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Chapter 4.
The effect of bisphosphonates on human periodontal-ligament-fibroblast-mediated osteoclastogenesis

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Manuscript in preparation

Abstract

Bisphosphonates (BPs) such as pamidronate are used to treat bone destructive diseases and act by inhibiting bone resorption by osteoclasts, thereby improving bone quality. In contrast, BPs were shown to stimulate osteoclast formation and resorption at the mouse molar root. Periodontal ligament (PDL) fibroblasts could be seen as the mediators of osteoclast formation at this location. Here, we investigated whether BP exposure of PDL fibroblasts isolated from molar roots alters osteoclast formation. Human PDL fibroblasts were isolated from healthy donors, and subjected to pamidronate (PAM; 1-100 µM) *in vitro* for 24 hours, after which cell viability was measured. PAM was removed, and freshly isolated peripheral blood mononuclear cells (PBMCs) were added to the PAM-treated or vehicle-treated fibroblasts. Osteoclasts were counted after 21 days of co-culture, and osteoclast-related gene expression was measured. Cell viability was not affected after 24 hours of treatment with any concentration of PAM. However, in the long term, transient exposure to 100 µM PAM was toxic to PDL fibroblasts. Osteoclast formation was induced by the vehicle-treated PDL fibroblasts, and this was not affected by pre-treatment with 1 or 10 µM PAM. Also, gene expression of the osteoclast marker tartrate-resistant acid phosphatase was unaffected by pre-treatment with PAM. Yet, 100 µM completely blocked osteoclast formation, probably due to the absence of PDL fibroblasts. Moreover, fewer PBMCs survived in this condition than in a mono-culture of PBMCs. In conclusion, BPs inhibited periodontal-ligament-fibroblast-mediated osteoclast formation, probably due to a toxic effect on PDL fibroblasts, however, there was no effect on osteoclastogenesis with a non-toxic BP concentration.

Introduction

Bone remodeling, a life-long process of bone renewal, is performed by bone-resorbing osteoclasts that decalcify and degrade bone matrix, and bone forming osteoblasts. The coupling between bone resorption and bone formation was shown to play an important role in maintaining bone homeostasis (reviewed in Sims and Martin 2014 [1]). Osteoblasts can signal and stimulate osteoclastogenesis by expressing M-CSF and RANKL [2,3]. Periodontal ligament fibroblasts were also shown to stimulate osteoclastogenesis *in vitro* [4,5].

When the balance between bone resorption and bone formation is disturbed and directed more towards bone resorption, such as in osteoporosis and bone metastasis, bisphosphonates (BPs) can be used as a treatment. BPs induce osteoclast apoptosis and inhibit bone resorption. The signaling towards osteoblasts however, is likely lost due to BP activity, and therefore long-term BP treatment results in reduced bone formation along with inhibition of resorption [6]. BPs were also shown to have a direct inhibiting effect on osteoblast viability and differentiation [7,8] and the viability of periodontal ligament cells [9,10]. Anti-apoptotic effects on osteocytes and osteoblast with low concentrations of BPs were also reported [11,12].

Recently, we have shown that BP administration can cause osteoclast formation at the molar root in mice (manuscript submitted). Surprisingly, and in contrast to the generally accepted mode of action of bisphosphonates, actively resorbing osteoclasts were observed at the molar roots, exclusively in the bisphosphonate-treated animals. We hypothesize that the periodontal ligament (PDL) may play a role in stimulating osteoclast formation at this site. The major cell type in the periodontal ligament, the PDL fibroblasts, were shown to stimulate osteoclastogenesis *in vitro* [4,5]. In direct contact with osteoclast precursors, PDL fibroblasts produced osteoclast stimulating factors such as RANKL and M-CSF [13].

In the current study, we investigated whether BP exposure to human PDL fibroblasts affects PDL-fibroblast-mediated osteoclastogenesis *in vitro*. PDL fibroblasts were pre-treated with pamidronate for 24 hours, rinsed with PBS, and human peripheral blood mononuclear cells (PBMCs) were seeded on top of the fibroblasts. The effect of BP treatment on markers of osteoclastogenesis and on osteoclast formation was studied. With this study, we aim to get more insight into the effect of bisphosphonates on PDL fibroblasts and their ability to induce osteoclastogenesis.

