Basel, Switzerland) were used to synthesize cDNA. The end products were used directly for PCR in a PerkinElmer GeneAmp PCR System 2400 (PerkinElmer, Wellesley, MA, U.S.A.) in the presence of 25 pmol primers, 10 pmol deoxyribonucleoside triphosphate, 75 pmol High Fidelity PCR buffer and 1.75 U Expand High Fidelity DNA polymerase (Roche Molecular Biochemicals, Summerville, NJ, U.S.A.). The conditions for amplification were 5 min at 96°C, followed by 35 cycles of denaturation for 30 s at 96°C, annealing for 30 s at 52°C, and extension for 60 s at 72°C. Reaction products were analyzed by gel electrophoresis using 2% agarose containing ethidium bromide (15 μ g to 100 mL gel).

In situ hybridization histochemistry. A 50-bp oligonucleotide probe for HMGB1 (5'- TCTTCTTCCTCCTTCCTCATCCTCTTCATCCTCCTCGTCTTCCTC-3') and a random probe having no similarities to known sequences (GenBank, National Institutes of Health, Bethesda, MD, U.S.A.) were synthesized (DNA Technology, Aarhus, Denmark). The probes were radiolabeled with [³⁵S] deoxyadenosine 5'-[α -thio]triphosphate at the 3' end using terminal deoxynucleotidyl transferase (Amersham Pharmacia Biotech). *In situ* hybridization histochemistry was performed according to Dagerlind et

al. (25). Briefly, sections were hybridized for 18 h at 42°C in humidified boxes and rinsed 5 \times 15 min in 1 \times SSC at 60°C. Tissue sections were dehydrated, air-dried, dipped in photographic emulsion (NTB-2, Eastman Kodak, Rochester, NY, U.S.A.), exposed for 3–4 wk at –20°C, developed, counterstained with carmalum and mounted (Entellan, Merck, Darmstadt, Germany). Sections were evaluated using bright- and dark-field microscopy (Nikon Eclipse E800, Nikon, Tokyo, Japan).

RESULTS

Isolation and identification of HMGB1. The lyophilized extracts from adenoid glands were dissolved in water and separated by reversed-phase HPLC. Fractions were collected every minute and lyophilized. After this, fractions were dissolved in water (10–20 μ L) and analyzed by inhibition zone assay using primarily *B. megaterium* as the test organism.

The most prominent antibacterial activity was detected in two complex peaks eluted after about 40 min elution time (Fig. 1A). Rechromatography of the main peak at 38–39 min gave three to four peaks, with the antibacterial activity detected only in the fraction corresponding to the last of these peaks (data not included). A second rechromatography of this bioactive fraction using a prolonged gradient between 32% and 35% acetonitrile gave a single symmetrical peak (Fig. 1B). The bioactive material resulting from several rounds of three chromatographic steps (Fig. 1B) were pooled and used for studies of antibacterial activity and for protein-chemical identification.

N-terminal sequence analysis of the component corresponding to the active HPLC peak revealed the 15 first amino acid residues to be GKGDPPKPRGKMSSY. This sequence is identical to the N-terminal sequence of human HMGB1, except that the initial methionine residue deduced from cDNA analysis (26) is not present. Preliminary matrix-assisted laser desorption/ionization mass analysis indicated a protein with a size of 24,670 D. Using electrospray for a refined mass analysis, a main peak of 24,630 D was detected, which corresponds well to HMGB1 lacking the C-terminal Glu residue, and a peak of 24,758 D, which corresponds to full-length HMGB1 lacking the initial methionine residue, with a theoretical mass of 24,762 D.

The identity of HMGB1 was further confirmed by gel electrophoresis and Western blot analysis. Both recombinant HMGB1 and purified adenoid material exhibited protein bands of the expected size of HMGB1 (Fig. 2, A and B). The product reacting with polyclonal HMGB1 antibodies was blotted to a polyvinylidene fluoride membrane for Edman degradation. The first 11 residues obtained were GKGDPPKPRGK. Thus, results from N-terminal sequence analysis, mass analysis, and Western blot analysis are consistent with the conclusion that the purified antibacterial adenoid-derived protein is HMGB1 (26).

