Expression of human α-defensin 5 (HD5) mRNA in nasal and bronchial epithelial cells

M Frye, J Bargon, N Dauletbaev, A Weber, T O F Wagner, R Gropp

Abstract

***Background/Aims*—Human defensins are antibiotic peptides expressed in myeloid and epithelial cells. Human α-defensin 5 (HD5) has been detected in Paneth cell granules in the crypts of Lieberkühn and has recently been identified in the female reproductive tract. The aim of this study was to investigate the presence of HD5 mRNA in nasal and bronchial epithelial cells.**

***Methods/Results*—Semiquantitative reverse transcription polymerase chain reaction (RT–PCR) analysis showed that HD5 mRNA was expressed infrequently and to varying degrees in bronchial and nasal epithelial cells. In situ hybridisation resulted in a positive signal in the epithelial layer of nasal polyps. HD5 mRNA was locally restricted to a specific area of epithelial cells and also occurred in submucosal glands.**

***Conclusions*—HD5 mRNA expression in nasal and bronchial epithelial cells is rare and seemed to be locally induced. The results indicate that HD5 might play a role in innate defence in nasal and bronchial epithelia.**

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Defensins are small cationic peptides with high antimicrobial activity against a broad spectrum of pathogenic agents, including bacteria, fungi, and viruses.[1] They are thought to act by selectively disrupting target membranes.[2] Defensins are particularly abundant in the microbicidal granules of polymorphonuclear leucocytes.[1] Since their discovery in epithelial cells they also have to be considered an important part of the mucosal barrier against invading microbes.

To date, four different human defensins have been identified in various epithelial organs. Two belong to the subfamily of β-defensins (hBD1 and hBD2) and have a complex expression pattern. hBD1 is found predominantly in the kidney, but has also been detected in all other epithelial cells analysed thus far.[3 4] hBD2 is found abundantly in skin, lung, uterus, and trachea. In contrast to the widely distributed expression pattern of β-defensins, the expression of epithelial α-defensins seem to be much more localised. α-Defensins are abundant constituents of mouse and human Paneth cells.[6–8] Therefore, epithelial α-defensins were often described as markers for Paneth cells.

However, recently α-defensin 5 (HD5) has also been found in the female reproductive tract, and mouse homologues (cryptdins) have been identified in the skin.[9 10] In light of these studies, the expression pattern of epithelial α-defensins becomes more complex.

In our study, we detected HD5 mRNA in nasal and bronchial epithelia by semiquantitative reverse transcription polymerase chain reaction (RT–PCR) analysis as well as DNA sequencing, and identified the epithelial cells expressing HD5 mRNA in tissue sections of nasal polyps by in situ hybridisation.

Materials and methods

TISSUE PREPARATION

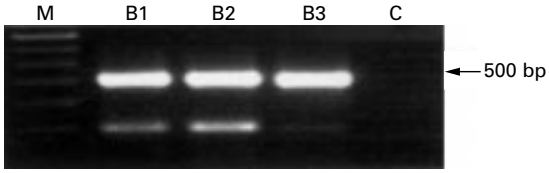
Normal respiratory epithelium was obtained from second order bronchi by fiberoptic bronchoscopy with a standard cytology brush and from nasal brushings of the inferior turbinate with Curaprox LS brushes (Curaden AG, Teningen, Germany). Intact human nasal epithelia were obtained from nasal polyp surgical specimens. Control specimens of colon were obtained at routine endoscopy (Olympus 1T130 gastroscope, Olympus colonoscope; Olympus, Hamburg, Germany). Our study protocol was approved by the local ethics committee and all subjects gave informed consent. All mucosal specimens were immediately transferred to ice, washed in phosphate buffered saline (PBS), and dissected in RNAzol B™ (WakChemie, Bad Homburg, Germany), or fixed in 4% paraformaldehyde, embedded in paraffin wax, sectioned to 5 μm thickness, and mounted on to glass slides.

RNA PREPARATION AND PCR ANALYSIS

Total RNA was isolated from the nasal and bronchial brushings by means of RNAzol B and 1–5 μg of total RNA was reverse transcribed into cDNA using the Superscript™II system (Gibco BRL, Eggenstein, Germany), according to the manufacturer's instructions. HD5 expression was measured by semiquantitative PCR analysis, using mRNA encoding glyceraldehyde 3 phosphate dehydrogenase (GAPDH) as an internal standard.

