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Human neutrophil peptide-1 affects matrix metalloproteinase-2, -8 and -9 secretions of oral squamous cell carcinoma cell lines *in vitro*

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ABSTRACT

Objectives: The present study aimed to investigate the effect of HNP-1 on the matrix metalloproteinase (MMP)-2, -8 and -9 secretions of two oral squamous cell carcinoma (OSCC) cell lines (UT-SCC-43A and UT-SCC-43B).

Design: In all experiments, the two OSCC cell lines were incubated with graded concentrations (0, 1, 5, and 10 µg/ml) of HNP-1 for 24 and 48 h. Cell viability was measured using a colorimetric proliferation test and cell death was analyzed with a colorimetric cytotoxicity detection kit. Enzyme activity of MMP-2 and MMP-9 was detected by using gelatin zymography, and molecular weight forms of MMP-8 were determined by Western-blot and a densitometric quantitation method.

Results: Both cell lines showed a significant increase in LDH toxicity at 24 h (UT-SCC-43A: $p = 0.005$ & UT-SCC-43B: $p = 0.014$). Reduced gelatinolytic activities of proMMP-2 were detected in UT-SCC-43B cell line after 24 and 48 h of incubation with HNP-1 (1 µg/ml: $p < 0.001$, 5 µg/ml: $p < 0.001$, and 10 µg/ml: $p = 0.0225$). MMP-8 levels of both cell lines decreased at 200–250 kDa after 24 h of incubation, while after 48 h only UT-SCC-43B decreased at 45–50 kDa.

Conclusions: Our results indicate that HNP-1 suppresses the secretion of MMP-2, -8, and -9 in OSCC cell lines.

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1. Introduction

Defensins are naturally occurring cationic polypeptides, widely involved in innate immunity (Evans and Harmon, 1995). Their main function is to act as an antimicrobial agent against a broad spectrum of microbes; however, they also serve as a bridge between the innate and acquired immune responses (Lillard, Boyaka, Chertov, Oppenheim, & McGhee, 1999). There are three classes of defensins: alpha (α), beta (β), and theta (θ), which are active against most bacteria, mycobacteria, enveloped viruses, fungi, and some parasites (Selsted and Ouellette, 1995). In the present study, we were interested especially in α -defensins, which are called also human neutrophil peptides (HNPs). HNPs are cysteine-rich proteins of six subtypes; the first four subtypes are produced by human polymorphonuclear leukocytes (neutrophils), while two subtypes are produced by the Paneth cells of the

intestine as well as by the human female reproductive tract (Ganz et al., 1985; Mallow et al., 1996; Quayle et al., 1998).

Oral squamous cell carcinoma (OSCC) represents more than 90% of all cancers in the oral cavity, and it is the sixth most common cancer worldwide (Güneri and Epstein, 2014). At high concentrations, defensins are cytotoxic on eukaryotic and tumor cells (Lichtenstein, Ganz, Selsted, & Lehrer, 1986; Lichtenstein, 1991). For instance, HNPs impair tumor cell proliferation in renal cell carcinoma and mediate tumor cell lysis in human neuroblastomas (Barker and Reisfeld, 1993; Müller et al., 2002). In oral cancer, HNP-1 has been detected in saliva of patients with OSCC and oral mucopidermoid carcinoma, exerting a cytotoxic effect on the cellular architecture of the cancerous lesion (Mizukawa et al., 1998, 2001). Although the cytotoxic non-oxidative mechanism of action of HNP-1 remains partly unclear, a common hypothesis suggests that these positively charged peptides interact electrostatically with the negatively charged surface molecules on the target cells, inducing the permeability of lipid bi-layered membranes and creating channels of regulated voltage (Peschel, 2002). However, breaking a single strand of the target cell DNA, leading to DNA

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injury, is considered a fundamental event as well (Gera and Lichtenstein, 1991a). The purified form of HNP-1 has been found to be capable of lysing tumor cells in dose- and time-dependent manners (Lichtenstein et al., 1986).