Reverse-transcriptase(RT)-PCR analysis confirmed a strong expression of HMGB1 mRNA in the adenoid gland (Fig. 3A) and *in situ* hybridization histochemistry revealed a distinct expression of HMGB1 mRNA in the germinal centers of adenoids (Fig. 3, B and C) compared with control sections in

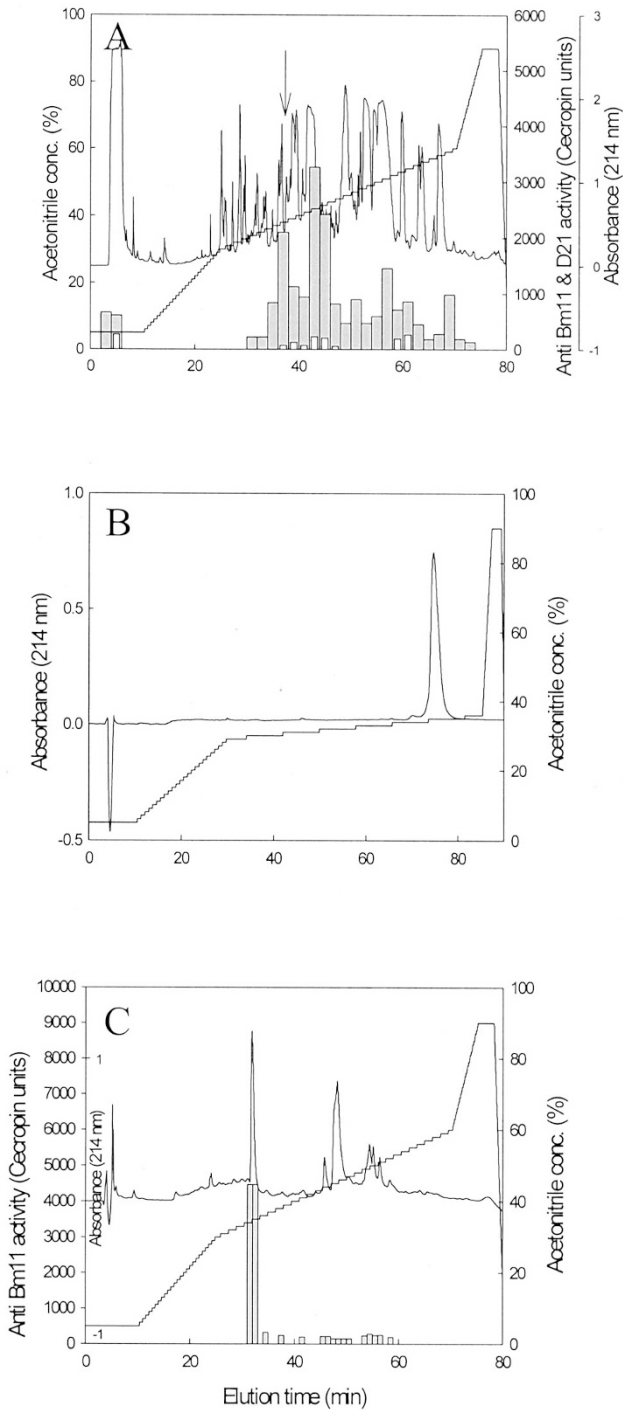


Figure 1. Reversed-phase HPLC purification of HMGB1 and α -defensins from the adenoid gland. Chromatograms illustrating (A) the first and (B) the third step of HMGB1 purification from adenoid gland by C_{18} reversed-phase HPLC. Shaded columns indicate the bactericidal activity against *B. megaterium*, open columns the killing activity against *E. coli*, expressed as cecropin A units against the respective bacteria. C illustrates a chromatogram of adenoid secretion extracted in the same way as the homogenized adenoid tissue. The main peak eluted at 31–32 min was analyzed by N-terminal sequence analysis and identified as a mixture of three separate α -defensins, HNP-1, HNP-2, and HNP-3.

which no signal was evident using a random probe (Fig. 3, D and E). These results thus indicate a local production of HMGB1 in the adenoid gland tissue.