All PCR reactions were performed as follows: 1 μl of cDNA was used for amplification with 0.5 U Taq DNA polymerase (Qiagen, Hilden, Germany), 1× PCR buffer, 0.1 mM dNTPs (each), and 1 pmol of each specific upstream and downstream primer, HD5: 5'-ATGAGGACCATCGCCATCC and 3'-TCAGCGACAGCAGAGTCTG; GAPDH: 5'-ATCTTCCAGGAGCGAGATCC and 3'-ACCACTGACACGTTGGCAGT (MWG Biotech, Ebersberg, Germany). To exclude false positive PCR signals for HD5 as a result of DNA contamination, the primers were derived from two different exons at the 5' and 3' end of the gene. PCR conditions were as

A



B

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     101                                                        150
N1   AGTCTGGGGA  AGACAACCAG  GACCTTGCTA  TCTCCTTTGC  AGGAAATGGA
B1   AGTCTGGGGA  AGACAACCAG  GACCTTGCTA  TCTCCTTTGC  AGGAAATGGA
S1   AGTCTGGGGA  AGACAACCAG  GACCTTGCTA  TCTCCTTTGC  AGGAAATGGA
N11  AGTCTGGGGA  AGACAACCAG  GACCTTGCTA  TCTCCTCTGC  AGGAAATGGA
B2   AGTCTGGGGA  AGACAACCAG  GACCTTGCTA  TCTCCTCTGC  AGGAAATGGA
B10  AGTCTGGGGA  AGACAACCAG  GACCTTGCTA  TCTCCTTTGC  AGGAAATGGA

C    AGTCTGGGGA  AGACAACCAG  GACCTTGCTA  TCTCCTYTGC  AGGAAATGGA

     151                                                        200
N1   CTCTCTGCTC  TTAGAACCTC  AGGATCCCAG  GCAAGAGCCA  CCTGCTATTG
B1   CTCTCTGCTC  TTAGAACCTC  AGGATCCCAG  GCAAGAGCCA  CCTGCTATTG
S1   CTCTCTGCTC  TTAGAACCTC  AGGTTCTCAG  GCAAGAGCCA  CCTGCTATTG
N11  CTCTCTGCTC  TTAGAACCTC  AGGTTCTCAG  GCAAGAGCCA  CCTGCTATTG
B2   CTCTCTGCTC  TTAGAACCTC  AGGTTCTCAG  GCAAGAGCCA  CCTGCTATTG
B10  CTCTCTGCTC  TTAGAACCTC  AGGTTCTCAG  GCAAGAGCCA  CCTGCTATTG

C    CTCTCTGCTC  TTAGAACCTC  AGGYTCTYAG  GCAAGAGCCA  CCTGCTATTG

     201                                                        250
N1   CCGAACCGGC  CGTTGTGCTA  CCCGTGAGTC  CCTCTCCGGG  GTGTGTGAAA
B1   CCGAACCGGC  CGTTGTGCTA  CCCGTGAGTC  CCTCTCCGGG  GTGTGTGAAA
S1   CCGAACCGGC  CGTTGTGCTA  CCCGTGAGTC  CCTCTCCGGG  GTGTGTGAAA
N11  CCGAACTGGC  CGTTGTGCTA  CCCGTGAGTC  CCTCTCCGGG  GTGTGTGAAA
B2   CCGAACTGGC  CGTTGTGCTA  CCCGTGAGTC  CCTCTCCGGG  GTGTGTGAAA
B10  CCGAACTGGC  CGTTGTGCTA  CCCGTGAGTC  CCTCTCCGGG  GTGTGTGAAA

C    CCGAACYGGC  CGTTGTGCTA  CCCGTGAGTC  CCTCTCCGGG  GTGTGTGAAA
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Figure 1 (A) Semiquantitative reverse transcription polymerase chain reaction (RT–PCR) analysis with specific primers for human α-defensin 5 (HD5) (286 bp) and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) as internal standard (500 bp) of bronchial brushings (B1–B3). A PCR reaction in the absence of cDNA was used as a negative control (lane C). Lane M contains a 100 bp ladder as marker. (B) Nucleotide alignment of HD5 cDNA sequences of nasal (N1 and N11), bronchial (B1, B2, and B10) epithelial cells as well as a sample obtained of human small intestine (SI). The nucleotide positions with inter-individual polymorphism are outlined at nucleotides 134, 174, 177, and 207. C defines the consensus sequence.

follows: denaturation for one minute at 94°C, annealing for one minute at 60°C, and extension for one minute at 72°C.

