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Immune regulatory functions of human beta-defensin-2 in odontoblast-like cells

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Abstract

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Aim To investigate the effects of human beta-defensins on the expression of genes involved in the host immune response of the dental pulp.

Methodology Human odontoblast-like cells were cultured in Dulbecco's modified Eagle's medium. Cells were stimulated by recombinant human beta-defensins (rhBDs) up to 4 h. RNA was extracted followed by cDNA synthesis (oligo-(dT)-primer). Samples were analysed by real-time polymerase chain reaction (PCR) technology. Genes of interest were: human beta-defensin-1, -2, interleukin (IL)-6, IL-8, tumour necrosis factor-alpha, cyclooxygenase-2, leukotriene-A4-hydrolase, cytosolic phospholipase-A-2 ($cPLA_2$), and dentine sialophosphoprotein. Gene expression of beta-actin served as internal standard for normalizing real-time PCR data. Two-way ANOVA and the paired *t*-test were applied for comparison of the gene expression.

Results In odontoblast-like cells rhBD-2 stimulation led to a down-regulation of the gene expression of hBD-1 ($P < 0.05$), whilst the mRNA expression of IL-6 ($P < 0.05$), IL-8 ($P < 0.05$) and $cPLA_2$ was increased in response to rhBD-2.

Conclusion The results of the present study suggest immune regulatory functions of human beta-defensin-2 in odontoblast-like cells.

Keywords: cytosolic phospholipase-A-2, human beta-defensins, innate immunity, interleukins, odontoblast-like cells, real-time PCR.

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Introduction

During the progression of a carious lesion the diffusion of bacterial antigens or the invasion of bacteria into the dental hard tissues is accompanied by inflammatory reactions of the dentine–pulp complex (Izumi *et al.* 1995) displaying a variety of immune mechanisms against invading bacteria (Jontell *et al.* 1998). After bacterial attack increased levels of many cytokines were identified in pulps cells, e.g. interleukin (IL)-6, IL-8 (Hahn *et al.* 2000, Engels-Deutsch *et al.* 2003). Cytokines and mediators such as arachidonic acid derivates play an important role in the regulation of the immune response against pathogenic agents (Akira *et al.* 1990, Schroder & Christophers 1992, Boyce 2005). More recently it has been found that antimicrobial peptides (human beta-defensins) are also present in dental pulp tissue (Dommisch *et al.* 2005b, 2006).

Human beta-defensins are positively charged antimicrobial peptides with molecular weights ranging from 3.5 to 6.5 kDa (Schroder 1999). These peptides are involved in the innate host defence (Lehrer 2004). Human beta-defensins have been detected not only in various epithelial tissues (oral cavity, intestine, respiratory tract, urinary tract, vagina) (Krisanaprakornkit *et al.* 1998, Dale & Krisanaprakornkit 2001, Dunsche *et al.* 2001, 2002, Islam *et al.* 2001, Takahashi *et al.* 2001, Ganz 2003), but also in monocytes, macroph-

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ages, lymphocytes and mast cells (Duits *et al.* 2002, Lehrer 2004).

Human beta-defensin-1 (hBD-1) was isolated from dialysate haemofiltrate of patients with advanced kidney infections (Bensch *et al.* 1995). HBD-1 was identified as a constitutively expressed gene and protein in epithelial tissues (Harder *et al.* 1997, Krisanaprakornkit *et al.* 1998, Sahasrabudhe *et al.* 2000, Islam *et al.* 2001), which shows a high activity against Gram-negative bacteria (Harder *et al.* 1997).

Psoriatic skin was the source for the isolation of human beta-defensin-2 (hBD-2) (Harder *et al.* 1997). Human beta-defensin-2 exhibits strong antimicrobicidal effects against Gram-negative bacteria as well as a high antimycotic potency, but only a weak bacteriostatic activity against Gram-positive bacteria (Harder *et al.* 1997).