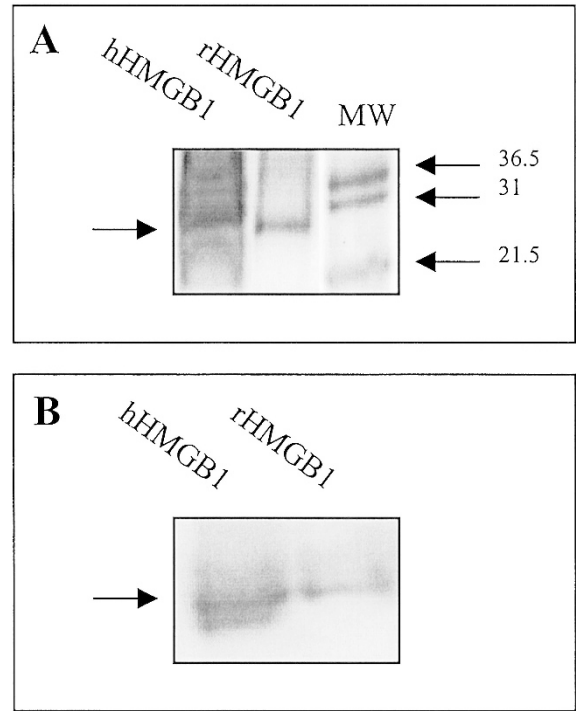


Figure 2. Protein staining (A) and Western blot analysis (B) of rHMGB1 and adenoid-derived HMGB1. (A) Silver staining shows protein bands of an apparent molecular size of slightly below 30 kD for both recombinant and adenoid-derived HMGB1. (B) In Western blot analysis, the immunoreactive bands of hHMGB1 and rHMGB1 both correspond to a molecular mass of approximately 30 kD.

Secretion from the surface of the adenoid glands was extracted and HPLC fractionated in the same manner as for the dispersed glandular tissue. Figure 1C depicts the HPLC chromatogram of secretion from the adenoid surface. The pattern differs significantly from that obtained for the intracellular extract (Fig. 1A). The first large peak at 31–32 min revealed significant antibacterial activity against *B. megaterium*, and N-terminal sequence analysis identified three different α -defensins, defensin-1 (HNP-1), defensin-2 (HNP-2) and defensin-3 (HNP-3), in the relative proportions 4:4:1. However, we could not detect any HMGB1 in the extracellular surface secretion.

Antibacterial activity. Antibacterial activities against the test organisms *B. megaterium* (Bm11) and *E. coli* (D21) and against the airway pathogen *M. catarrhalis* (Mc) are given in Table 1. Adenoid-derived HMGB1 and rHMGB1 were equally efficient in inhibiting bacterial growth, with lethal concentrations below 1 μ M for *B. megaterium*. The antibacterial effect of HMGB1 was in the same concentration range as for the well-characterized antibacterial peptide cecropin A regarding *B. megaterium* and *M. catarrhalis*, but slightly less efficient versus *E. coli* (Table 1). Furthermore, rHMGB1 was capable of stimulating cytokine synthesis in cultured human blood monocytes at the same extracellular concentration (20) that was bactericidal (data not shown).

The rate of the bacterial killing corresponded in a dose-dependent manner to the amount of added adenoid-derived HMGB1. Killing rates of HMGB1 purified from human ade-

