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RESEARCH ARTICLE

Role of HSP70 protein in human periodontal ligament cell function and physiology

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ABSTRACT

Objective: Heat pre-treatment of mechanically loaded human periodontal ligament cells (hPDL) dampens the inflammatory cellular response, as evidenced by a reduced expression of pro-inflammatory cytokines, inhibition of monocyte adhesion and osteoclastic differentiation. These findings imply heat shock proteins (HSP) as cell protective molecules acting in the PDL that are up-regulated upon ischemia caused by mechanical loading. HSP70 and its inhibition by VER155008 as the active agent in several pharmaceuticals are established targets and strategies, respectively, in the treatment of neoproliferative diseases. However, the effect of both players on periodontal remodeling in unknown. Therefore, we analyzed the role of HSP70 and its frequently used inhibitor VER155008 in the regulation of physiological hPDL cell functions and immune cell interaction.

Materials and Methods: Fifth passage hPDL cells were cultured in the presence of 25 μm HSP70 inactivating agent VER155008. At harvest, HSP70 expression, cell proliferation, and parameters of cell interaction, colony formation and wound healing were analyzed by means of real-time PCR, immunohistochemistry, Western blot, biochemical MTS assay, microscopy, and functional assays for monocyte adhesion and differentiation.

Results: Basal HSP70 expression and hPDL cell morphology were not affected by HSP70 inhibitor VER155008. In contrast, cell proliferation, tissue defect healing, and colony formation were reduced significantly following HSP70 inhibition, whereas apoptosis and necrosis, monocyte adhesion and osteoclastic differentiation were markedly increased.

Conclusions: The present data indicate a regulatory role for HSP70 protein in hPDL cell biology.

Clinical relevance: These findings identify HSP70 as a promising target in the attempt to modify periodontal remodeling and point to potential periodontal side effects of HSP70 pharmaceutical usage.

1. Introduction

Mechanical loading of the periodontal ligament (PDL) leads to ischemic conditions of the local microenvironment followed by a cascade of signaling events which eventually result in enhanced periodontal remodeling allowing for orthodontic tooth movement. Within this complex process, circulatory disturbances and cell stress evoke necrosis of a certain proportion of cells with subsequent initiation of a host immune response. This response is characterized by a chemoattraction of immune competent cells and their differentiation along the monocyte/macrophage lineage to clear the cellular debris and facilitate a structural reorganization of the periodontium in the first phase and tooth movement in a later stage (Jäger et al., 1993; Kim et al., 2010; Wolf et al., 2016). In case of an immune response going to excess, loss of alveolar bone height and tooth root resorption have been reported as adverse effects of orthodontic treatment (Yamaguchi et al., 2006; Koide et al., 2010). Recently, heat pre-treatment of human PDL cells (hPDL) was demonstrated to result in the retention of proinflammatory cytokines such as interleukin-6, high-mobility-group-box protein 1 and interleukin-8 and, thereby, limited the inflammatory response in vitro (Wolf et al., 2016). Those findings indicate a cytoprotective role for heat shock proteins in hPDL cells which has already been described comprehensively for other cell sys-

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tems and tissues (Collier and Schlesinger, 1986; Knowlton, 1995; Larson et al., 1995). Heat shock proteins (HSP) are released into the cytoplasm upon thermal stimulation, ischemic or hypoxic conditions and support protein folding and stabilization, facilitate protein transportation across cell membranes, and unfold denatured proteins and, thereby, contribute to cell survival under stress conditions (Wong et al., 1997a, 1997b). Despite of several reports in the literature addressing PDL cell response to various stimuli including mechanical loading (Kanzaki et al., 2006a; Kanzaki et al., 2006b; Nakao et al., 2007; Pinkerton et al., 2008), little information is available on the regulatory role of HSP in hPDL cells under pathological conditions and even less regarding their physiological function. Arai and co-workers reported on elevated HSP1A1 expression, which is the encoding gene for HSP70, in the pressure zone of experimental tooth movement and concluded on the activation of an intracellular defense system to secure cell survival (Arai et al., 2010). Kwon et al. proved the involvement of HSP27 in the mediation of transforming growth factor-β1 induced migration of hPDL cells and, thereby, identified HSP27 as a potential target for the regeneration process related to cell migration (Kwon et al., 2011). In another study, the temporal expression of HSP25 in regenerating nerve fibers and denervated Schwann cells was observed in the periodontal ligament following transection of the inferior alveolar nerve (Iijima et al., 2003). Yoshimatsu et al. reported on an enhanced expression of HSP47, which is actively involved in the regulation of collagen I synthesis, in the tension zone during orthodontic tooth movement (Yoshimatsu et al., 2008). Finally, a differential expression pattern of HSPs has been described in different endodontic periapical lesions (Goodman et al., 2014).