Human beta-defensin-3 (hBD-3) was also isolated from keratinocytes (Harder *et al.* 2001) and exhibits a strong potency against Gram-negative bacteria, i.e. *Pseudomonas aeruginosa* and *Escherichia coli*, as well as against Gram-positive pathogens, i.e. *Staphylococcus aureus* and *Streptococcus pyogenes* (Harder *et al.* 1997). A differential gene expression of hBD-1, -2, and -3 could be demonstrated in healthy and inflamed gingival tissues (Dommisch *et al.* 2005a). The results showed an increased gene expression of hBD-2 in inflamed gingival biopsies compared with hBD-1, which has been revealed as a constitutively expressed antimicrobial peptide in keratinocytes (Zhao *et al.* 1996, Krisanaprakornkit *et al.* 1998, Dommisch *et al.* 2005a). Furthermore, a differential gene expression of hBD-1 and -2 was also shown in healthy and inflamed dental pulps (Dommisch *et al.* 2006). In healthy dental pulp the gene expression of hBD-1 was significantly higher than for hBD-2. In contrast, the mRNA expression of both hBD-1 and hBD-2 was significantly decreased during the clinical stage of an irreversible pulpitis (Dommisch *et al.* 2006).

It has been described that human beta-defensins possess direct and indirect activities in response to microorganisms (Yang *et al.* 2002, Brogden 2005). Their direct antimicrobicidal effects leading to pore formation or the formation of membrane disrupting structures ('carpet like') result in the osmotic cell death of microorganisms (Brogden 2005). In addition, it has been shown that human defensins exert regulative effects onto surrounding tissue cells. This implies that defensins are able to affect monocytes, mast cells, immature dendritic cells, lymphocytes and cytokine-activated neutrophils, and thereby modulate or enhance the host immunity (Lehrer 2004). HBD-2 caused the induction of histamine release and prostaglandin D2 production in rat peritoneal mast cells (Niyonsaba *et al.* 2001). Moreover, hBD-2 was able to increase the level of protein expression of dentine sialophosphoprotein (DSPP) in dental pulp cells (Shiba *et al.* 2003).

The aim of the present study was to investigate whether human beta-defensins affect the expression of genes involved in the immuno-inflammatory response of odontoblast-like cells.

Methods

Cell culture

To culture human odontoblast-like cells, freshly extracted, intact third molars were obtained from healthy patients (age 20–25) during routine surgical procedures. All patients had been informed about this study and had signed a letter of informed consent. The study was conducted in full accordance with the declared ethical principles (World Medical Association Declaration of Helsinki, version VI, 2002) and was approved by the Ethics Committee of the University of Bonn. The teeth were prepared for culturing according to described protocols (Tjaderhane *et al.* 1998, Dommisch *et al.* 2006). In brief, the cleaned tooth surfaces of third molars were disinfected with 70% (v/v) ethanol. The teeth were cut perpendicular to their long axis apically to the cemento-enamel junction and the root fragment was dissected from the crown. After removing the pulp with sterile forceps the crowns with attached odontoblasts were placed in Dulbecco's modified Eagle's medium (PAA, Cölbe, Germany, 10% fetal calf serum, Penicillin, Streptomycin and Amphotericin). The aim of this approach was to cultivate cells from the odontoblast layer lining the pulp chamber. Only third molars with immature roots were used.

Stimulation of odontoblast-like cells

Cells were cultured at 37 °C in a humidified atmosphere (5% CO_2). For stimulation experiments, the cells were grown to 80% of confluence. Subsequently, the cells were exposed to various concentrations of recombinant hBD-1, -2 and -3 (rhBDs at 15, 50, 150 ng mL^{-1}) (Promocell, Heidelberg, Germany). The cells were harvested after a time-period of 0.5, 1, 2 and 4 h. Unstimulated control cells were simultaneously harvested at the given time-points. Each set of experiments was performed in triplicate including the controls. Total RNA was isolated using RNeasy-Mini-Kit®

according to the manufacturer's instructions (Qiagen, Hilden, Germany). CDNA synthesis was carried out using standard protocols (Dommisch *et al.* 2005a).

Quantification of gene expression by real-time PCR

Expression of transcripts was determined by means of real-time polymerase chain reaction (PCR) using 1 μL of the cDNA-solution as a template. PCR amplification was performed using the iCycler® (Bio-Rad, Hercules, CA, USA). Reactions were carried out in a total volume of 25 μL with SYBR Reaction Green-Mix (Bio-Rad).