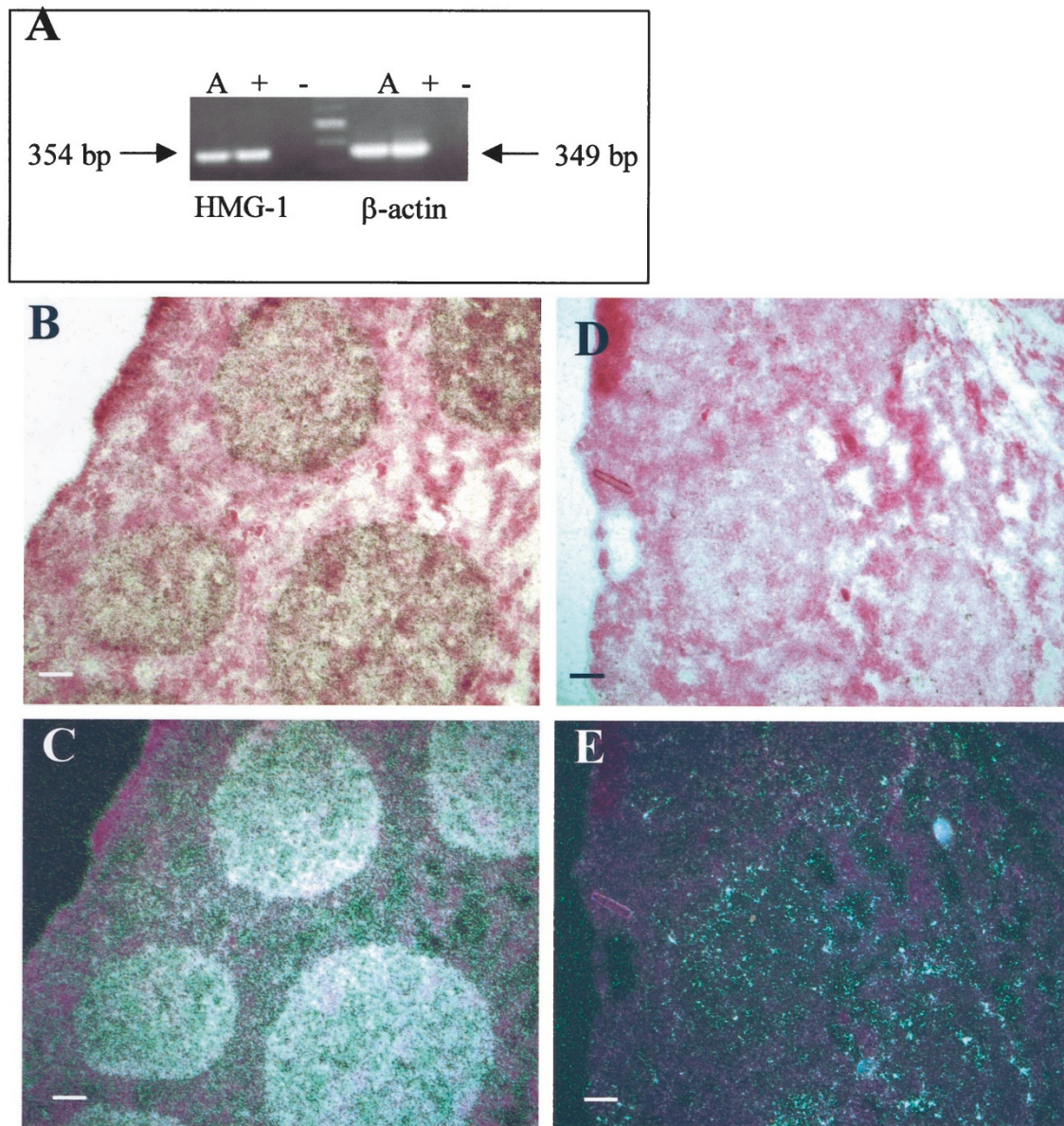


Figure 3. RT-PCR (A) and *in situ* hybridization histochemistry (B–E) analysis of HMGB1 mRNA expression in human adenoid. HMGB1 mRNA is strongly expressed in the adenoid (A). Human unstimulated monocytes were used as positive control (+) and RT-PCR reaction without addition of mRNA were used as negative control (–). Product size 354 bp. β -actin were used as reference gene (product size 349 bp). Bright- (B and D) and dark-field (C and E) photomicrographs of *in situ* hybridization of HMGB1 mRNA and random probe (D and E). A distinct expression of HMGB1 mRNA is localized to the germinal centers of the human adenoid gland (B and C). Use of a random probe show no localized expression (D and E). Scale bars: 100 μ m.

Table 1. Antibacterial activity of HMGB1

Test substance	Strain		
	<i>B. megaterium</i> (Bm11)	<i>M. catarrhalis</i> (ATCC #25238)	<i>E. coli</i> (D21)
aHMGB1	0.1 \pm 0.0 (n = 4)	1.2 \pm 0.2 (n = 3)	2 (n = 1)
rHMGB1	0.5 \pm 0.0 (n = 4)	0.3 \pm 0.0 (n = 2)	3 (n = 1)
Cecropin A	0.6 \pm 0.1 (n = 3)	0.5 \pm 0.1 (n = 3)	0.5 \pm 0.1 (n = 3)

Antibacterial activities are given as lethal concentrations (LC values in μ M). 0.4 μ M recombinant HMGB1 induced tumor necrosis factor- α production in 25% of cultured human monocytes after 8 h (data not shown).

noid tissue and recombinant HMGB1 were identical (Fig. 4). Concentrations of 3.5 μ M for hHMGB1 and rHMGB1 applied to *B. megaterium* killed approximately 80% of the bacteria

within 2 min and >95% after 5 min (Fig. 4). If no substance was added, the bacterial density was clearly increased due to normal growth.

