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ARTICLES

Adenoviral Down-Regulation of Osteopontin Inhibits Human Osteoclast Differentiation In Vitro

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Abstract Although osteopontin (OPN) is highly expressed in osteoclasts, OPN-deficient mice have a near-normal bone phenotype and its role in osteoclast differentiation and function remains uncertain. We used an adenoviral OPN-antisense vector (AdOPN-AS) to down-regulate OPN expression in a human in vitro osteoclastogenesis model employing CFU-GM precursors treated with RANKL and M-CSF. Cultures infected with AdOPN-AS showed reduced secretion of OPN compared to cultures infected with a control adenoviral vector expressing β-galactosidase. Infection with AdOPN-AS co-incident with exposure to RANKL was associated with substantial (approximately 50%) inhibition of osteoclast formation with a concomitant reduction in dentine resorption. There was also a small reduction in the size of generated osteoclasts but no significant effect on the size of resorption pits/tracks nor on the amount of resorption per osteoclast. When the cultures were infected with AdOPN-AS after 4 days exposure to RANKL only minor effects on osteoclastogenesis were seen. Our data demonstrate that early down-regulation of OPN in vitro inhibits human osteoclastogenesis. Since mice totally lacking OPN do not have reduced osteoclast numbers our results imply the existence in vivo of an alternative molecular pathway(s). J. Cell. Biochem. 93: 896–903, 2004.

Key words: osteopontin; osteoclast differentiation; adenoviral gene transfer

Osteopontin (OPN)is a non-collagenous secreted phosphorylated glycoprotein containing an RGD sequence [Oldberg et al., 1986]. It was first described in bone, where it is one of the most abundant non-collagenous proteins [Oldberg et al., 1986] and is expressed by both osteoclasts [Reinholt et al., 1990; Ikeda et al., 1992; Tezuka et al., 1992] and osteoblasts [Moore et al., 1991; Prince et al., 1991]. It is also expressed by a variety of inflammatory cells, including macrophages [Denhardt et al., 2001; Gravallese, 2003]. Expression of OPN in the early stages of differentiation of both osteoclasts and osteoblasts has been demonstrated by in situ hybridization [Yamate et al., 1997]. The RGD sequence of OPN has been shown to be important for binding to integrins, especially the $\alpha v\beta_3$ integrin expressed by osteoclasts [Ross et al., 1993]. Blocking the binding of $\alpha_v\beta_3$ using peptidomimetic antagonists inhibits osteoclastic bone resorption [Engleman et al., 1997; Carron et al., 2000] and chemotactic migration of precursors [Nakamura et al., 1999]. The RGD site has been implicated in cell migration [Xuan et al., 1995] and attachment of osteoclasts to the bone surface [Flores et al., 1992; Ross et al., 1993; Xuan et al., 1995]. The binding of OPN to the $\alpha v\beta_3$ integrin has been shown to stimulate phosphatidylinositol 3-hydroxy kinase activity [Hruska et al., 1995] which has been linked to the formation of podosomes in osteoclasts that are important in cell motility and bone resorption [Chellaiah et al., 2003]. The binding of OPN to its receptor CD44 is RGD-independent [Weber et al., 1996; Katagiri et al., 1999] and antibodies to CD44 inhibit osteoclast formation and fusion of precursors [Kania et al., 1997] although do not affect the ability of mature osteoclasts to resorb

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bone. Intracellular association of OPN with CD44 has been implicated in both osteoclast formation and resorption [Kania et al., 1997].

Inhibition of OPN using antibody or antisense oligonucleotide has been shown to inhibit osteoclast formation in vitro. In mouse bone marrow cultures treated with 1,25 dihydroxyvitamin D_3 for 7 days, Yamate et al. [1997] demonstrated substantial inhibition of osteoclast-like cell formation with anti-OPN antibody, provided that the antibody was added early in the culture period. They also showed inhibition with an RGD peptide in vitro and that estrogen deficiency increased OPN expression in vivo. Tani-Ishii et al. [1997], found that treatment of co-cultures of mouse bone marrow cells and stromal cells with an OPN antisense oligonucleotide inhibited the number of TRAP+ cells generated by approximately 50%, with an increase in the proportion of mononuclear cells, but decreased resorption by only 20%.