However, information on the function of HSP in hPDL cell physiology, also in conditions requiring its inhibition for medical reasons, is still missing. Therefore, in our study, we focused on HSP70 since this protein is well-characterized in various cell systems (Knowlton, 1995; Larson et al., 1995) and has already been attributed important regulatory roles in PDL cells (Wolf et al., 2016). HSP70 is a member of the actin class of ATPase proteins. HSP70 possesses a specific ATP binding domain and its function is dependent on energy release by cleavage of adenosine triphosphate (ATP). We, therefore, conducted specific inhibitor experiments to throw light on the role of HSP70 in the regulation of physiological cell functions in PDL remodeling. The HSP70 inhibitor VER155008 blocks the ATP binding site and, therefore, acts as an ATP-competitive inhibitor that prevents allosteric control between the nucleotide binding domain (NBD) and the substrate binding domain (SBD) by binding to the NBD of HSP70 and arresting it in a half-open conformation (Schlecht et al., 2013).

We hypothesized that an inhibition of HSP70 by HSP70 inhibitor VER155008 would result in a modification of important hPDL functional parameters including cell proliferation and viability via changes of cell necrosis and apoptosis. To gain further knowledge of potential periodontal side effects when HSP70 targeting pharmaceutics are used hPDL cell interaction and monocyte physiology was addressed by analyzing colony formation, defect healing, monocyte adhesion and osteoclastic differentiation.

2. Materials and methods

2.1. PDL cell culture and HSP70 inhibitory treatment

Fifth passage human PDL cells (Lonza, Verviers, Belgium) were seeded in duplicate into 24-well plates (n = 6) in a density of 10000 cells/well and cultured to confluence prior to further experimental stimulation. Cells were cultured in DMEM containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000 U/ml penicillin and 5000 U/ml streptomycin; Biochrom AG, Germany) at 37 °C in an atmosphere of 100% humidity, 95% air, and 5% CO_2 (Lossdörfer et al., 2011; Wolf et al., 2014a; Wolf et al., 2014b). Prior to experimental use, cells were characterized for their mesenchymal origin as described previously (Lossdörfer et al., 2011; Wolf et al., 2014a; Wolf et al., 2014b). To investigate the effect of HSP70 protein and its inhibition HSP70 inhibitor and pharmaceutically used agent VER155008 was applied according to the protocol of Wen and co-workers using a working concentration of 25 μm (Wen et al., 2014).

2.2. Gene expression experiments and real-time PCR

To determine the effect of HSP70 protein and its inhibition on HSP70 mRNA expression and on proliferative marker genes with an expected peak occurring earlier at the transcriptional level than at the functional level, fifth passage hPDL cells were seeded in a density of 30000 cells/well in 6-well plates using the protocol mentioned above. At subconfluence, hPDL cells were exposed to HSP70 inhibitor for 24 h (Wen et al., 2014). Vehicle-treated cultures (DMSO) served as controls. At harvest, the expression of Ki-67 as a candidate gene associated with proliferation were analyzed by real-time PCR as described previously [25]. The primer sequences used were as follows: Ki-67 sense 5′-AAA-TTC-AGA-CTC-CAT-GTG-CCT-GAG-3′, antisense 5′-TCA-AAT-ACTT-CAC-TGT-CCC-TAT-GAC′ (Winter et al., 2012). The house keeping gene β-actin served as an endogenous reference (sense 5′-CAT-GGA-TGA-TGA-TAT-CGC-CGC-G-3′, antisense 5′-ACA-TGA-TCT-GGG-TCA-TCT-TCT-CG-3′) (Kraus et al., 2012).

2.3. Proliferation assay

The importance of HSP70 function and its inhibition by HSP70 inhibitor treatment on hPDL cell proliferation was examined by means of MTS assay according to the manufacturer's instructions. Briefly, hPDL cells were seeded into 96-well plates in a density of 1500 cells/well in the presence of HSP70 inhibitor for 3 d. Vehicle-treated cultures (DMSO) served as controls. Thereafter, the reagents for MTS (Promega GmbH, Mannheim, Germany) were added and cells were incubated at 37 °C for another 2 h prior to a reading of colorimetric changes using a photometer at 490 nm and 450 nm with a reference wavelength of 690 nm (Wolf et al., 2016).

2.4. Colony forming assay

To analyze possible changes in colony forming activity, hPDL cells were seeded on 6-well plates at a density of 1500 cells/well and stimulated with HSP70 inhibitor for 5 w. Vehicle-treated cultures (DMSO) served as controls. Formed colonies were stained with crystal violet (Sigma Aldrich, Schnelldorf, Germany). To quantify the size of the colonies at the bottom of the wells, three images of each well were captured at a magnification of ×100 and the colony formation areas were quantified as a function of total area.

