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Radiotherapy for oral cancer decreases the cutaneous expression of host defence peptides

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ABSTRACT

Introduction: Bacterial resistance against antibiotics has become an increasing challenge in the treatment of cutaneous infections. Consequences can be severe, especially in infected wounds following previous local radiotherapy. Certain endogenous peptide antibiotics, the host defence peptides (HDPs), exhibit broad-spectrum antimicrobial activity and promote wound healing. Their use as supplements to conventional antibiotics is a current topic of discussion; however, knowledge of their quantities in healthy and compromised tissue is a prerequisite for such discussion. To date, no data concerning HDP quantities in irradiated skin are available.

Methods: Expression profiles of the genes encoding HDPs, namely human beta-defensin-1 (DEFB1, hBD-1), beta-defensin-2 (DEFB4A, hBD-2), beta-defensin-3 (DEFB103, hBD-3) and S100A7, were assessed in samples of non-irradiated and irradiated neck.

Results: A reduction in the expression of all of the examined genes was observed in irradiated skin when compared with non-irradiated skin (statistically significant in the case of *S100A7*, $P = 0.013$). Immunohistochemistry revealed differences in HDP distribution with respect to the epithelial layers.

Conclusion: The study demonstrates a significant reduction in HDP gene expression in neck skin as a result of radiotherapy. These findings might represent a starting point for novel treatments of cutaneous infections in irradiated patients, such as topical supplementation of synthetic HDP.

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1. Introduction

Host defence peptides (synonym: HDPs, antimicrobial peptides (AMP)) are “natural peptide antibiotics”. They defend epithelial surfaces against microbial infestation (Braff et al., 2005; Fulton et al., 1997; Kaus et al., 2008). HDPs destroy bacteria, for example, by membrane permeabilization, and also promote wound healing (Kagan et al., 1990; Lehrer et al., 1989; Steinstraesser et al., 2008).

Human beta-defensins (hBDs) form a major HDP subgroup. DEFB1 (also known as human beta-defensin-1, hBD-1, encoded by the *DEFB1* gene) is produced by keratinocytes and $CD4^+/CD8^+$ T cells. It is efficient against Gram-positive and Gram-negative bacteria. DEFB4A (also known as human beta-defensin-2, hBD-2, encoded by the *DEFB4A* gene) is produced in keratinocytes, mast cells and $CD4^+/CD8^+$ T cells and shows activity against Gram-positive/Gram-negative bacteria and fungi. DEFB103 (also known as human beta-defensin-3, hBD-3, encoded by the *DEFB103A* gene) is synthesized by keratinocytes, monocytes and $CD4^+$ T cells and disrupts Gram-positive/Gram-negative bacteria and fungi (Harder et al., 1997; Schittek et al., 2008; Steinstraesser et al., 2008). S100A7, another HDP, is encoded by the *S100A7* gene and is

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produced in keratinocytes. S100A7 exhibits activity against Gram-positive/Gram-negative bacteria (Fulton et al., 1997; Schittek et al., 2008; Steinstraesser et al., 2008). It is also known as "psoriasin", because of its identification in psoriatic skin (Madsen et al., 1991).

The "natural antibiotic" properties of HDP have been used as a model for the development of alternatives to conventional antibiotics (Gilmore et al., 2009; Steinstraesser et al., 2009). This becomes relevant with respect to the emerging rates of bacterial resistance to antibiotics (Gould, 2008; Livermore, 2004; Wagenlehner et al., 2002; Wilson, 2003). Synthetic HDP derivatives have been produced and have been tested in clinical trials (Lipsky et al., 2008; Rennie et al., 2005; Rotem and Mor, 2009; Tew et al., 2010). Increased bactericidal activity has been reported in some studies but cytotoxity occurs as an adverse reaction (Vaara, 2009). Research is now focusing on the development of synthetic HDP with lower cytotoxity (Lipsky et al., 2008; Tew et al., 2010). Because of the narrow therapeutic window, the exact dose–response characteristics of HDPs are crucial for potential topical treatment.

Wound care and the treatment of cutaneous infections are challenges in previously irradiated tissue. The disruption of the protective 'shield' mechanism of HDP has been speculated to be causative for recurrent skin infections (Harder et al., 1997). A decrease in *DEFB4A* expression has been found as a reason for cutaneous infections in burn wounds (Milner and Ortega, 1999). In addition to their antimicrobial capacities, HDP promotes wound healing and neovascularization in infected and chronic wounds (Steinstraesser et al., 2008; Steinstraesser et al., 2006).

Accordingly, the use of HDP for the treatment of infected wounds in irradiated skin seems to be a promising new therapeutic option. However, knowledge of the underlying HDP quantities is essential. No data comparing HDP expression between non-irradiated and irradiated skin are to date available.

We have investigated the expression levels of the above-named HDP in samples of non-irradiated and irradiated skin by means of real-time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry to test the null-hypothesis that HDP expression is not different between non-irradiated and irradiated skin.