Kinetic studies revealed that under these conditions the PCR fragments were logarithmically amplified up to 32 cycles. Therefore, all cDNA targets were amplified for 30 cycles.

The most crucial aspect of gene expression analysis by PCR is the generation of false positive signals by contamination. Therefore, we performed several controls to verify our results. In all PCR reactions we routinely used negative and positive controls. Negative controls were PCR reactions omitting the cDNA. Positive controls comprised RNA samples obtained from human intestine. Furthermore, we repeated all PCR reactions independent of each other. To exclude false positive PCR signals, some HD5 fragments obtained by PCR analysis were confirmed by DNA sequencing.

IN SITU HYBRIDISATION

Full length cDNAs of HD5, hBD1, and hBD2 were gel purified and cloned into pGEM-T-easy Vector (Promega, Mannheim, Germany). The constructs were confirmed by DNA sequencing. The templates were linearised and ^{35}S labelled probes (sense and antisense) (Hartmann Analytics, Braunschweig, Germany) were prepared using the Riboprobe® Germini System II (Promega), according to the manufacturer's instructions.

The probes were purified by the nucleotide removal kit (Qiagen). Hybridisation steps were conducted according to conventional methods with some modifications. Briefly, before hybridisation, the slides were treated with 50 µg/ml proteinase K, rinsed with PBS, and acetylated with 0.1 M triethanolamine and 0.25% acetic anhydride. The slides were dehydrated in graded ethanol and air dried. The riboprobe was denatured at 80°C before adding to the hybridisation mix containing 10 mM dithiothreitol, 50% formamide, 0.3 M NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 10% dextran sulphate, l× Denhart's, and 0.5 mg/ml tRNA. The hybridisation solution was spread over the sections and allowed to hybridise at 60°C for 16–20 hours. After hybridisation, sections were washed with high stringency buffer (50% formamide, 2× saline sodium citrate, and 20 mM dithiothreitol) at 65°C for 30 minutes, rinsed with 0.5 M NaCl, 10 mM Tris, 5 mM EDTA, and treated with 20 µg/ml RNase A. After a second series of high stringency washes and dehydration steps the sections were coated with LM-1 autoradiography emulsion (Amersham, Braunschweig, Germany) and exposed at 4°C before being developed.

Results

PCR ANALYSIS

Nasal brushings of 24 volunteers and bronchial brushings of nine patients were analysed for HD5 mRNA expression. Figure 1A shows the PCR results obtained by semiquantitative RT–PCR of bronchial brushings using GAPDH as an internal standard. Whereas GAPDH (500 bp) was expressed to the same degree in different samples, HD5 mRNA (286 bp) expression varied between all samples analysed. A PCR reaction in the absence of cDNA was used as a negative control (fig 1A; lane C). In summary, four of nine bronchial and three of 24 nasal brushings analysed were positive for HD5 expression. We found no correlation between HD5 positive brushings and inflammatory markers, such as raised numbers of neutrophils in differential cell counts or raised hBD2 mRNA, which is known to be induced by proinflammatory mediators (data not shown).

HD5 NUCLEOTIDE ALIGNMENT

Six HD5 PCR fragments were confirmed by DNA sequencing. Figure 1B shows the nucleotide alignment of HD5 cDNA of nasal (N), bronchial (B), and intestinal (S1) epithelial samples. The PCR fragments obtained from different volunteers had low variability. The polymorphisms occurred in at least two samples and were clustered at nucleotide (nt) positions 137, 174, 177, and 207.

LOCALISATION OF HD5 IN NASAL POLYSS

To identify the cell types within nasal epithelia that express HD5 mRNA, ^{35}S-labelled sense and antisense RNA of full length HD5, hBD1, and hBD2 were used for in situ hybridisation

on sections of nasal polyps (fig 2A–H). In situ hybridisation with the antisense probe revealed a specific signal within the epithelial cells (fig 2A). The positive signal was restricted to specific areas and did not occur in all epithelial cells of the nasal polyp. No signal was detected in sections incubated with the HD5 sense probe (fig 2B). These results correlate well with the PCR analysis. Most of the nasal polyps analysed showed no HD5 mRNA signal (fig 2C and D). Furthermore, positive signals were found in submucosal glands, but not in sense controls (fig 2E and F). To exclude false positive or non-specific signals, we performed in situ hybridisation on tissue sections of colon (fig 2G and H). These experiments revealed a

