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Expression profile of drosomycin-like defensin in oral epithelium and oral carcinoma cell lines

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ABSTRACT

Objective: Drosomycin-like defensin (DLD) is a recently discovered antimicrobial peptide mainly active against filamentous fungi. The present study investigated the expression profile of DLD in oral epithelium and oral squamous cell carcinoma (SCC) cell lines.
Methods: Tissue sections of human oral mucosa, keratinocytes derived from oral mucosa (NOK) and eight kinds of SCC cell lines were used. In situ hybridization was performed on tissue sections of oral mucosa. Expression levels of DLD in the cells were observed by reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR assays. The cells were treated with IL-1β, IL-8 and TNF-α, and agonists for TLR2, TLR4 and β-glucan. DLD expression in cells was increased and decreased by the DLD gene and its siRNA transfection, respectively. The proliferation rates were assessed by cell counting.
Results: By means of in situ hybridization, DLD mRNA positive staining was detected in the epithelial layer of the oral mucosa. An RT-PCR assay confirmed the expression of DLD mRNA in keratinocytes derived from oral epithelium. Expression of DLD in two out of eight cell lines was significantly lower than in NOK cells. The expression levels of DLD mRNA were not significantly changed in the cells stimulated with any cytokines or agonists. The cell proliferation rate where there was decreased expression of DLD was significantly lower than in the control.
Conclusion: DLD may be partially involved in the defence against filamentous fungal infection in the oral mucosa, and may also serve other functions, such as contribution to cell growth.

1. Introduction

The oral epithelium functions as a protective barrier to resist microbial infection, acting not only as a mechanical barrier, but also through the innate immunity conferred by antimicrobial peptides in saliva, and through adaptive immunity.[1,2] Antimicrobial peptides are produced by many types of cells including blood cells, epithelial cells and mesenchymal cells.[3] Several types of antimicrobial peptides, including β-defensins,[4] cathelicidin,[5] calprotectin,[6] psoriasin,[7,8] RNase 7,[9] and CCL20/MIP-3a,[10] are produced by oral epithelial cells.[11] They have broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria, fungi and viruses.[11] The expression of these peptides is often regulated by stimulation with inflammatory cytokines and microbial infections.[12,13]

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The expression of β-defensin-2 and -3 is upregulated by certain types of cytokines and bacteria.[14,15] Some of these antimicrobial peptides may function as proto- or suppressor-oncogenes.[7,16,17] Psoriasin and β-defensin-1 may be proto- and suppressor-oncogenes, respectively.[18–22]

A new type of antimicrobial peptide was recently discovered, called drosomycin-like defensin (DLD).[23] DLD is a human homologue of Drosophila-derived drosomysin, which displays a broad spectrum of activity against filamentous fungi. A reverse transcription polymerase chain reaction (RT-PCR) assay revealed the expression of DLD mRNA in several human tissues including pancreas, testis, heart, muscle and skin. The highest expression level of DLD mRNA was found in the skin. The oral mucosa is morphologically similar to the skin, and may therefore also express DLD; however, this has not yet been demonstrated. The present study investigated expression of DLD in oral mucosa with RT-PCR and in situ hybridization using specific probes. We found that DLD was mainly localized in the stratified squamous epithelium. Since certain types of antimicrobial peptides may be involved in cancer development,[16,17,24] we observed the expression profiles of DLD in oral squamous cell carcinoma cell lines, and examined whether increased and decreased expression affected cell growth.

2. Materials and methods

2.1. Cell cultures

A spontaneously immortalized human keratinocyte cell line (HaCaT), nine kinds of oral cancer cell lines (SAS, BSC-OF, SCC9, OSC-19, HSC-2, HSC-3, HSC-4, KB) were incubated with Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, Saint Louis, MO) supplemented with 10% foetal bovine serum (FBS: Gibco, Invitrogen Corporation, Carlsbad, CA), 100 U/ml penicillin (Sigma–Aldrich) and 200 μg/ml streptomycin (Sigma–Aldrich).