2.5. Wound healing assay

To study wound healing, a well-established in vitro model was a used (Clericuzio et al., 2014; Zhan et al., 2014). Confluent monolayer hPDL cells in 6-well plates were linearly scratched in the midline from 12 to 6 o'clock with the tip of a sterile 1000 μl pipette resulting in a wound width of 813.15 μm ± 173.23 μm. Cells were gently rinsed with PBS to remove any remaining cells debris. Thereafter, HSP70 inhibitor was added for 7 d. Vehicle-treated cultures (DMSO) served as controls. On each day of culture, three images per well were captured at ×100 magnification, and the width of

the scratched cleft was quantified as a function of total width on day 0.

2.6. Cell viability

PDL cells and supernatants from non-treated/HSP70 inhibitor-treated cultures were harvested for analysis of apoptosis and necrosis by means of cell death detection ELISA (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) according to the manufacturer's instructions. With this photometric immunoassay, the amount of mono- and oligonucleosomes, i.e. histone-associated DNA fragments was detected qualitatively and quantitatively. The abundance of nucleosomes in the supernatant originating from ruptured cells from osmotic lysis determines the rate of necrosis, while the rate of apoptosis was determined by nucleosomes in the cytoplasm.

2.7. THP1 cell adhesion assay

To examine the influence of HSP70 inhibition on hPDL cell induced monocyte adhesion, CFSE labelled THP1 cells (DSMZ GmbH, Germany) were cultured in 24-well plates in the presence of the conditioned medium collected from hPDL cells pre-treated with HSP70 inhibitor for 24 h. After 4 h of incubation, non-adherent THP1 cells were removed. Adherent THP1 cells were documented photographically using a fluorescence microscope (Zeiss, Jena, Germany) and quantified using the cell counter freeware ImageJ (National Institute of Health, Bethesda, MD, USA) (Winning et al., 2010). Vehicle-treated cultures (DMSO) served as controls.

2.8. Osteoclastic differentiation assay

To analyse the effect of HSP70 inhibition on PDL cell ability to induce osteoclastic differentiation, mononuclear cells of the monocyte and macrophage lineage (RAW264.7 cells; CLS cell line services, Eppelheim, Germany) were pre-differentiated along the osteoclastic pathway in the presence of 30 ng/ml RANKL (Axxora, Lörrach, Germany) and 20 ng/ml M-CSF (BioCat, Heidelberg, Germany) and shown to form multinuclear osteoclastic cells after five days in culture staining positively for tartrate-resistent acid phosphatase (TRAP) (Lossdörfer et al., 2011). Thereafter, such pre-differentiated cells were seeded on cover slips in 24-well-plates at a seeding density of 1000 cells/well and cultured in the conditioned medium of HSP70 inhibitor pre-treated PDL cells supplemented with 15 ng/ml RANKL and 10 ng/ml M-CSF for 12 d. Medium was changed every three days. At harvest, TRAP staining was performed to visualize osteoclast differentiation. Vehicle-treated cultures (DMSO) served as controls. To quantify the number of TRAP-positive multinucleated cells, cover slips were photographed microscopically and analyzed by the help of Axiovision software (Zeiss, Jena, Germany). Four images were captured per specimen at x100 magnification and the TRAP-positive area was quantified as a function of total area.

2.9. Statistical analysis

All data were analysed by Student's t test. P values <0.05 were considered to be significant. The data are representative of two replicate experiments, which both yielded similar results.

3. Results

3.1. Effect on hPDL cell proliferation

To analyze the specific role of HSP70 in hPDL physiology, the possible impact on functional parameters was addressed. The addition of HSP70 inhibitory reagent VER155008 to the cultures for 24 h was used to analyze the regulatory role of HSP70 on hPDL cell proliferation. HSP70 inactivation led to a significant reduction of proliferation marker Ki-67 gene expression (~50%; Fig. 1A) as well as of a reduced proliferation activity in the functional MTS assay (~55%; Fig. 1B).