Materials and methods

Isolation of PDL fibroblasts and cell culture

Experiments with human PDL fibroblasts were approved by the Medical Ethical Committee of the VU University and informed consents from healthy donors undergoing third molar extractions were obtained. Cells were isolated and cultured as described previously with minor modifications in the culture conditions [4,13]. Briefly, fourth to sixth passage PDL fibroblasts ($1.5x10^4$ viable cells) were seeded in 48-well plates and allowed to attach for 24 h. Medium was replaced and pamidronate (PAM; Sigma-Aldrich, St. Louis, MO, USA) or vehicle was added in several concentrations. Another 24 h later, PAM was removed, cells were rinsed with PBS and freshly isolated PBMCs ($5x10^5$) were added on top. For mono-cultures without PBMCs, cells were also washed with PBS and complete medium (DMEM; GIBCO, Paisley, UK, containing 10% FCS; Hyclone, Logan, UT, USA, and 1% antibiotic antimycotic solution; Sigma, St. Louis, MO, USA) was added. Medium was replaced twice a week and micrographs were taken weekly. At 21 days, cells were fixed with 4% phosphate-buffered formaldehyde and stored at 4°C for TRACP staining, or lysed with RLT lysis buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol and stored at -80°C until RNA isolation.

Cell viability and CyQuant Assay

Directly after PAM treatment, PDL fibroblasts were detached from the plastic by a mild trypsin treatment, were counted and viability was determined using a Count and Viability Assay which is based on membrane permeability, and a Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany). Cells were also lysed and DNA content was measured using the CyQUANT Cell Proliferation Assay according to manufacturer's instructions (Invitrogen, Molecular Probes Carlsbad, CA, USA).

RNA isolation and real time quantitative PCR

RNA from co-cultures and PDL fibroblast mono-cultures was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Subsequently, cDNA was synthesized using the MBI Fermentas cDNA synthesis kit (Fermentas, Vilnius, Lithuania). Real time qPCR was performed on an ABI Prism 7000 using SYBR Green mastermix (Applied Biosystems, Foster City, CA, USA) as described previously [14]. Gene expression was normalized for the housekeeping gene β2-microglobulin and primers had the following sequences (5'→3'): β2-microglobulin forward (FW): AAgATTCAggTTTACTCACgTC, β2-microglobulin reverse (RV): TgATgCTgCTTACATgTCTCg; Ki67 FW:

CgAgACgCCTggTTACTATCAA, Ki67 RV: ggATACggATgTCACATTCAATACC; TRACP FW: CACAATCTgCAgTACCTgCAAgAT, TRACP RV: CCCATAgTggAAgCgCAgATA

TRACP staining

Tartrate-resistant acid phosphate (TRACP) staining was performed with the leukocyte acid phosphatase kit (Sigma-Aldrich) and nuclei were visualized with 4'6-diamidino-2-phenylindole dihydrochloride (DAPI). Five micrographs per well were taken in standardized regions, using a 10x magnification with an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). The number of TRACP-positive, multinucleated (≥3 nuclei) cells was assessed in those 5 fields.

Statistical analyses

Gene expression data were analyzed using a Repeated Measures ANOVA, followed by Dunnett's Multiple Comparison tests. All other data were analyzed with a Friedman test, followed by Dunn's Multiple Comparison to compare BP-treated samples with the controls. Graphpad Prism 5 Software was used (GraphPad Software, Inc., La Jolla, CA, USA) and graphs show the mean and standard deviations of 3-4 healthy donors.

Results

PAM does not affect PDL fibroblast viability in a short term

To investigate the effect of BP treatment on PDL fibroblast-mediated osteoclast formation, we pre-treated PDL fibroblasts for 24 hours with the bisphosphonate PAM. After 24 hours of pre-treatment, the viability of those cells was not affected by PAM (Figure 1A), neither were the DNA (Figure 1B) and RNA concentration (Figure 1C). Also, gene expression of the proliferation marker Ki67 was not affected by 24 hours of PAM treatment (Figure 1D). At this time point, PAM was washed away with PBS after which PBMCs were added. These data indicate that at the time PBMCs were added, the viability of PDL fibroblasts is not affected by the PAM treatment.