Normal human oral keratinocyte cells (NOK) were isolated from healthy gingival tissue overlying the impacted third molar of an adult human. Basically, according to the method described by Krisanaprakornkit et al.[25] Briefly, explants of the healthy gingival tissue obtained from the third molar surgical extraction were cultured in DMEM containing 10% FBS and antibiotics (100 Ug/ml penicillin; 200 μg/ml streptomycin; 5 μg/ml amphotericin-B: Sigma–Aldrich) and 30 mg/ml Fungizone (Bristol-Myers Squibb, Japan) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Outgrowth developed after 2 or 3 weeks of incubation. The two cell types were separated into epithelial populations that were more and less resistant to detachment with 10% dispase (Goudousyusei, Japan). The cells that were less resistant were detached, removed from the rest of the cell population, and discarded. The attached cells were cultured. The separation procedure was repeated two or three times so as to remove any fibroblasts. NOK cells were grown in keratinocyte basal medium (KBM: Lonza Walkersville, Inc., Walkersville, MD) supplemented with 7.5 mg/ml bovine pituitary extract (Lonza), 0.1 μg/ml hEGF (Lonza), 5 mg/ml insulin (Lonza), 0.5 ml aqueous solution of gentamicin sulphate (Lonza) and amphotericin-B containing 0.8 mM Ca^{2+} (low Ca^{2+} conditions) or 1.8 mM Ca^{2+} (high Ca^{2+} conditions).

To investigate whether inflammation or infection influences the expression of DLD, cells were stimulated with recombinant human cytokines and Toll-like receptor (TLR) agonists. Cells were stimulated with the following substances for 24 h: IL-8, IL-1β, TNF-α (Sigma–Aldrich), HKLM (TLR2 agonist: Invivogen, San Diego, CA), LPS (TLR4 agonist: Invivogen), Sonifilan (Kaken Pharmaceutical Co., Tokyo, Japan).

2.2. RT-PCR and quantitative real-time RT-PCR

The levels of DLD mRNA in human oral cells were analysed by RT-PCR and quantitative real-time RT-PCR. Total RNA was extracted from cells by the acid guanidine thiocyanate/phenol–chloroform method using TRIzol (Invitrogen). The RNA was reverse transcribed (SuperScript reverse transcriptase: Invitrogen), according to the manufacturer's instructions using oligo(dT)12–18 primers (Invitrogen). For RT-PCR, RT products were amplified using a PCR kit (AmpliTaq Gold: Applied Biosystems, Foster City, CA) and a thermocycler (Takara PCR Thermal Cycler MP, Osaka, Japan). The primer sets used were 5′-GCAACTCTTCACCAGTCCAGAGA-3′ (forward) and 5′-GCAGGCCACACTTGTACTTCCT-3′ (reverse) for DLD (product size, 70 bp); and 5′-CAACTACATGGTTTACATGTTC-3′ (forward) and 5′-GCCAGTGGACTCCACGAC-3′ (reverse) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (product size, 180 bp). The PCR conditions were 45 s at 94 °C, 45 s at 58 °C, and 2 min at 72 °C for 33 cycles. The PCR products were separated on 2% NuSieve (ICN Bio Products, Rockland, ME) agarose/ethidium bromide gels.

For quantitative real-time RT-PCR, primers and probes specific for DLD and GAPDH were designed using Primer Express software (Applied Biosystems). The probe and primer sets used were: probe, 5′-TGGGCCTGGCAGTGGCTTTCC-3′; and primers, 5′-CGTGGTCAGCATCCTCCTG-3′ (forward) and 5′-GTGAAGAGTTGCTGGATGCG-3′ (reverse) for DLD. The GAPDH probe and primer sets were purchased from Applied Biosystems. The reaction mixture was prepared by the TaqMan Universal PCR Master Mix (Applied Biosystems) with primer, probe sets, and RT products. Real-time PCR was performed using a GeneAmp 5700 Sequence Detection System instrument and software (Applied Biosystems). The expression level of DLD mRNA was standardized against GAPDH mRNA. The relative expression of DLD mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method described by Saitoh et al.[26] Data were expressed as the ratio of DLD mRNA to GAPDH mRNA.

2.3. In situ hybridization

The deparaffinized sections were pretreated with 10 μg/ml proteinase K and then treated with a triethanolamine buffer (pH 8.0) containing 0.25% acetic anhydride. The sections were hybridized with digoxigenin (DIG)-labelled antisense RNA probes for DLD for 16 h at 45 °C. Stringent washes were performed for 60 min at 45 °C with 50% formamide in sodium saline citrate (SSC), and the slides were treated with 20 μg/ml of RNase-A (Roche Diagnostics, Indianapolis, IN) for 30 min at 37 °C. The sections were incubated with anti-DIG antibody coupled to alkaline phosphatase (1/1000 dilution, Roche