Mice deficient in OPN have normal embryonic development [Liaw et al., 1998], and in an earlier study were reported to have normal bones [Rittling et al., 1998]. However, a recent study [Chellaiah et al., 2003] reported an abnormal bone phenotype in OPN-deficient mice with delayed bone resorption in metaphyseal trabeculae, reduced eroded perimeters, and increased bone rigidity. These investigators also reported increased osteoclast numbers in vivo and that osteoclasts generated in vitro were smaller, hypomobile, and produced smaller resorption pits. Yoshitake et al. [1999] also found increased numbers of osteoclasts in the long bones of OPN-deficient mice, but their numbers did not increase after ovarectomy, as found in wild type mice. In contrast, Ihara et al. [2001] found that the number of osteoclasts was similar in untreated bones of wild type and OPN-deficient mice. However, PTH and RANKL did not stimulate osteoclast numbers and Ca^{++} release from cultured bones of OPN-deficient mice, as seen in the wild type. Asou et al. [2001] found that resorption was reduced by 80% and osteoclast numbers by 50% when bone discs from OPN-deficient mice were implanted into the muscle of OPN-deficient mice, which was rescued by exogenous OPN. Bone discs from normal mice were resorbed at an intermediate level when implanted into OPD-deficient mice. Hemopoietic precursors from OPD-deficient mice have been reported to generate normal [Ihara et al., 2001] and increased [Rittling et al., 1998] numbers of tartrate-resistent acid phosphatase positive (TRAP+) multinucleate cells in vitro, but neither of these studies assessed the bone resorption capacity of the cells.

In summary, the available data indicate that, in the basal state in vivo, OPN-deficiency in mice is associated with increased or normal numbers of osteoclasts despite the presence of a subtle osteopetrotic phenotype, indicating that the osteoclasts have mildly impaired resorptive ability. However, OPN-deficient mice exhibit markedly impaired osteoclastic response to acute resorptive stimuli, indicating an impairment in acute osteoclastogenesis. Why the chronic in vivo defect appears to be a mild impairment in resorptive ability yet acutely there is a marked impairment of osteoclastogenesis has not been explained. Also unexplained is why some in vitro studies using precursors from OPN-deficient mice showed normal or increased TRAP+ multinucleate cell generation, yet OPN antisense is inhibitory.

The effect of OPN-deficiency in a human model has never been reported and there has been no systematic evaluation of the relative effects of OPN-deficiency on the in vitro differentiation of osteoclasts versus their capacity to resorb. In a human osteoclastogenesis model employing CFU-GM precursors, we used an OPN antisense adenoviral vector (AdOPN-AS) to down-regulate OPN expression and to determine the effects of this on osteoclast generation and resorption capacity. When precursors were treated with RANKL for 4 days prior to infection with AdOPN-AS, osteoclast formation was only mildly affected. However, when precursors were infected with AdOPN-AS simultaneously with RANKL treatment, osteoclast formation and resorption were inhibited by approximately 50%. Although the generated osteoclast were slightly smaller, down-regulation of OPN did not significantly reduce the resorption rate of generated osteoclasts, nor the size of resorption pits/tracks. We conclude that the predominate effect of OPN deficiency on in vitro human osteoclastogenesis is to impair early differentiation events.

MATERIALS AND METHODS

Osteoclastogenesis Cultures

Human umbilical cord blood was obtained from healthy donors under a protocol approved

by Barwon Health Research and Ethics Advisory Committee. The isolation of mononuclear cells from cord blood, preparation of CFU-GM colonies, generation of osteoclasts from CFU-GM, and the quantification of formation and resorption has been previously described [Hodge et al., 2004]. Recombinant human M-CSF (hM-CSF) was generously provided by Genetics Institute. Soluble human RANKL coupled to GST fusion protein (sRANKL) was generously provided by Dr. Matthew Gillespie and Dr. Julian Quinn, St. Vincent's Institute of Medical Research, Melbourne, Australia. All osteoclastogenesis cultures were treated with hM-CSF at 25 ng/ml and sRANKL 62.5 ng/ml.

Adenoviral Gene Transfer

The recombinant, replication deficient adenovirus vectors were constructed using the Adeno-X^{TM} Expression System (Clontech, Palo Alto, CA). The LacZ adenovirus expressing *E. coli* β-galactosidase (AdLacZ) was used to optimize infection conditions and as the normal control. The OPN antisense adenovirus (AdOPN-AS) was constructed by cloning a 300 bp antisense region of OPN that incorporated the ATG start codon at 109 bp in the 5′-coding region of human OPN mRNA (Accession no. J04765.1). Recombinant virus were produced in the human embryonic kidney 293 cell line and purified according to instructions in the Adeno-X^{TM} Expression System Users manual (Clontech). Viral stock was diluted with α-MEM and added directly to the cultures. Cultures were infected with a multiplicity of infection (MOI) of 100 unless otherwise specified. To quantify the efficacy of infection with AdLacZ, the cultures were fixed in 0.05% glutaraldehyde and half of the dentine slices reacted for β-galactosidase (X-gal, Promega, Madison, WI) to produce a blue product, or for tartrate-resistant acid phosphatase (TRAP, Sigma, St. Louis, MO) to produce a red product. Efficacy was expressed as the ratio β-galactosidase-positive/TRAP-positive multinucleated cells. The diameter of resorption pits and width of resorption tracks were quantified using MICD software (Imaging Research, Inc., Ontario, Canada).