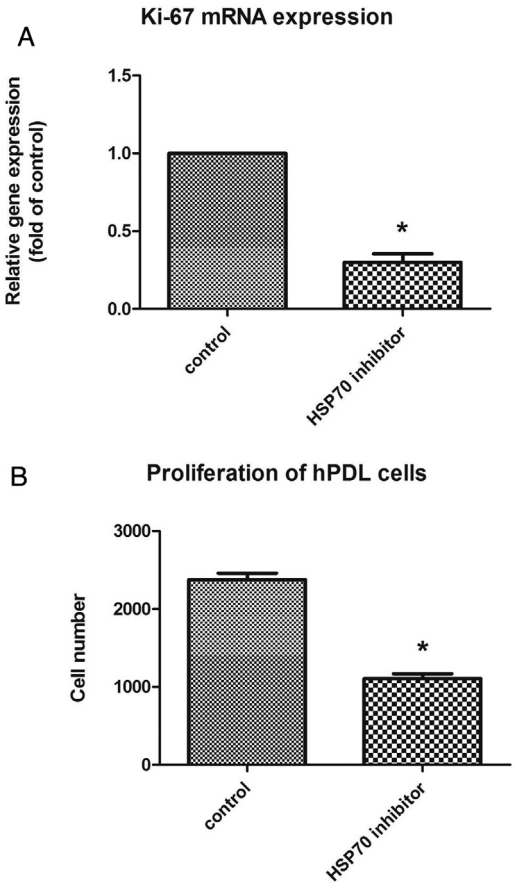


Fig. 1. Role for HSP70 in the regulation of hPDL cell proliferation. Following culture of the cells in the presence of HSP70 inhibitor, real-time PCR for Ki-67 (A) and MTS assay (B) both revealed a significant reduction in proliferative activity as compared to the untreated control. Each value represents the mean ± SD for 6 independent experiments. *P < 0.05, experimental group vs. vehicle-treated control.

3.2. Regulatory effect on colony formation

To gain further information on the capability of HSP70 to organize cell interconnection as an important factor for tissue formation, a colony formation assay was carried out. Following the addition of the HSP70 inhibitor to the culture medium, colony formation was visibly inhibited (Fig. 2A). Compared to untreated control cell cultures, tissue formation and interconnection of hPDL cells was significantly reduced by ~78% when HSP70 inhibition treatment was applied (Fig. 2B).

3.3. Impact on tissue regeneration

To address the physiological relevance of cell interconnection and tissue formation, a tissue defect regeneration assay was performed. Investigating the process of tissue repair, confluent interconnected hPDL cell cultures were scratched and then cultured for another 7 days in the presence or absence of HSP70 inhibitor VER155008. As a result of HSP70 inactivation, hPDL "tissue regeneration" was slowed down significantly. In contrast to

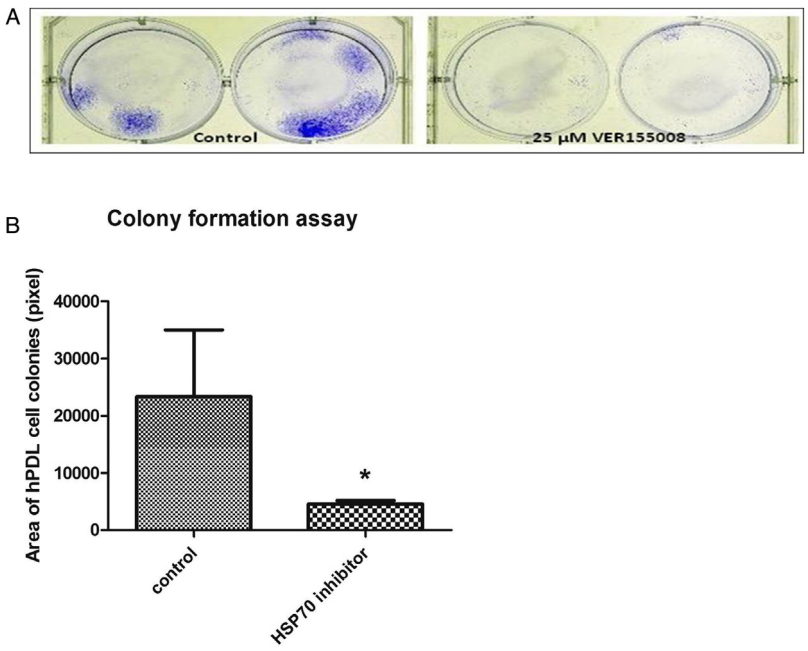


Fig. 2. Influence of HSP70 activity on hPDL cell interconnectivity. Inactivation of HSP70 protein in cell cultures resulted in a significantly reduced colony forming activity over 5 d compared to control cultures as visualized by crystal violet staining of the cultures (A) and quantification of the staining results (B). Each value represents the mean ± SD for 6 independent experiments. *P < 0.05, experimental group vs. vehicle-treated control;.

control cultures, in which wound healing was completed within 3d, inhibitor treated hPDL cells needed 5 d on average (Fig. 3).

3.4. Influence on hPDL cell necrosis and apoptosis

Examining the effect of HSP70 on hPDL cell viability, enhanced cell necrosis was noticed when the HSP70 inhibitor was applied. Compared to untreated hPDL cells, necrosis was increased approximately 2.76-fold when HSP70 inhibitor was added (Fig. 4). Likewise hPDL cell necrosis, mechanisms inducing programmed cell death were also upregulated in the course of HSP70 inactivation as evidenced by a significant increase in hPDL cell apoptosis in the presence of HSP70 inhibition treatment (3.71-fold) (Fig. 5).