2. Material and methods

2.1. Patients and specimens

Samples were collected from 80 patients at the maxillofacial unit of the Technical University Munich, Germany (2012–2013). The methods were approved by the local ethics committee (No. 212108) and are in accordance with the Helsinki Declaration. All patients gave written informed consent.

The following parameters were recorded: age, sex, alcohol/nicotine abuse and history of previous cervical radiotherapy. Skin samples were collected from incision margins during routine procedures being undergone by patients requiring craniofacial free flap reconstruction. In such cases, the vessels for microsurgical anastomoses were accessed by using a standard neck incision. Each sample consisted of a skin strip of 2 × 10 mm. One half of each sample was placed in Allprotect™ (Qiagen, Hilden, Germany) and stored at −80 °C for PCR experiments, whereas the other half was stored in 4.5% buffered formalin for immunohistochemistry.

2.2. RNA isolation and reverse transcription

Samples were homogenized by means of a rotor-stator system (Miccra, ART Labortechnik, Muellheim, Germany) and ultrasonification. Total ribonucleic acid (RNA) was isolated by using the RNeasy® Protect Mini Kit (Qiagen). The amount of extracted RNA was determined by measuring the optical density (BioPhotometer, Eppendorf, Hamburg, Germany). For reverse transcription (RT) of 1 μg isolated RNA per sample, the SuperScript™ First Strand Synthesis System (Invitrogen, Karlsruhe, Germany) and random primers were used.

2.3. Real-time RT-PCR

Real-time RT-PCR was performed in a LightCycler® 1.0 system (Roche, Mannheim, Germany) with 2 μl cDNA sample, 2 μl LightCycler® FastStart DNA Master SYBR Green I reaction mix (Roche), 1 μl forward and reverse primer (0.5 μM), 1.6 μl MgCl (3 mM) and 12.4 μl RNase-free water, resulting in a volume of 20 μl/sample. Primer sequences are detailed in Table 1. Primer specificity was verified by the electrophoretic separation of the PCR products. Amplification algorithms were: 10 min at 95 °C, 40 cycles of 15 s at 94 °C, 10 s at 60 °C (*S100A7*: 58 °C) and 10 s at 72 °C. A melting curve analysis was recorded in order to test for cDNA fragment consistency. The RNA amount was calculated by a comparison of measured threshold cycles with a standard curve being included in each run and normalized with *GAPDH* (*D-glyceraldehyde-3-phosphate dehydrogenase*) as a housekeeping gene. A no-template control was included in each run. Amplifications were carried out in triplicate.

2.4. Histochemistry and immunohistochemistry

Samples were stored in buffered formalin (4.5%) and embedded in paraffin. Sections (5 μm) were stained with the following histological staining reactions: haematoxylin/eosin (HE) for histological orientation, Masson's Trichrome for connective tissue and Elastica van Gieson for elastic and collagen fibres. Immunohistochemical staining was performed according to the avidin–biotin horseradish-peroxidase-complex method by using the Histostain Plus Kit (Zymed, San Francisco, CA, USA). The following primary antibodies were used and were diluted as follows: human beta-defensin-1 and human beta-defensin-2 (DEFB1/DEFB4A), 1:100 (polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA); human beta-defensin-3 (DEFB103), 1:100 (polyclonal, Novus Biologicals, Littleton, CO, USA); S100A7, 1:50 (monoclonal, Imgenex, San Diego, CA, USA). Sections were pre-incubated with microwave irradiation and blocked with 3% goat serum. The second biotinylated antibody (Vector Laboratories Inc., Burlingame, CA, USA) was diluted 1:200. Diaminobenzidine was used as the chromogen. Controls in which the primary antibody was omitted were treated identically. Sections were analysed with a Nikon microscope and images were captured with a digital camera (Nikon, Duesseldorf, Germany). Two investigators scored the staining independently. For each sample, a staining intensity value (− = negative; + = weak; ++ = medium; +++ = strong)

Table 1
Primer sequences and GenBank® accession numbers of examined genes.

Gene	Acc.-no.	Sequence 5′–3′	
DEFB1	NM_005218.3	Forward	TTGTCTGAGATGGCCTCAGGTGGTAAC
		Reverse	ATACTTCAAAAGCAATTTTCCTTTAT
DEFB4A	NM_004942.2	Forward	CCAGCCATCAGCCATGAGGGT
		Reverse	GGAGCCCTTTCTGAATCCGCA
DEFB103A	NM_018661.3	Forward	CTGTTTTTGGTGCCTGTTCC
		Reverse	CTTTCTTCGGCAGCATTTTC
S100A7	NM_002963.3	Forward	TGCTGACGATGATGAAGGAG
		Reverse	ATGTCTCCCAGCAAGGACAG
GAPDH	NG_007073.2	Forward	GAGTCAACGGATTTGGTCGT
		Reverse	TTGATTTTGGAGGGATCTCG

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