Degradation of the extracellular matrix (ECM) and basement membrane components provides access for cancer cells to the blood circulation and lymphatics. Matrix metalloproteinases (MMPs), the zinc-dependent proteolytic enzymes, are endopeptidases having a pivotal oncogenic role in the ECM degradation process by allowing the tumor to grow, invade, and metastasize into surrounding tissues (Vihinen and Kähäri, 2002). Usually, MMPs are expressed and activated in trace amounts in human adult tissues, and they are mainly involved in tissue development, remodelling, and wound healing (Uitto, Overall, & McCulloch, 2003). Overexpression of MMPs is a common characteristic in almost all human cancers and is often strongly associated with the activation cascade of invasion, metastasis, angiogenesis, and poor prognosis (Cox & O'Byrne, 2001). For instance, some studies have reported the expression of MMP-2 and MMP-9 in tissue samples of oral cancer (Aparna, Rao, Kunhikatta, & Radhakrishnan, 2015; de Vicente, Fresno, Villalain, Vega, & Hernández Vallejo, 2005). Moreover, others have suggested their identification as prognostic indicators of the aggressiveness of cancerous behavior (Nanda et al., 2014; Swain, Kumar, Richa, & Pathak, 2012). However, traces of MMP-8 have been demonstrated in many OSCC cell lines, and MMP-8 has seemingly an anti-tumor effect on breast carcinoma tissues (Agarwal, Goodison, Nicholson, Tarin, & Urquidi, 2003).

Although HNP-1 concentrations of $>20 \mu\text{g/ml}$ demonstrate cytotoxic effects on OSCC cell lines in *in vitro* conditions, its tissue or saliva levels are below cytotoxic concentrations (Mizukawa et al., 1998). In the present study, we hypothesized that even low non-cytotoxic concentrations of HNP-1 ($\leq 10 \mu\text{g/ml}$) demonstrate anti-cancer effect, probably on cancer invasion. Therefore, the aim of this *in vitro* study was to examine the effect of HNP-1 on the levels of MMP-2, -8, and -9 expressed by the OSCC cell lines.

2. Materials and methods

2.1. Cell lines and cultures

In all experiments, two oral squamous cell carcinoma cell lines (UT-SCC-43A, UT-SCC-43B) were used (Räsänen, Virtanen, Salmenperä, Grenman, & Vaheri, 2009). UT-SCC-43A was derived from a primary tumor of a 75-year-old female and UT-SCC-43B was derived from a recurrent tumor of the same patient. Methods of tissue sampling and cell line establishment procedures were previously described (Takkunen et al., 2006). Cells were grown in 96-well plates in a culture medium of RPMI 1640 (R0883, Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum, 1% antibiotic, and 1% L-glutamine.

2.1.1. Preparation of HNP-1 suspensions

Culture medium with synthetic HNP-1 (Peptide Institute Inc., Osaka, Japan) was added to the cell culture wells. OSCC cells were incubated with HNP-1 concentrations of 1, 5, or $10 \mu\text{g/ml}$. Culture medium without HNP-1 served as a control. For each HNP-1 containing test group and the control, wells were prepared in triplicate immediately before the experiments.

2.1.2. Cell viability measurements

After incubation of cells for 24 and 48 h, the cell viability was analyzed using a colorimetric proliferation test (Cell Titer 96 Kit, Promega, Madison, WA, USA) and the cell death was measured with a colorimetric cytotoxicity detection kit (Lactate

dehydrogenase [LDH], Roche, Mannheim, Germany). All experiments were done in triplicates.

2.1.3. Assay of gelatinolytic activity

The gelatinolytic activity assay of cell culture samples was performed by using zymography in 11% sodium dodecyl sulphate (SDS)-polyacrylamide gels with 1 mg/ml fluorescent dye using 2-methoxy-2,4-diphenyl-3-(2H) furanone (MDPF, Fluka, Buchs SG, Switzerland) labeled gelatin as a substrate (Gursoy et al., 2010). Briefly, after 2 h of preincubation with Laemmli's buffer without reducing reagent, the zymography was performed, and then the gels were washed with 50 mM Tris-HCl buffer (pH 7.5, containing 2.5% Tween 80 and 0.02% $\text{Na}_2\text{S}_2\text{O}_5$) for 30 min. After three different washings, the gels were incubated overnight in 50 mM Tris-HCl buffer (pH 7.5, containing 0.02% $\text{Na}_2\text{S}_2\text{O}_5$, 0.5 mM CaCl_2 and $1 \mu\text{M}$ ZnCl_2) at 37°C . The degradation of gelatin was visualized under UV light, and then gelatin was stained with 1% Coomassie Brilliant Blue R-250. The gelatinolytic activity was visualized as clear bands against the blue background on stained gels. The evaluation of gelatinolytic activity was performed with the Bio-Rad Model GS-700 Imaging Densitometer using the Bio-Rad Quantity One-program (Bio-Rad laboratories, Hercules, CA, USA).