3.5. Role for HSP70 and it's inhibition treatment on the regulation of monocyte adhesion

In the attempt to analyze the effect of HSP70 inhibition pre-treatment on hPDL cell and osteoclastic precursor cell interaction, a monocyte adhesion assay was performed. Compared to untreated controls, monocyte adhesion was enhanced by factor ~1.25 (Figs. 6A, B) when hPDL cells received HSP70 inhibition treatment initially. The difference between both groups reached the level of significance.

3.6. Effect of HSP70 and it's inhibition treatment on osteoclastic differentiation

The conditioned medium from hPDL cell cultures induced the differentiation of mononuclear RAW264.7 cells along the osteoclastic line on a basal level. A significant increase in TRAP positive cell formation was noted when monocytes were cultured in the presence of conditioned medium of hPDL cells which received HSP70 inhibition treatment (Figs. 7A, B).

4. Discussion

The present study investigated the role of HSP70 in the regulation of crucial cell functions under physiological, unstimulated conditions using VER155008 as a competitive inhibitor of HSP70 ATPase activity. Data revealed a cell protective effect of the protein. In the presence of HSP70 inhibitor treatment, important hPDL physiological factors such as proliferation, colony formation and wound healing were significantly reduced, whereas necrosis and programmed cell death markedly increased. Likewise, monocyte adhesion and osteoclastic differentiation were enhanced.

It has to be acknowledged that VER155008 inhibits HSP70 but also glucose-related protein 78, another chaperone of the HSP family involved in the process of protein folding and glucose metabolism (Macias et al., 2011; Biswas et al., 2014). Thus, the observed effects might result from the inhibition of targets other than HSP70.

The agent used to down regulate HSP70 protein to analyse its function in hPDL cells is well-established not only in HSP70 basic research, but also frequently used as an active agent in pharmaceuticals for HSP70 inhibition treatment of neurodegenerative and neoproliferative diseases (Jiang et al., 2013). As demonstrated by real-time PCR and Western blot data HSP70 inhibition by VER155008 has a specific interaction with the ATPase binding domain of HSP70, thus inhibiting its activity (Wen et al., 2014).

Proliferation of hPDL cells markedly decreased in the presence of the HSP70 inhibitor in our study and this observation is also supported by observations by Wen *et al.* in non-small-cell lung carcinoma cells (NSCLC). These authors even provide an explanatory mechanism of action. Performing cell cycle analysis, they found that the HSP70 inhibitor VER155008 dramatically inhibited cell cycle progression with treatment. Loss of HSP70 activity induced an increase of the number of NSCLC cells in G0/G1, whereas the percentage of cells in the S phase decreased significantly. In support of these results, markers of cell cycle progression including cyclin A, extracellular-signal regulated kinase, and phosphorylated Akt were also reduced in the presence of the inhibitor (Wen et al., 2014). From these findings, a pro-proliferative effect of HSP70 can