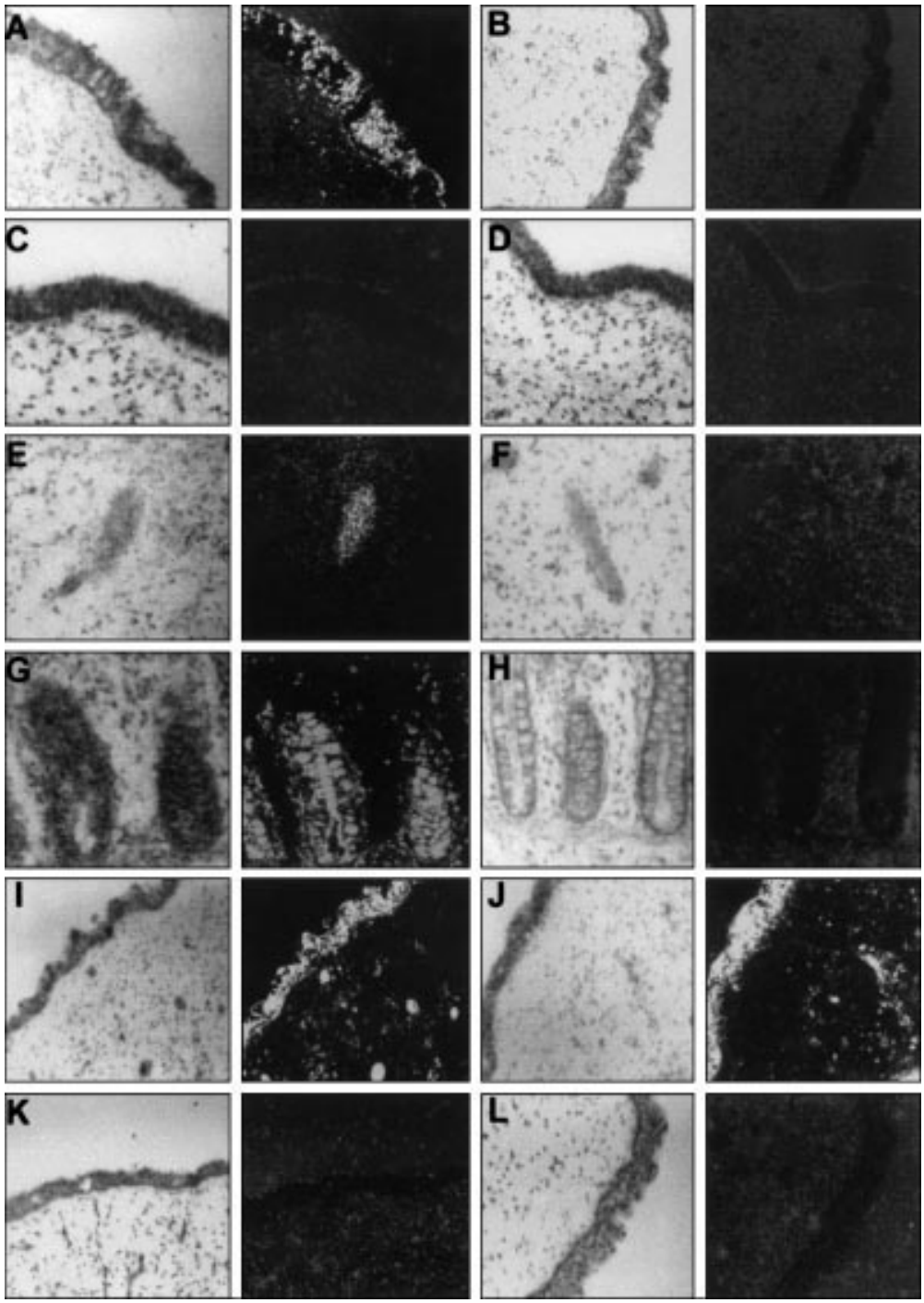

Figure 2 In situ hybridisation experiments to localise human α-defensin 5 (HD5) mRNA expression in nasal polyps. All results are shown in bright and dark field. The HD5 antisense mRNA probe revealed a specific signal in restricted areas of epithelial cells of the nasal polyp (A). Hybridisation with the sense HD5 mRNA probe as control showed no non-specific staining (B). In most nasal polyps analysed, no HD5 mRNA was detectable with antisense probes (C and D). HD5 mRNA was detected in submucosal glands with antisense (E), but not with sense riboprobes (F). Localisation of HD5 mRNA in the crypts of Lieberkühn of human colon with HD5 antisense probe, as positive control (G). No non-specific staining was detectable with the HD5 sense probe (H). In situ hybridisation experiments with hBD1 and hBD2 antisense probes (I and J) revealed a specific signal in epithelial cells and submucosal glands. No signal was detected with sense controls for hBD1 (K) or hBD2 (L).

specific signal restricted to the crypts of Lieberkühn. In addition, we performed in situ hybridisation on HD5 positive tissue with hBD1 and hBD2 probes as controls for intact epithelium (fig 2I–L). As described previously in sections of bronchial tissue, positive signals for hBD1 and hBD2 were detected in epithelial cells and submucosal glands (fig 2I and J). No signal were detected with sense controls (fig 2K and L).

To exclude the possibility that positive signals obtained by in situ hybridisation were caused by other cell types (such as incoming leucocytes), we selectively stained neutrophils and tissue mast cells. The stained neutrophilic myeloid cells and mast cells did not colocalise with the HD5 signal and gave no indication of inflammation (data not shown).

Discussion

Whereas several recent studies have shown a wide distribution expression pattern for the β-defensins, hBD1 and hBD2,[3–5] the expression of the epithelial α-defensins seemed to be locally restricted. Human as well as mouse epithelial α-defensins were described as localised exclusively to intestinal Paneth cell granules.[6–8] However, this assumption was refuted by localisation of HD5 in the female reproductive tract and the detection of mouse homologues (cryptdins) in skin.[9 10] In our study, we found HD5 mRNA in samples of nasal and bronchial epithelial cells.

Our semiquantitative RT–PCR results indicate that HD5 mRNA occurs infrequently in the airways. Although samples with positive HD5 signals exhibited inter-individual variability of HD5 expression (fig 1A), HD5 mRNA was absent in most cases. These data indicate an on/off regulation of HD5 in nasal and bronchial epithelia by factors that are yet to be determined.