2.1.4. Western immunoblotting

For detecting the molecular forms of MMP-8, a modified method of enhanced chemiluminescence (ECL) Western blotting kit was used according to the protocol recommended by the manufacturer (GE Healthcare, Amersham, UK) (Hästbacka et al., 2007). Briefly, the cell culture samples were mixed with Laemmli's buffer without the reducing reagent and heated for 5 min, and protein separations were performed with 11% sodium dodecyl sulphate (SDS)-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes (Protran, Whatman GmbH, Dassel, Germany), which were incubated overnight with a polyclonal primary antibody, anti-MMP-8 (Hanemaaijer et al., 1997). Target detection was performed according to the manufacturer's protocol. The quantitation of immunoblots was discharged by using the GS-700 Imaging Densitometer Scanner (Bio-Rad, Hercules, CA, USA) and the Bio-Rad Quantity One program.

2.2. Statistical analyses

Out of the triplicate measurements, multiple comparisons of the levels of proliferation, cell death, both gelatinolytic activities, and expression of different molecular weight forms of MMP-8 among the controls and test groups by using the ANOVA test. To demonstrate only the dose-dependence effect, non-significant outcomes obtained by ANOVA test were discarded even if they were significant between certain test groups at the individual level. P value of <0.05 was accepted as statistically significant.

3. Results

3.1. Cell proliferation and death

No significant change was noticed in the cell proliferation of both cell lines when incubated with any of the tested concentrations of HNP-1 for either time intervals. However, as for cell viability, both cell lines showed a significant increase in LDH toxicity at 24 h (UT-SCC-43A: $p=0.005$ & UT-SCC-43B: $p=0.014$) (Fig. 1). After 48 h, the cell toxicity of only UT-SCC-43B cell line was found to decrease, but the level of decrease varied slightly among the different test concentrations of HNP-1 ($1 \mu\text{g/ml}$: $p=0.025$, $5 \mu\text{g/ml}$: $p=0.007$, and $10 \mu\text{g/ml}$: $p=0.003$) (Fig. 1).

CELL DEATH

24 h

48 h

Fig. 1. Effect of human neutrophil defensin (HNP)-1 on the proliferation of oral squamous cell carcinoma cell lines UT-SCC-43A and UT-SCC-43B. Following the incubation of each cell line with different concentrations of HNP-1 for 24 h and 48 h, the cell proliferation was measured. In addition, the lactate dehydrogenase level (LDH) was measured as a marker of cell death (asterisk indicates a significant difference from the control (0 µg/ml HNP-1) group with a *p* value of <0.05).

3.2. Effect on MMP-2 and -9 secretion

In Figs. 2 and 3, where the relative enzyme levels are given, the data are presented as percentages (%). The enzyme levels were measured and the difference was assessed in comparison to all test concentrations. The enzyme levels in the test groups (1, 5, and 10 µg/ml of HNP-1) are demonstrated in percentage of increase or decrease, when the enzyme level of control group (0 µg/ml of HNP-1) was accepted as 100%. A zymogram shows the relative densities of these MMPs according to HNP-1 concentrations (Fig. 2A). After 24 and 48 h of incubation of the two OSCC cell lines with HNP-1, a decrease in the overall enzyme activity of proMMP-2 was noticed

in UT-SCC-43B (1 µg/ml: *p* < 0.001, 5 µg/ml: *p* < 0.001, and 10 µg/ml: *p* = 0.022) (Fig. 2B and C). No significant increase or decrease was found in other enzyme groups.