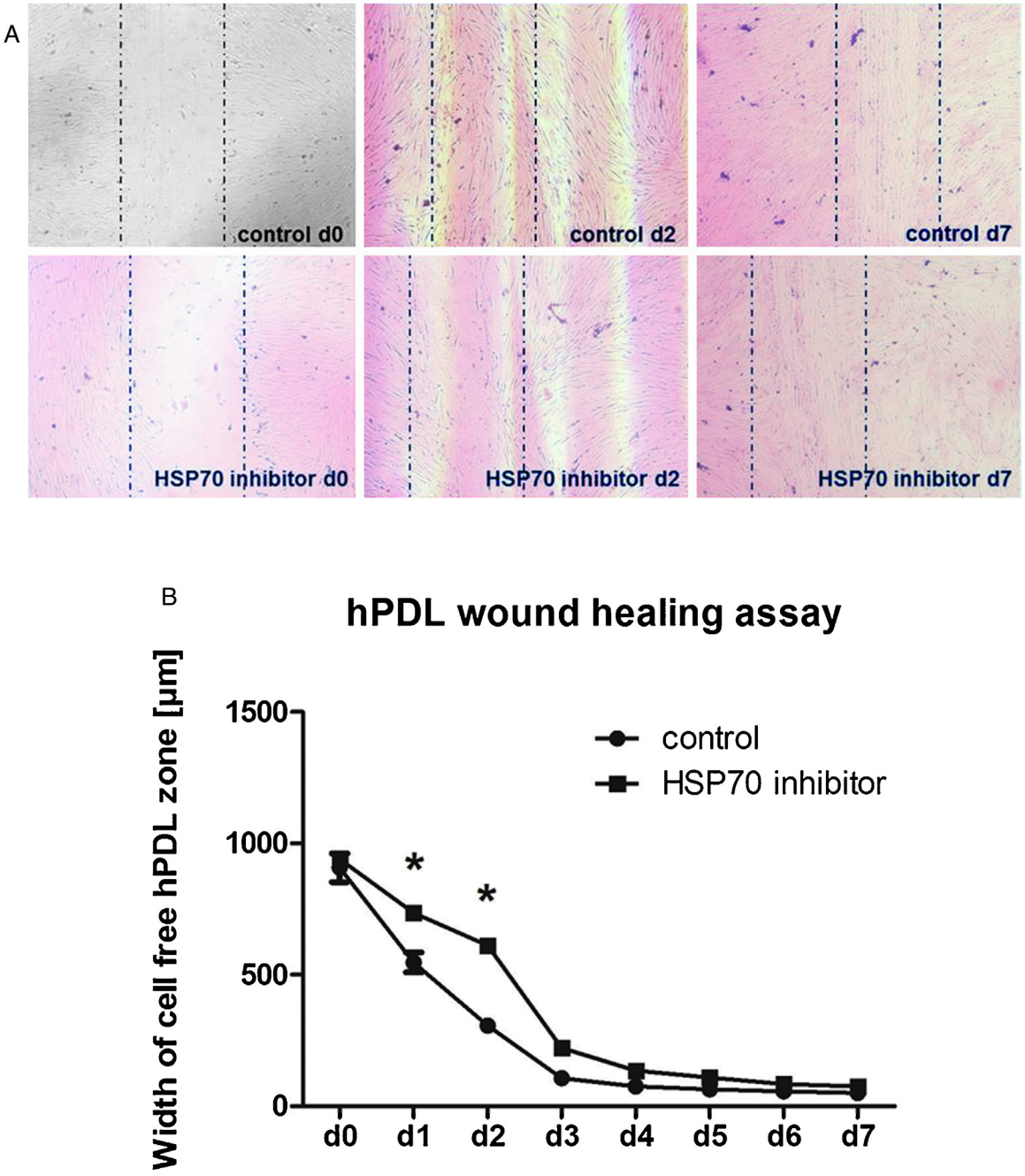


Fig. 3. HSP70 regulates defect regeneration in hPDL cells. The cell free zone (area between the dashed lines) generated by scratching the wells gradually filled until wound healing was completed and this process was slowed down significantly during the first three days in the absence of active HSP70. Thereafter, from day 4, the wound fill rate in both groups was comparable (A: immunohistochemical staining; magnification ×100; B: quantitative analysis). Each value represents the mean ± SEM for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

be presumed not only for malignantly transformed cells, but also for normal cells underlining the importance of hPDL cells in periodontal tissue remodelling and regeneration.

In the light of the findings for proliferation, the results of the colony formation and regeneration experiments seem reasonable, since HSP70 inhibition reduced proliferation significantly and, thereby, prolonged the period needed for the cells to establish cell-to-cell contacts. Once those contacts were established, the artificially induced gap filled quickly until complete closure of the wound was reached within a few days only. These findings underline the importance of cellular interconnectivity and communication and of a possible role for HSP70 for a prompt reestablishment of the periodontal architecture in case of physiological remodeling and of injury. The results of the colony forming assay are confirmed by the results of Massey et al. obtained in colon carcinoma cells (Massey et al., 2010). Interestingly, in the cited experiments, a minimum of 40 µm VER155008 or greater was necessary for a robust reduction of colony formation in cancer cells, whereas a concentration of 25 µm was sufficient for significant inhibition in hPDL cells, suggesting a higher sensitivity of non-transformed cells to HSP70 inhibition. An additional explanation might be the commonly reported overexpression of HSP correlated with a wide range of tumors (Jaattela, 1995; Vargas-Roig et al., 1997; Ravagnan et al., 2001) requiring higher concentra-

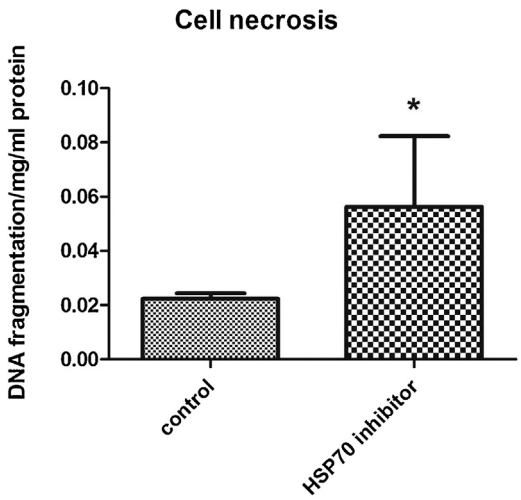


Fig. 4. Inhibitory effect of HSP70 on hPDL cell necrosis. The abundance of nucleosomes in the supernatant originating from ruptured cells from osmotic lysis determined the rate of necrosis. As depicted, necrosis was enhanced ~2.76-fold when cells were exposed to HSP70 inhibitor. Each value represents the mean ± SD for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