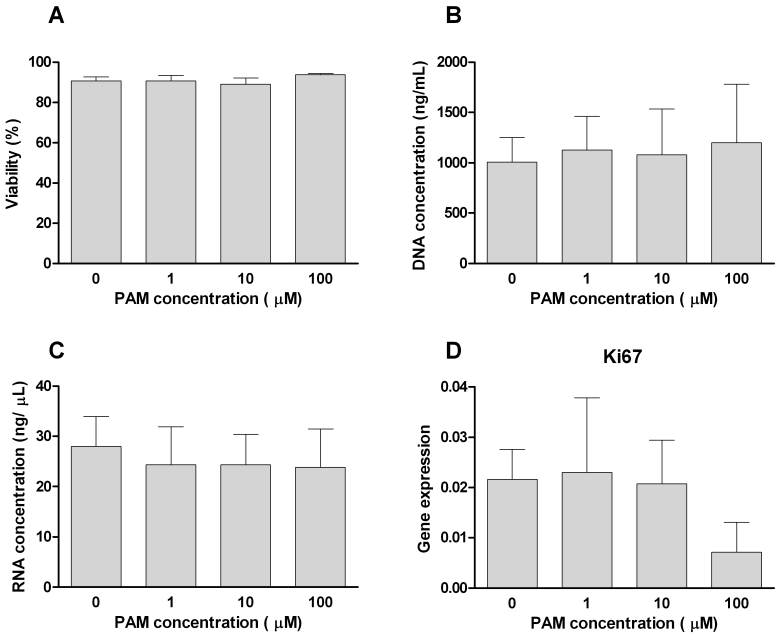

Figure 1. PDL fibroblast viability was unaffected after 24 h of PAM. (A) The viability of PDL fibroblast was unaffected by 24 h of PAM. (B) The DNA and (C) RNA content were also unaffected after 24 h. (D) Gene expression of the proliferation marker Ki67 was not significantly affected by 100 μM PAM. One representative experiment of 2 is shown (A-C), the mean and standard deviations of n=4 donors are shown (A-D).

PAM reduces the number of PDL fibroblasts in the long term

After 24 hours of treatment with 100 µM PAM, no striking differences were observed compared to control cultures (Figure 2A,B). After 7 days of co-culture with PBMCs, no PDL fibroblasts could be identified anymore after the pre-treatment with 100 µM PAM (Figure 2D). After 21 days of culture, all the PDL fibroblasts, as well as the PBMCs had died (Figure 2E,F). This indicates that a short-term treatment with PAM reduces the number of PDL fibroblasts in the long term. With lower concentrations of PAM, no effect on the number fibroblasts was noted (not shown).