3.3. Effect on MMP-8 secretion

Western-blot results demonstrated that, after 24 h, a decrease in MMP-8 levels of UT-SCC-43A cell line occurred at 200–250 kDa (1 µg/ml: *p* = 0.013, and 10 µg/ml: *p* = 0.048) (Fig. 3A). On the other hand, UT-SCC-43B showed a decrease in all of MMP-8 and its associated domains at the ranges 200–250 (5 µg/ml: *p* = 0.008, and 10 µg/ml: *p* = 0.003) and 250–300 (5 µg/ml: *p* = 0.018, and

Fig. 2. A–C: Gelatinolytic enzyme activities of oral squamous cell carcinoma cell lines (UT-SCC-43A and UT-SCC-43B) when incubated with human neutrophil defensin (HNP)-1. Following the incubation of each cell line for 24 h and 48 h, the MMP-2 and MMP-9 activities were measured with gelatin zymography (2A). All enzyme assays were done in triplicate, only one representative gel is given in figure. Enzyme activities were measured densitometrically and were given as box plots according to their kDa levels (2B–C). In densitometric analysis, enzyme activity of control group was taken as 100% and test groups were presented as percentages of increase or decrease (asterisk indicates a significant difference from the control (0 µg/ml HNP-1) group with a *p* value of <0.05).

Fig. 3. A–C: Matrix metalloproteinase (MMP)-8 activities of oral squamous cell carcinoma cell lines (UT-SCC-43A and UT-SCC-43B) when incubated with human neutrophil defensin (HNP)-1. Following the incubation of each cell line with different concentrations of HNP-1 for 24 h and 48 h, the enzyme activity was measured with western-blot (3A). All enzyme assays were done in triplicate, only one representative gel is given in figure. MMP-8 enzyme activities were measured densitometrically and are given as box plots according to their kDa levels (3–C). In densitometric analysis, enzyme activity of control group was taken as 100% and test groups were presented as percentages of increase or decrease (asterisk indicates a significant difference from the control (0 µg/ml HNP-1) group with a p value of <0.05).

10 $\mu\text{g/ml}$: $p < 0.001$) (Fig. 3B). After 48 h, the only significant difference was noticed in UT-SCC-43B where a decrease occurred at the range of 40–50 kDa (10 $\mu\text{g/ml}$: $p < 0.001$) (Fig. 3C).

4. Discussion

To our knowledge, this study is the first to show that HNP-1 can regulate enzyme activities of gelatinases, MMP-2 and -9, and expression of MMP-8 in OSCC cell lines at relatively non-cytotoxic concentrations.

In the present study, we tested the effect of different HNP-1 concentrations between 1–10 $\mu\text{g/ml}$. This concentration range of HNP-1 was chosen, since it is in line with the salivary concentrations of this peptide found in OSCC and various oral diseases (Mizukawa et al., 1998, 1999). At concentration of 25 $\mu\text{g/ml}$ or higher, HNP-1 can inhibit DNA synthesis and thus reduce the viability of tumorigenic and non-tumorigenic cell lines (Nishimura et al., 2004). Use of two cell lines which were derived from the same person can be seen as a limitation of this project. Nevertheless the selection criteria of these two cell lines were based on their characterization. However, we agree that inclusion of other cell lines would bring additional power to our study. It is true that in studies with enzyme activities, alpha-tubulin or actin can be used to confirm the identical load of proteins into each well. This procedure is commonly used in experiments where the cells are lysed to get intracellular proteins. In the present study, we referred to the number of cells because after we collected the media, cells were left alive. Since no difference was found in the number of viable cells at the time the experiment was stopped (as measured by cell proliferation) we did not use additional measures to confirm protein levels. Moreover, the present work stems from the inherent nature of *in vitro* experiments where the latter cease to simulate the physiological behavior of HNP-1 and its effect on the expression pattern of MMPs from cells in the intraoral environment. From a prospective aspect, it has been estimated that infection and inflammation are responsible for the occurrence of 15–20% of human tumors (Meurman, 2010). Since the production of HNP-1 is linked to the presence of oral infection, we urge the need of *in vivo* research to assess further the role of infectious microbes in the etiology of oral cancer.

To evaluate the behavior of cells against HNP-1, we ran two independent tests, cell viability and cell death. Cell death experiment is based on the level of LDH activity in the media. Cell proliferation, on the other hand, measures the number of viable cells at the time of assay. Cell viability, as measured by cell proliferation, did not demonstrate difference between groups, while cell death showed slight (10–15%) increases at different concentrations. One explanation of this finding can be attributed to increased metabolism of OSCC cells; HNP-1 at low levels may induce cell death and cell proliferation simultaneously, which ends up at same number of cells in each group. Previous studies have demonstrated that low concentrations of HNP-1 exert a proliferative effect, while high doses induce cell death (Han, Wang, Mohammed, & Su, 2009; McKeown et al., 2006). However, in the present work no significant effect of HNP-1 on cell proliferation was noticed. Several different mechanisms of HNP-1 induced cell death have been proposed in the literature, *i.e.* HNP1-3 may induce apoptosis in Jurkat cell through activation of caspases -3/-7 (Aarbiou et al., 2006) or cause a direct action on the mitochondrial membrane (Gera and Lichtenstein, 1991b).