Beta-actin-PCR was performed for normalizing the threshold cycle (C_t), whilst H_2O served as negative control. All measurements were performed in duplicate. Every set of experiments comparing the expression of the beta-defensins (hBD-1, -2) was carried out with cDNA from the same sample.

Cloned cDNA of beta-actin, hBD-1, -2, IL-6, IL-8, leukotriene-A4-hydrolase (LTA_4H), cyclooxygenase-2 (Cox-2), and cytosolic phospholipase-A-2 ($cPLA_2$) served as positive controls and was amplified in dilution series of 1 : 1, 1 : 10; 1 : 100; 1 : 1 000; 1 : 10 000; 1 : 100 000 to construct standard curves based on the relationship between the threshold cycle and the logarithm of the cDNA concentration. Standard curves were calculated by the iCycler software® (Bio-Rad) for all PCR experiments. The slope of every specific standard curve was used to determine the primer efficiency. Primer sequences are displayed in Table 1.

The relative expression level was verified by normalizing the threshold cycle using an established mathematical method according to the literature and presented in fold change (Pfaffl 2001).

Statistical analysis

The data were analysed using two-way ANOVA, followed by the paired *t*-test (SPSS, Version 14, Munich, Germany). The significance level was set at $P \leq 0.05$.

Results

Studies on dilution series (10-fold) of the cloned cDNA showed that a deviation of three real-time PCR cycles (threshold cycles, C_t) was equivalent to a 10-fold difference in cDNA concentration.

The mRNA expression of beta-actin was shown in all investigated samples. The gene expression of three different components involved in the host immunity was analysed, including the gene expression of hBD-1, -2, IL-6, IL-8, tumour necrosis factor-alpha (TNF-α), and genes representing enzymes ($cPLA_2$, Cox-2, LTA_4H) involved in the biosynthesis of eicosanoids in odontoblast-like cells. Additionally, the mRNA expression of DSPP, as a marker gene for odontoblast cells, was analysed in each investigated sample.

The data were analysed using two-way ANOVA to determine differences for the gene expression at the given time-points and the separate concentrations. The statistical analysis demonstrated significant differences for either group (time or dose) in the case of the gene expression of hBD-1, IL-6, IL-8 and $cPLA_2$. In addition, the paired *t*-test was performed to specify significant differences in mRNA expression for these particular genes.

The mRNA of hBD-1 was significantly decreased in the presence of rhBD-2 ($P = 0.045$) (Fig. 1a). This effect was shown after stimulation for 2 h at the concentrations of 15 ng mL^{-1} ($P = 0.009$) and 50 ng mL^{-1} ($P = 0.047$), but not at 150 ng mL^{-1} (Fig. 1b). The gene expression of hBD-2 was not affected by any of the rhBDs at any concentration (data not shown).

In response to rhBD-2 (15 ng mL^{-1}) the gene expression of IL-6 was significantly up-regulated after 0.5 h ($P = 0.045$) and 1 h ($P = 0.036$) in odontoblast-like cells (Fig. 2a). A dose-dependent relationship

Table 1 Primer sequences

Gene	Forward	Reverse
beta-actin	5′-CAT GGA TGA TGA TAT CGC CGC G-3′	5′-ACA TGA TCT GGG TCA TCT TCT CG-3′
hBD-1	5′-CAT GAG AAC TTC CTA CCT TCT GC-3′	5′-TCA CTT GCA GCA CTT GGC CTT-3′
hBD-2	5′-CAT GAG GGT CTT GTA TCT CCT CT-3′	5′-CCT CCT CAT GGC TTT TTG CAG C-3′
IL-6	5′-ATG AAC TCC TTC TCC ACA AGC-3′	5′-CTA CAT TTG CCG AAG AGC CC-3′
IL-8	5′-ATG ACT TCC AAG CTG GCC GTG G-3′	5′-TGA ATT CTC AGC CCT CTT CAA AAA C-3′
TNF-α	5′-CTG CTG CAC TTT GGA GTG AT-3′	5′-CAG CTT GAG GGT TTG CTA CA-3′
Cox-2	5′-ATT GAC CAG AGC AGG CAG AT-3′	5′-CAG GAT ACA GCT CCA CAG CA-3′
LTA_4H	5′-CAG TGG CTC ACT CTC CTG AAC A-3′	5′-TCT GGG TCA GGT GTT TCT CC-3′
$cPLA_2$	5′-CCA AGG GAA ACT GAG GAA GA-3′	5′-AGG GAA ACA GAG CAA CGA GA-3′