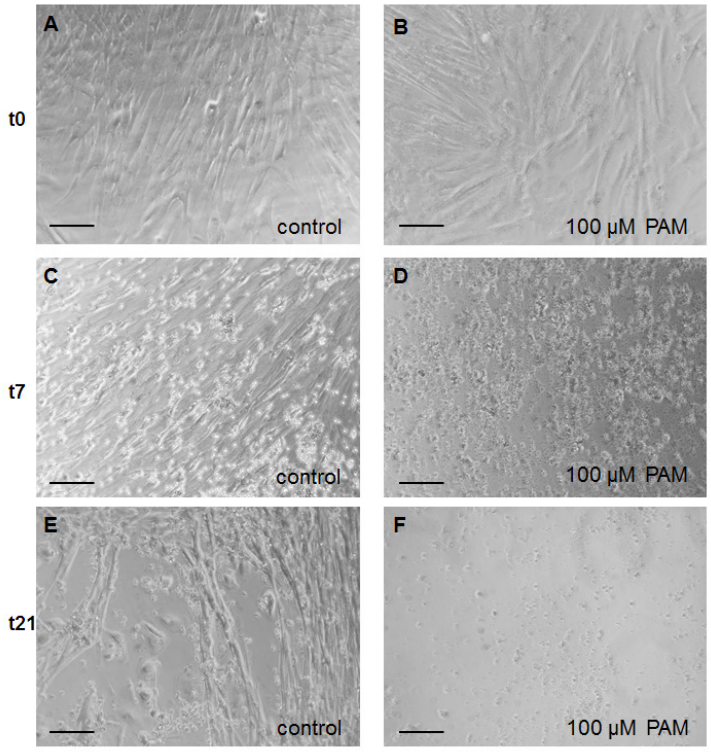

Figure 2. Micrographs of PDL fibroblasts (t0) and co-cultures of fibroblasts with PBMCs (t7,t21). (A-B) Directly after PAM treatment, PDL fibroblasts were not strikingly different from the controls. (C-D) After 7 days of culture in the absence of PAM, pre-treated PDL fibroblasts were almost absent from cultures. PBMCs were clearly visible at this time point. (E,F) PBMCs were also inhibited after a longer culture period. Bars: 100 µm.

Pre-treatment of PDL fibroblasts with 100 µM PAM inhibits osteoclast formation in co-culture

PDL fibroblasts were treated for 24 hours with PAM, rinsed with PBS, and PBMCs were added. After 21 days, the number of osteoclasts (TRACP-positive cells with 3 or more nuclei, Figure 3A) was counted. Low concentrations of 1 and 10 µM PAM did not affect PDL fibroblast-mediated osteoclastogenesis (Figure 3B). Yet, a pre-treatment of the fibroblasts with 100 µM PAM completely blocked osteoclastogenesis (Figure 3B). Likely, this was related to the PDL fibroblasts disappearing under the influence of PAM (Figure 2). The 10-fold lower amount of RNA ($p<0.01$) isolated from these cell is further indicative that most cells had disappeared after treatment with 100 µM PAM. The cells that were still present in the culture, did express a similar level of TRACP mRNA as controls, indicating that the few remaining cells were most likely PBMCs directed towards the osteoclast lineage.

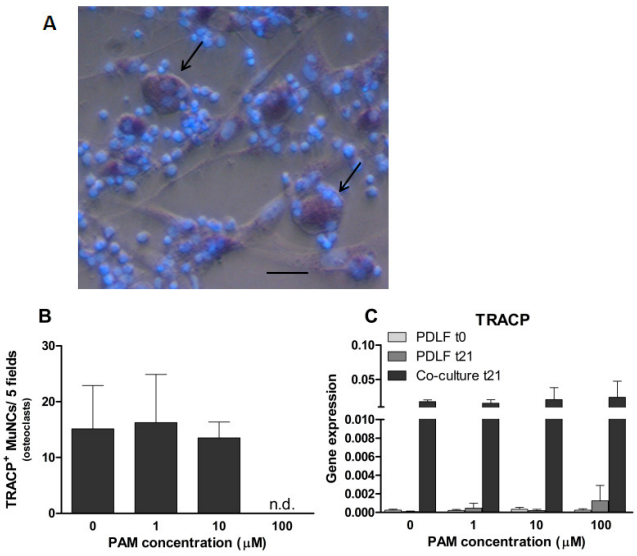

Figure 3. Osteoclast formation was completely abolished in co-culture with 100 µM PAM pre-treated PDL fibroblasts. (A) Osteoclasts (arrows) formed from PBMCs in co-culture with PDL-fibroblasts, bar: 50 µm. (B) Pre-treatment of PDL fibroblasts with 1 and 10 µM PAM did not affect their osteoclast-inducing potential, however, 100 µM completely abolished osteoclast formation (n.d.: not detectable). (C) TRACP expression by mono-cultures and co-cultures was not affected by PAM pre-treatment of PDL fibroblasts. The mean and standard deviations of n=4 donors are shown.

Apart from the absence of multinucleated osteoclasts (Figure 3A), PDL fibroblasts caused a dramatic decrease in the number of TRACP-positive mononuclear cells when 100 µM PAM was added in pre-culture (Figure 4A). In this condition, fewer cells were present after 21 days of culture than in mono-cultures of PBMCs (Figure 4B). These data suggest that PDL fibroblasts that are pre-treated with BPs can be lethal to PBMCs.