Measurement of OPN Secretion

To quantify the effect of RANKL-induced osteoclastic differentiation on expression of OPN protein, media from cultures of CFU-GM treated with rhM-CSF alone or rhM-CSF plus sRANKL were collected at days 4, 7, and 11 of culture and replaced with fresh media. To determine the effect of AdOPN-AS infection on OPN expression, cultures of CFU-GM treated with rhM-CSF and sRANKL were infected with AdLacZ or AdOPN-AS (100 MOI) on day 4, the media changed at day 7, and the replacement media collected at day 11. Secreted OPN was measured in these media using a human OPN Elisa kit (Array Designs, Ann Arbour, MI).

RESULTS

Increased OPN Secretion During Early Osteoclast Differentiation

OPN concentrations were significantly higher in the media from osteoclastogenic CFU-GM cultures (i.e., hM-CSF and sRANKL-treated) compared to macrophage cultures (i.e., hM-CSF-treated) at 4 days (+44%) and 8 days (+57%), but there was no significant difference at day 11 (Table I). Activated macrophages are known to secrete OPN [Denhardt et al., 2001; Gravallese, 2003] and under the in vitro culture conditions existing in our experiments, mature macrophages and osteoclasts appear to secrete similar amounts of OPN.

Characterization of AdLacZ Infection of CFU-GM Cultures

Individual osteoclasts infected with AdLacZ were readily identified using β-galactosidase cytochemistry (Fig. 1A, left panel) and efficacy of infection could be estimated by relating the number of osteoclasts expressing LacZ to the total number expresssing TRAP (Fig. 1A, right panel). The efficacy of infection with AdLacZ increased with increasing dose of adenoviral infection (MOI) (Fig. 1B) and also with the maturity of the cultures (Fig. 1C). Immature osteoclast precursors were not efficiently

TABLE I. Osteopontin Concentrations in Media of CFU-GM Cultures Treated With hM-CSF Alone or hM-CSF Plus sRANKL

	Osteopontin concentration (μg/ml)	
Day	hM-CSF alone	hM-CSF plus sRANKL
4	6.1 ± 0.46	8.8 ± 0.57*
8	4.4 ± 0.16	6.9 ± 0.54*
11	6.5 ± 0.18	6.8 ± 1.46

Results expressed as mean ± SEM, n = 5.
*Significantly greater than hM-CSF alone, 2 sample *t*-test, $P < 0.01$.

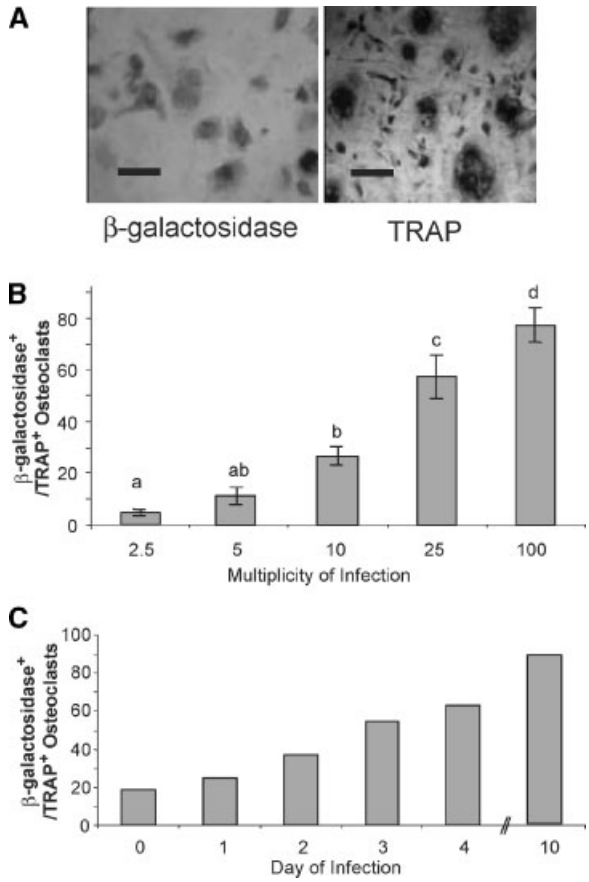

Fig. 1. Infection of CFU-GM cultures with AdLacZ. CFU-GM cultured in 96-well plates with $4 \times 4 \times 0.1$ mm sperm whale dentine slices were treated with hM-CSF and sRANKL and infected with AdLacZ at the MOI or times indicated. At day 14 the cells were reacted for either β-galactosidase or TRAP. **A**: Photomicrograph showing β-galactosidase-positive and TRAP-positive osteoclasts on dentine slices. Bar = 100 μm. **B**: Cultures were infected with 2.5–100 MOI AdLacZ at day 4. Results expressed as mean ± SEM of three separate experiments, n = 8 dentine slices per group in each experiment. Groups with different annotations are significantly different, $P < 0.001$, one way ANOVA, Fishers multiple comparison test. **C**: Cultures were infected with 100 MOI AdLacZ at the days indicated. Representative experiment, n = 8 dentine slices per group.