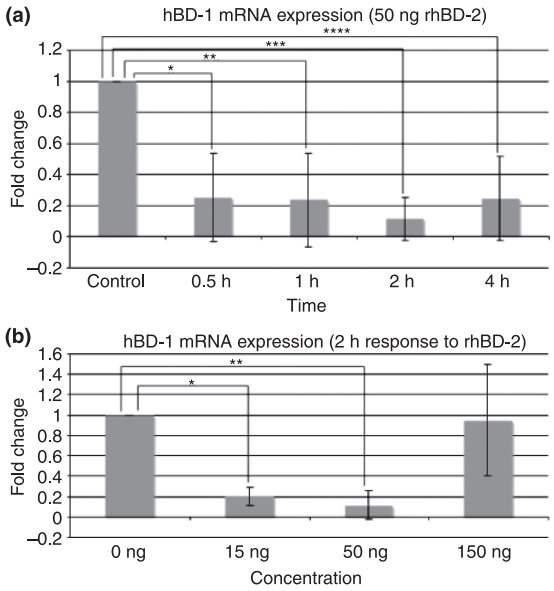

Figure 1 Gene expression of the human beta-defensin-1 (hBD-1) in odontoblast-like cells after stimulation with rhBD-2 analysed by real-time PCR. The *Y*-axis represents fold changes of gene expression. (a) The expression of hBD-1 mRNA after stimulation with 50 ng mL^{-1} rhBD-2. The expression was significantly down-regulated after 0.5 ($^{*}P = 0.045$), 1 ($^{**}P = 0.047$), 2 ($^{***}P = 0.008$) and 4 h ($^{****}P = 0.041$). (b) The dose-dependently decreased hBD-1 mRNA expression at 2 h after stimulation. A significant down-regulation of the gene expression of hBD-1 was demonstrated using 15 ng mL^{-1} ($^{*}P = 0.004$) and 50 ng mL^{-1} ($^{**}P = 0.008$).

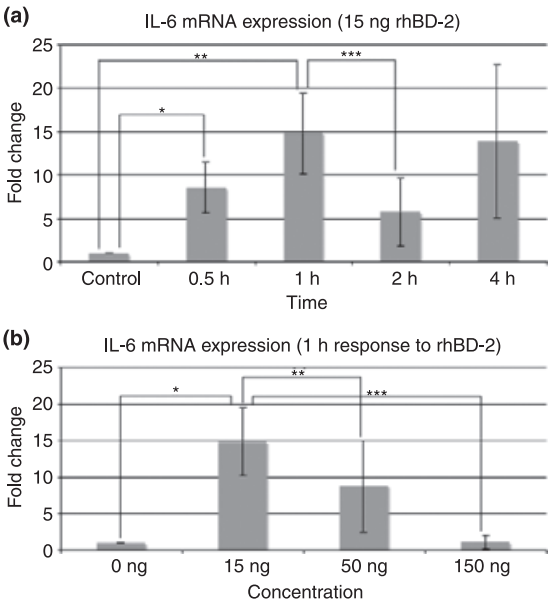

Figure 2 Gene expression of interleukin-6 (IL-6) in odontoblast-like cells after stimulation with rhBD-2 analysed by real-time PCR. The *Y*-axis represents fold changes of gene expression. (a) The analysis over the time after stimulation with 15 ng mL^{-1} rhBD-2 ($^{*}P = 0.045$; $^{**}P = 0.036$; $^{***}P = 0.02$). (b) The dose-dependent gene expression of IL-6 ($^{*}P = 0.036$; $^{**}P = 0.025$; $^{***}P = 0.05$).

between rhBD-2 and the mRNA expression of IL-6 was demonstrated in all experiments (Fig. 2). The use of 15 ng mL^{-1} rhBD-2 for 1 h ($P = 0.036$) showed increased mRNA levels, whereas higher concentrations up to 150 ng mL^{-1} restored the gene expression of IL-6 to its regular basal level according to the control cells (Fig. 2b).