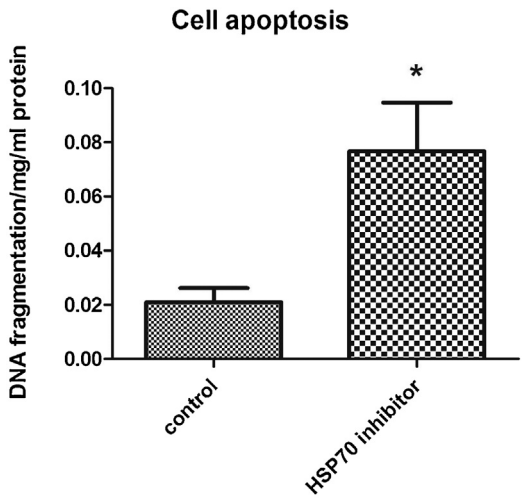
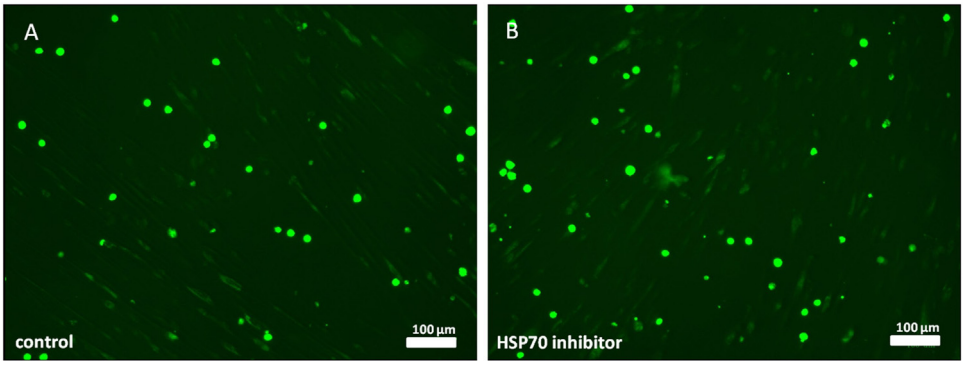


Fig. 5. Inhibition of HSP70 enhances programmed cell death of hPDL cells. As determined by photometric assay, HSP70 inactivation resulted in a significant increase in hPDL cell apoptosis (~3.71-fold). Each value represents the mean ± SD for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

tions of the inhibitor for successful inhibition. The data of our regeneration assay are further supported by those from Boroughs et al. who also reported on attenuated defect closure upon stimulation of cells with VER155008 (Boroughs et al., 2011). In the light of the present data and its reports in literature, a significant role of HSP70 biology and in regenerative capacity can be assumed.



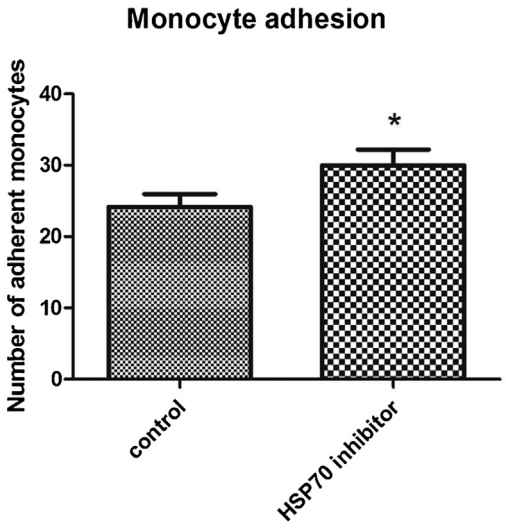


Fig. 6. Influence of HSP70 inhibitory treatment on monocyte adhesion. Monocyte adhesion increased significantly, when cells were cultured in the presence of the conditioned medium collected from hPDL cells that had been exposed to HSP70 inhibitor as compared to those exposed to the supernatant of untreated hPDL control cultures. The visual impression (A) was confirmed by quantitative analysis (B). Each value represents the mean ± SD for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