DISCUSSION

The present work demonstrates that the human adenoid tissue produces several antibacterial factors. Extracellular components comprised the well-characterized antibacterial peptides α -defensins 1–3, which were detected in the surface secretion from the adenoid gland. From previous studies, these three defensins are known to appear as a single peak on reversed-phase HPLC (27). The intracellular antibacterial factor detected turned out to be HMGB1. This is, to our knowledge, the first report on an antibacterial activity of HMGB1,

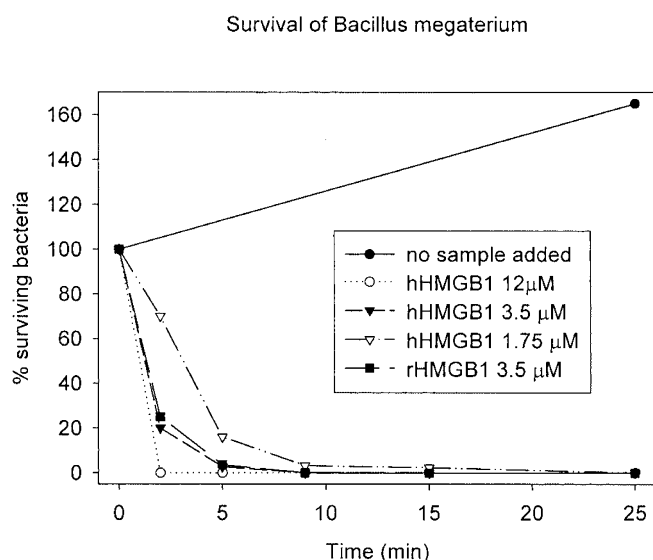


Figure 4. Kinetics of bacterial killing by HMGB1. Rate of killing of *B. megaterium* by human HMGB1 purified from adenoid tissue (*hHMGB1*) and rHMGB1. A dose-dependent killing rate was scored. *hHMGB1* and rHMGB1 displayed a dose-equivalent killing rate. More than 95% of bacteria were killed within 5 min. When no sample was added, bacterial density was clearly increased.

which now adds to the recently demonstrated proinflammatory role of HMGB1 (17, 20). However, other research groups have reported difficulties in expressing rHMGB1 in *E. coli*, indicating that HMGB1 might be toxic to bacteria (28) and that the choice of vector when expressing rHMGB1 is crucial for the yield of recombinant protein (10, 17, 28).

The isolation of human HMGB1 from patients without acute infectious symptoms, such as fever, otitis, or airway infections, was unexpected, as recent reports indicate that HMGB1 is a late mediator of the proinflammatory response (17, 29). The demonstration of HMGB1 mRNA in the germinal centers of the adenoid confirms that the protein is locally synthesized in the gland. HMGB1 lacks a signal peptide such as the cytokines in the IL-1 and fibroblast growth factor families. This may indicate that HMGB1 protein is normally not designed to leave the cells. Many airway pathogens are encapsulated and are phagocytosed, and thus intracellular antibacterial peptides might be of great importance in eradication of these pathogens. However, it has recently also been clearly demonstrated that extracellular HMGB1 release occurs at inflammatory sites (17–19). It is thus plausible that HMGB1 may enact dual intracellular and extracellular bactericidal activities. The resected adenoid tissue in this study arose from children without clinical signs of ongoing infectious disease and therefore does not allow conclusions about a putative extracellular bactericidal role of HMGB1.

The kinetics of bacterial killing by HMGB1 was rapid, even compared with other well-known antibacterial peptides (4, 30, 31), and HMGB1 eliminated bacteria within a few minutes in a dose-dependent fashion.

The discovery of HMGB1 in the adenoid prompted us to search for HMGB1 in surface secretion from the adenoid gland. No secreted HMGB1 was detectable from noninfected

adenoid tissue. However, high HMGB1 concentrations could be expected after necrotic cell damage caused by an infection with strong inflammatory response. The capacity of HMGB1 to bind DNA is interesting inasmuch as it has been determined that other proteins binding DNA or RNA, such as the ribosomal protein L1 and the histone protein H2A, can be processed to mediate antibacterial activity (31–33). The mechanisms by which HMGB1 exerts its bactericidal function thus remain to be defined.

We conclude that HMGB1 is a potent antibacterial protein produced and harbored in the adenoid. By delaying or inhibiting bacterial penetration, it may contribute to the barrier defense system in the upper respiratory tract mucosa.

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