Recombinant hBD-2 also affected the gene expression of IL-8, which was significantly increased over time up to 4 h ($P = 0.031$) (Fig. 3a). Similar to IL-6 the gene expression of IL-8 reached its regular level at higher concentrations (1 h stimulation) ($P = 0.009$) (Fig. 3b). A similar dose-dependent gene expression of IL-6 and IL-8 was shown for all measured time-points (data not shown).

In contrast to the pro-inflammatory cytokines IL-6 and IL-8, the expression of TNF-α mRNA in odontoblast-like cells was not affected by rhBDs (data not shown).

The gene expression of $cPLA_2$ showed a significantly higher expression at 0.5 h after stimulation ($P = 0.029$) (Fig. 4a). An increase in the mRNA expression of $cPLA_2$ was demonstrated at the highest concentration used in this study (150 ng mL^{-1}) (Fig. 4b).

The genes Cox-2, LTA_4H and DSPP were detected in the investigated cells, however, their expression was not affected by rhBDs. No significant influences could be shown in odontoblast-like cells in response to rhBD-1 and -3 (data not shown).

Discussion

In the present study, the immune reaction of odontoblast-like cells was analysed in response to human beta-defensins. The cells were stimulated by rhBD-1, -2, -3 at different concentrations (15, 50 and 150 ng mL^{-1}) over a time-period of 0.5–4 h. The expression of hBD-1, -2, IL-6, IL-8, TNF-α, $cPLA_2$, Cox-2 and LTA_4H was observed as representative markers.

The aim was to determine regulations of the expression of genes that coordinate immune responses in the dental pulp. The results show that hBD-2 affects gene expression of hBD-1, IL-6 and IL-8 but does not significantly affect the other markers tested.

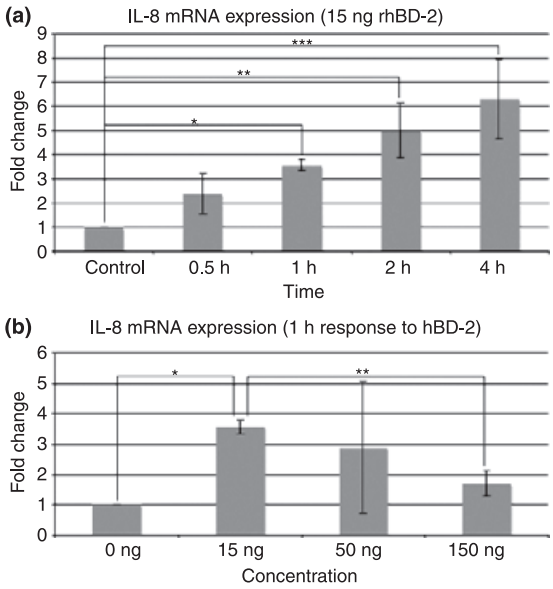

Figure 3 Gene expression of interleukin-8 (IL-8) in odontoblast-like cells after stimulation with rhBD-2 analysed by real-time PCR. The *Y*-axis represents fold changes of gene expression. (a) The time-dependent up-regulation of the IL-8 mRNA in odontoblast-like cells ($^{*}P = 0.003$; $^{**}P = 0.025$; $^{***}P = 0.031$). (b) The dose-dependent mRNA expression at the 1-h time-point ($^{*}P = 0.03$; $^{**}P = 0.009$).