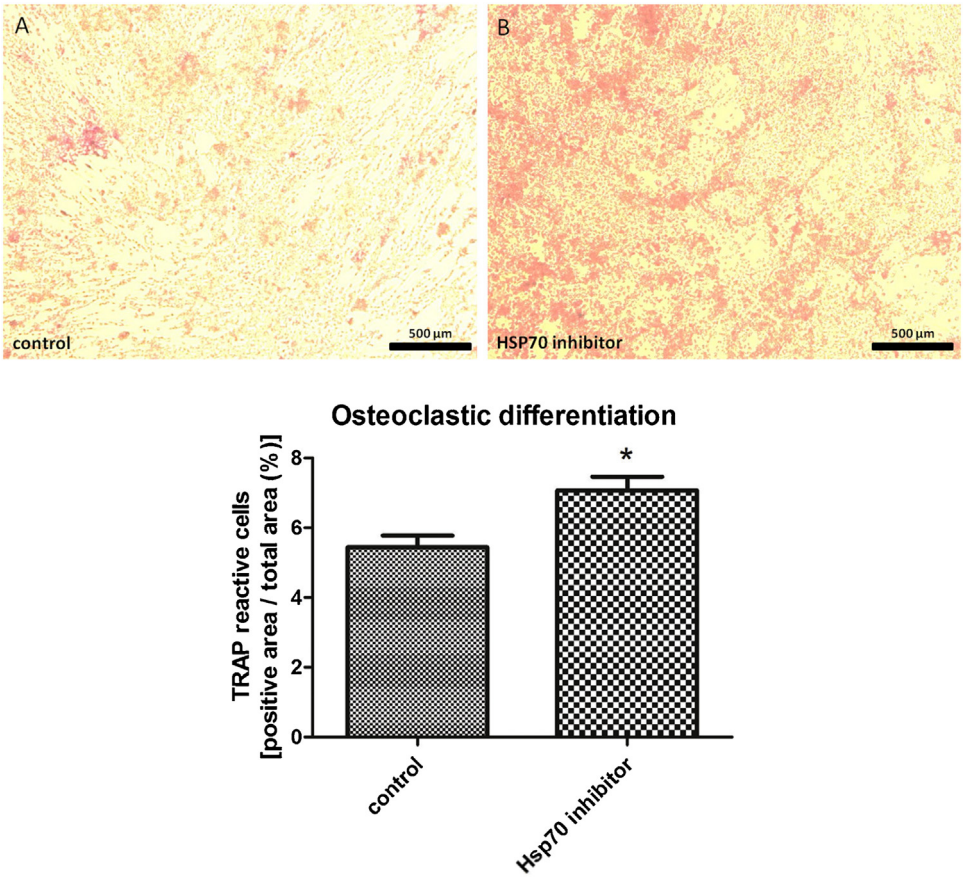


Fig. 7. HSP70 inhibition resulted in enhanced osteoclastic differentiation.Likewise monocyte adhesion, osteoclastic differentiation was enhanced when precursors where challenged with the conditioned medium of HSP 70 inhibitor pre-treated hPDL cells. The visual impression (A) was confirmed by quantitative analysis (B). Each value represents the mean ± SD for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

Previous reports indicate that HSP70 inhibition using the pharmaceutical agent VER155008 induces apoptosis in several cell line including HCT116 cells (Massey et al., 2010), myeloma cells (Chatterjee et al., 2013), and lung cancer cells (Nylandsted et al., 2000), while having minimal effects on non-cancer cells. Furthermore, recent studies demonstrate that selective depletion of HSP70 induced cell death in lung cancer cells (Nylandsted et al., 2000), but not in normal lung cells (Frese et al., 2003). Strategies to increase the radiosensitivity of tumor cells include the selective inhibition of cytoprotective proteins including HSP70. Apart from that, HSP70 inactivation has been shown to enhance the sensitivity of tumor cells to apoptosis induced by certain anticancer drugs (Fani et al., 2016; Schilling et al., 2017). Here, we demonstrate a pro-apoptotic as well as a necrosis inducing effect of HSP70 inhibition in normal hPDL cells supporting its potential harming effect the periodontal system in patients receiving HSP70 targeting pharmaceutics.

Summarizing the cited reports on the effect of HSP70 and its inhibition, it becomes apparent that the described effects were mostly observed in tumor cell lines while the same treatment regimen had only minimal or even no effect in peritumorous healthy tissue or normal cells in vitro. Here, we describe a hPDL cell response to HSP70 inhibition that demonstrates that besides tumour cells also the non-tumours transformed periodontal system shows an increased sensitivity to HSP70 inhibitory treatment regimens and establishes HSP70 protein as an important factor to maintain hPDL cell physiology. This might represent a special characteristic of this cell type that warrants further exploration in future experiments.

In summary, the present results indicate a regulatory role of HSP70 in the maintenance of periodontal tissue homeostasis and control of PDL physiology. The present findings provide further evidence that HSP70 might play a crucial role in periodontal remodeling and identify this chaperone as a promising target for future intervention strategies to influence this process. Such intervention strategies might comprise the therapy of inflammatory periodontal disease or help minimize side effects of orthodontic tooth movement.

Furthermore, the present data indicate the risk for unwanted side effects within the periodontal system when systemic pharmaceutical interventions addressing HSP70 are carried out.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aanat.2018.09.006.

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