The nucleotide alignment of HD5 cDNA has high sequence similarity between different individuals (fig 1B). We detected four polymorphisms, three of them representing silent mutations, probably reflecting the highly conserved amino acid sequence of HD5 in humans. All mutations were found in at least two individuals. Therefore, the nucleotide substitutions detected by sequencing can be considered as a synapomorphic marker. Interestingly, the nucleotide polymorphisms at positions 174 and 177 are localised at the splicing recognition site of exon 1 (nt 174) and exon 2 (nt 177). The substitution at nt 177 has been detected previously by sequencing HD5 fragments derived from endometrium.[9] The mutation at nt 137 affects the propeptide and results in an amino acid substitution from serine to phenylalanine. In conclusion, we detected two different isoforms of HD5 in nasal and bronchial epithelia.

To localise the HD5 mRNA in nasal epithelia cells we performed in situ hybridisation. In situ hybridisation with tissue sections of nasal polyps revealed a positive signal in cells of the epithelium (fig 2A) and submucosal glands (fig 2E). This specific expression pattern in the epithelium and submucosal glands has been described previously for β-defensin mRNA in the upper airways.[11 12] However, in most nasal polyps we were not able to detect HD5 mRNA (fig 2C and D).

In contrast to hBD1 and hBD2 localisation in the epithelium of nasal polyps (fig 2I–L), HD5 mRNA was not distributed throughout the whole nasal epithelia, but was patchy and restricted to a specific area. Therefore, the concentrations of mRNA and the peptide were very low. Most probably because of these low mRNA expression levels, HD5 was not detectable by immunocytochemistry (data not shown). Alternatively, the epithelial cells might secrete HD5 peptides, which would reduce the intracellular concentration to a level that prevents HD5 localisation.

Because of the lack of correlation of inflammation with HD5 mRNA expression in nasal and bronchial epithelia in our study, our data indicate a complex expression pattern of α-defensin HD5. Our data might reflect the ability of the HD5 gene to be induced rapidly in various epithelial organs by different regulatory factors. Thus, the absence or presence of epithelial defensins might depend predominantly on the endogenous and/or exogenous factors in these organs.

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