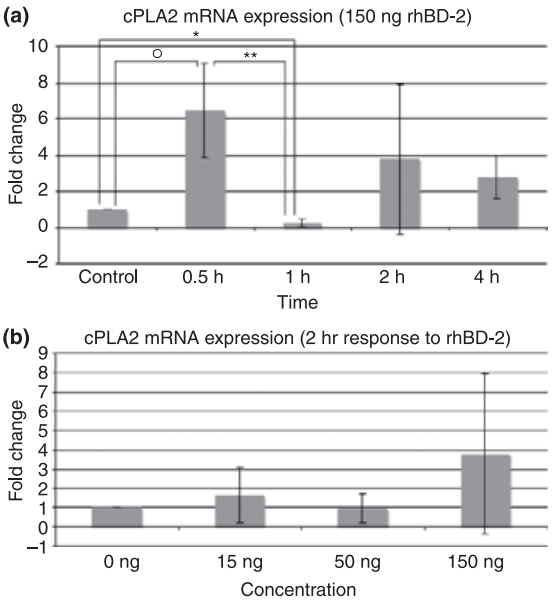

Figure 4 Gene expression of the cytosolic phospholipase-A-2 ($cPLA_2$) in odontoblast-like cells after stimulation with rhBD-2 analysed by real-time PCR. The *Y*-axis represents fold changes of gene expression. (a) The gene expression of cPLA2 at the 0.5 h time-point. The cells were stimulated with 150 ng mL^{-1} rhBD-2 ($^{\circ}P = 0.06$; $^{*}P = 0.029$; $^{**}P = 0.05$). (b) The changes of gene expression in response to different doses of recombinant human beta-defensin-2. No significant changes were found at any concentration. Only a trend could be shown using 150 ng mL^{-1} of rhBD-2.

Therefore, odontoblast-like cells were cultured from patient samples (third molars) according to the established method by Tjaderhane *et al.* (1998). The odontoblast phenotype was confirmed by the proof of the gene expression of DSPP. Different cell densities in the cell culture dishes might cause variation in the levels of the gene expression. Thus, the cells were grown to only 80% of confluence to reduce general differentiation, which could alter the mRNA expression. The concentration of rhBD-2 for stimulation experiments was based on previous findings that hBD-2 demonstrates low gene and protein expression in healthy dental pulps (Dommisch *et al.* 2005b, 2006). Thus, lower concentrations of rhBDs were used in this study when compared with investigators that worked on mast or epithelial cells (Niyonsaba *et al.* 2002, Sakamoto *et al.* 2005). Real-time PCR technology was used to analyse a differential gene expression. The results display relative changes in expression (fold change). A well-established mathematical method was used to determine the correct relative expression levels. The mathematical equation calculates the threshold cycles (C_t-values) and determines a correlation between the expression of the control and the sample group, including the primer efficiency and the normalization using the data from the housekeeping gene (Pfaffl 2001).

It has been shown previously that hBD-2 affects gene expression of DSPP in dental pulp cells (Shiba *et al.* 2003), however, alteration of DSPP in these odontoblast cultures did not occur in the present study. Odontoblasts are considered as dentine-producing cells, which represent the border between dentine and the pulp tissue. Thus, odontoblasts are the first cells of the pulp tissue, which might have early antigen contact due to microorganisms that are present in a carious lesion. It was therefore of interest whether odontoblast-like cells express genes that are known to be involved in the early inflammatory response (Akira *et al.* 1990, Schroder & Christophers 1992). Recently, it has been shown that hBD-1 and -2 are expressed in the odontoblast cell layer of the human dental pulp (Dommisch *et al.* 2005b).

In addition, hBD-2 possesses cytokine-like attributes in mast cells and neutrophils (Niyonsaba *et al.* 2002,

2004). The combination of these findings raised the question whether odontoblast-like cells might respond to human beta-defensins that are secreted by their neighbour cells.