MMPs are a group of zinc-dependent endopeptidases whose main function is to degrade certain extracellular proteins. Many studies have highlighted the importance of MMPs in the pathogenesis of oral and neck cancers (Pérez-Sayáns García

et al., 2012; Vilen, Salo, Sorsa, & Nyberg, 2013). Out of the 23 genetically distinct human MMPs, only MMP-2 and MMP-9 have been mainly implicated in oral carcinogenesis in terms of staging and metastasis (Katayama et al., 2004). To support the view of metastatic role of MMP-9 further, it has been reported that interleukin-4 (IL-4), through its anti-inflammatory action, inhibits cancer progression by downregulating MMP-9 (Tanaka and Tanaka, 2011). These two members of the MMP family, MMP-2 and -9, have been detected in sera and histological samples from patients with OSCC in higher quantities than in normal subjects (Lotfi et al., 2015; Monteiro-Amado et al., 2013). In the present study, the production of MMP-2 and MMP-9 was mainly reduced in both cell lines after being incubated with HNP-1. It has been previously demonstrated that HNP-1 increase the invasiveness of cancer cell lines, *in vitro* (Al-Rayahi and Sanyi, 2015), however, up to our knowledge this study is the first to demonstrate antigelatinolytic effects of HNP-1 on OSCC cell lines, so it was not possible for us to compare our results with the literature. The mechanism of induction of some MMP members by HNP-1 was tested on rheumatoid arthritis synovial fibroblasts by Ahn et al. (2013). They suggested that HNP-1 regulates ERK and JNK pathways culminating in the activation of the transcription factor NF- κ B, thereby leading to production of MMP-1 and -3 beside other proinflammatory cytokines, *e.g.*, IL-6 and IL-8. We believe that further studies are needed to describe the suppressive effect of HNP-1 on gelatinolytic activities of OSCC cell lines.

An initial hypothetical theme of the present results suggests that this selective inhibition may reflect a protective role of HNP-1 against the spread of metastatic cells, since the gelatinolytic action of these enzymes is needed in order to facilitate the movement of cancerous cells between normal cells and through the extracellular matrix (Kurahara et al., 1999).

On the contrary to other members of MMPs, the role of MMP-8 has been suggested to impede the progress of oral cancer, thus playing a protective role (Pradhan-Palikhe et al., 2010). We found that the expression of MMP-8 was reduced at a concentration (dose)-dependent manner, especially in the UT-SCC-43B cell line. The reduction of MMP-8 expression in UT-SCC-43B was at a broader range, *i.e.*, 200–250 and 250–300, compared with only 200–250 in UT-SCC-43A, possibly reflecting the recurrent nature of the former in which an antitumorigenic is inhibited. In marked contrast to its role in inhibiting the cancer invasion-associated enzymes, *i.e.*, MMP-2 and -9, HNP-1 seems to play a protumorigenic action by suppressing the release of MMP-8, which is known to be associated with less cancerous invasion and better survival (Pradhan-Palikhe et al., 2010).

In this regard, the actions of HNP-1 in oral carcinogenesis can be exemplified with other similar substances having dual opposite effects in the pathogenesis of oral cancer. Nitric oxide (NO) is one of such biological compounds; at a low concentration it can act as a protumorigenic agent whereas at a high concentration, it tends to demonstrate an anticancerous effect (Chinje and Stratford, 1997). Therefore, NO is considered to be a double-edge sword (Korde Choudhari, Sridharan, Gadbail, & Poornima, 2012). The same approach may hold true for HNP-1 in the sense that it can inhibit several pro- and antitumorigenic MMPs. In addition, our results suggest that oral inflammatory conditions, such as periodontitis, could modify oral cancer progression through an increased α -defensin-1 concentration.

To conclude, in *in vitro* conditions, HNP-1 tends to have an inhibitory effect on MMP-2, -8, and -9 secretions of OSCC cells.

Conflict of interest

None.

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