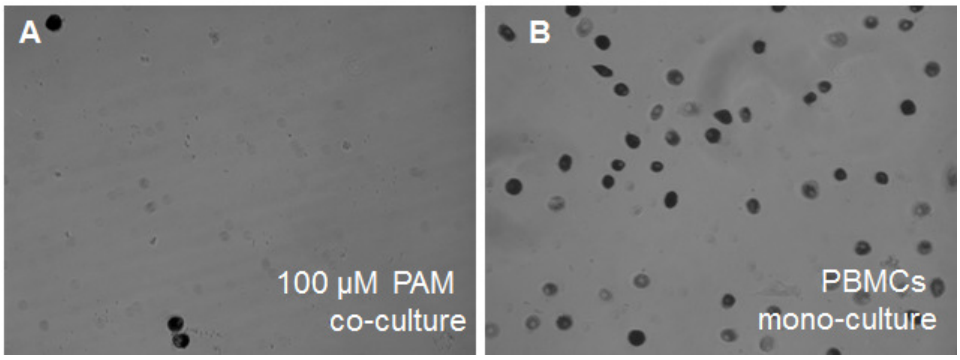

Figure 4. PAM pre-treatment of PDL fibroblasts is toxic to PBMCs. (A) Co-culture of PBMCs with pre-treated PDL fibroblasts, where PBMCs have almost completely died. (B) Mono-culture of PBMCs where more cells have survived. Bars: 100 µm

Discussion

Human periodontal ligament fibroblasts are capable of inducing osteoclastogenesis from human PBMCs *in vitro* [4,5,13]. We investigated whether this process was affected by bisphosphonates. Treatment with PAM for 24 hours did not affect the viability of PDL fibroblasts. Similar results were found in a previous study, where 30 and 100 μM zoledronic acid were not toxic to PDL fibroblast after 24 hours, whereas they were toxic after 48 hours [9]. Agis and coworkers showed that transient exposure to ZOL for only 1 hour resulted in toxicity after 48 hours. This is in line with our findings, where PAM was not toxic after 24 hours, whereas at later time points and after removal of PAM, the majority of the PDL fibroblasts had died. These data indicate that although PAM did not affect cell viability after 24 hours of treatment, the PDL fibroblasts are affected later on. Possibly, we were not able to detect decreased cell viability at 24 hours yet, since the viability test we used is based on membrane permeability, which is a late marker of cell death [15]. Also, we found that proliferation as measured by Ki67 gene expression was slightly reduced by 24 hours of treatment with 100 μM PAM.

So far, no reports have described the effect of BPs on PDL-fibroblast-mediated osteoclastogenesis. Osteoblasts from human bone biopsies were shown to secrete more OPG [16,17], and express less RANKL after BP treatment [16]. Yet, it is not known whether these changes affect osteoclastogenesis. Here, we showed that PDL fibroblast death after 1 day of 100 μM PAM resulted in a completely abolished osteoclastogenesis, whereas lower concentrations did not affect PDL fibroblasts or their effect on osteoclast formation. These data may indicate that after treatment of PDL fibroblasts with 100 μM PAM, osteoclastogenesis did not occur due to the absence of PDL fibroblasts. There was no additional effect of BPs on osteoclastogenesis through PDL fibroblasts.

Based on our previous study we questioned whether osteoclast formation at the murine molar root induced by BPs is regulated by the adjacent periodontal ligament. The data provided in the current study may indicate that BPs do not affect PDL-fibroblast-induced osteoclast formation *in vitro*. BP treatment, however, increases bone volume *in vivo*, thereby likely increasing the compressive loading on the PDL. Therefore it would be of interest to study the effect of BPs on the osteoclast-stimulating potential of PDL fibroblasts under compressive loading [18].

Interestingly, after BPs were washed away, the fibroblasts treated with 100 μM PAM were toxic to PBMCs. Perhaps, this was the result of BP that was released from dying fibroblasts, making the BP available for uptake by PBMCs. Another explanation could be that BP-treated and dying PDL fibroblasts express or release factors that are toxic to PBMCs. The

mechanism behind this, and the possible effect of lower BP concentrations on PDL-mediated toxicity to PBMCs requires further investigation.

Taken together, we demonstrated that a high concentration of BPs was toxic to PDL fibroblasts in a long term, leading to completely abolished osteoclast formation and toxicity to precursors. Non-toxic concentrations of BPs to PDL fibroblasts did not affect osteoclast formation induced by those cells.

Acknowledgments

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