The protein expression of human beta-defensins in dental pulp biopsies has been examined previously and the expression of hBD-1 was strongly evident in the odontoblast cell layer, whereas a weaker signal was detected for hBD-2 by immunohistochemistry (Dommisch *et al.* 2005b). The gene expression of hBD-1 and hBD-2 was compared in healthy and inflamed dental pulp biopsies to reveal a significantly lower gene expression of hBD-1 in inflamed compared with healthy dental pulps (Dommisch *et al.* 2006). The comparison of the data from the present *ex vivo* study with the data from previous work on biopsies reveals a similar trend with regard to the down-regulation of the hBD-1 mRNA expression. The data showed an early decrease of the gene expression of hBD-1 in response to recombinant hBD-2 in-between the investigated 0.5–4 h, whilst no change was observed for the mRNA expression of hBD-2. Human beta-defensin-1 is characterized by a constitutive expression, e.g. in epithelial cells (Harder *et al.* 1997). Unlike hBD-1, hBD-2 has been recognized as an antimicrobial peptide that is up-regulated in the presence of microorganisms (Krisanaprakornkit *et al.* 2000, Chadebech *et al.* 2003). In addition, antimicrobial activity of hBD-2 has been shown against *Streptococcus mutans* (Shiba *et al.* 2003, Nishimura *et al.* 2004). During the progression of a carious lesion, the expression of hBD-2 might be increased in response to metabolic mediators or exotoxins of microorganisms. *Streptococcus mutans* has been strongly associated with dental caries (Donkersloot & Hoerman 1974). Over the time measured, the mRNA expression of hBD-1 was decreased compared with the control group. The application of different amounts of rhBD-2 showed an effect on the expression at lower concentrations (15, 50 ng mL^{-1}), whereas the highest concentration (150 ng mL^{-1}) was ineffective in altering hBD-1 gene expression. These data lead to the suggestion that the constitutive gene expression of hBD-1 may be partially regulated dependent on the presence of hBD-2.

At the same time, rhBD-2 also stimulated the gene expression of the pro-inflammatory cytokines IL-6 and IL-8. Interleukin-6 is co-responsible for B-cell differentiation and an activation factor for T cells (Akira *et al.* 1990), whereas IL-8 possesses the ability to specifically activate neutrophil granulocytes (Schroder & Christophers 1992). Both interleukins lead to a pro-inflammatory reaction. Whilst IL-8 showed an almost eightfold up-regulation at the 4-h time-point, the mRNA for IL-6 was increased almost 19-fold measured after 1 h of stimulation. IL-6 and IL-8 gene expression were comparably stimulated at lower concentrations of rhDB-2 in a time-dependent manner (Fig. 2). The mRNA expression of both IL-6 and IL-8 was most affected by a concentration of 15 ng mL^{-1}. IL-6 as well as IL-8 mRNA expression was increasingly restored at higher concentrations of rhBD-2. These data suggest a biological effectiveness of hBD-2 at concentrations between 15 and 50 ng mL^{-1}. Higher amounts of hBD-2 did not alter the gene expression of hBD-1, IL-6 and IL-8.

In addition to the genes of the antimicrobial peptides and cytokines, the mRNA expression of enzymes that are involved in the eicosanoid biosynthesis ($cPLA_2$, LTA_4H, Cox-2) were investigated. Unlike the previously discussed genes (hBD-1, IL-6, IL-8) a different pattern of gene expression was demonstrated for the cytosolic phospholipase-A-2. $CPLA_2$ mRNA expression was increased only at very early time-points (0.5 h) and rapidly restored to its regular expression level, however this phenomenon was only seen at the highest concentration used (150 ng mL^{-1}). As the IL-6 and IL-8 mRNA expression was already highly up-regulated at lower concentrations of hBD-2, the biological relevance of an up-regulation of $cPLA_2$ only by high concentrations of hBD-2 appears to be questionable.

The results of the present study provide evidence that hBD-2 may initiate or enhance the cytokine-induced pro-inflammatory reaction of odontoblast-like cells. In contrast, rhBD-1 and rhBD-3 had no significant effects on the gene expression neither on human beta-defensins and cytokines nor on genes of enzymes belonging to the eicosanoid biosynthesis. Thus, these data implicate that hBD-1 and hBD-3 exhibited no defined immune regulatory function for the investigated genes in odontoblast-like cells.

In summary, the present study indicates stimulative effects on the gene expression of pro-inflammatory mediators in response to hBD-2. The mRNA for the related antimicrobial peptide hBD-1 was down-regulated in the presence of rhBD-2. The results suggest immune regulatory functions of hBD-2 in odontoblast-like cells in an autocrine or paracrine fashion.

Conclusion

In addition to their antimicrobial function human beta-defensins may exert immune regulatory functions in

dental pulp cells. Odontoblast-like cells showed a potent ability to respond to hBD-2, which is an inducible antimicrobial peptide in relation to a microbial challenge. It is suggested that the synthesis of hBD-2 in odontoblast cells may enhance the immuno-inflammatory reactivity of the dental pulp.

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