infected with AdLacZ but 65–75% of osteoclasts expressed β-galactasidase when infected at 4 days with 100 MOI.

Effect of AdOPN-AS Infection in CFU-GM Cultures Pre-Exposed to RANKL

As infection rates were low before 4 days of culture, in initial experiments we infected cultures that had been treated with sRANKL and hM-CSF for 4 days. Compared to the AdLacZ control, infection with AdOPN-AS at day 4 resulted in a modest (approximately 44%), although significant ($P < 0.001$), reduction in secretion of OPN protein into the media at day 11 (AdLacZ 4.24 ± 0.58 μg/ml versus AdOPN-AS 2.39 ± 0.12 μg/ml, n = 3 experiments, one way ANOVA, Fishers multiple comparison test).

When CFU-GM cultures treated with sRANKL and hM-CSF were infected with adenoviral constructs at day 4 (Fig. 2), AdOPN-AS infection did not have any significant effect on osteoclast formation, dentine resorption, osteoclast size (mean plan area) or the resorption/osteoclast ratio at 14 days of culture. However, statistically significant inhibition of resorption was seen at days 6 and 10 and reduction in the resorption/osteoclast ratio was seen at 10 days (2 sample *t*-test with Bonferoni correction).

Effect of AdOPN-AS Infection in CFU-GM Cultures not Pre-Exposed to RANKL

In view of the possibility that down-regulation of OPN expression after 4 days exposure to sRANKL is too late in the differentiation process to substantially modulate osteoclastogenesis, we next investigated the effect of infecting the cultures simultaneously with sRANKL treatment. Since infection efficiency is poor in immature cells (Fig. 1) and it is known that M-CSF induces the expression of the adenovirus receptor, $\alpha_v\beta_3$ integrin [Cappellen et al., 2002], we elected to infect CFU-GM precursors that had been treated with hM-CSF alone for 4 days. Under these conditions, AdOPN-AS infection resulted in significant inhibition (approximately 50%) of osteoclast number (Fig. 3A) and dentine resorption (Fig. 3B). The size (mean plan area) of the osteoclasts formed was approximately 25% smaller in the AdOPN-AS group (Fig. 3C). The ratio of resorption to osteoclast number was also approximately 25% lower in the AdOPN-AS group, but this was not a significant difference. To determine whether down-regulation of OPN affects the size of resorption areas, we measured the diameter of individual resorption pits and the width of resorption tracks on dentine slices randomly selected from AdLacZ and AdOPN-AS cultures and found no difference (Table II and Fig. 4).

DISCUSSION

The use of adenovirus gene transfer to modulate osteoclast function was first used by

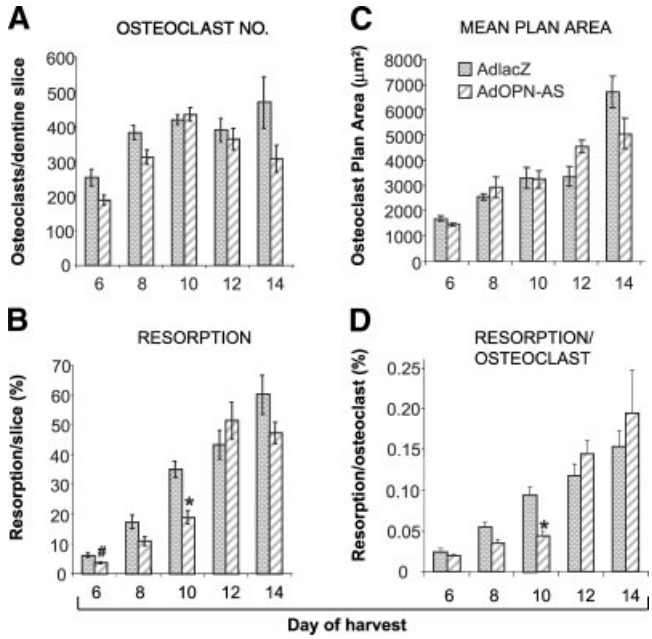

Fig. 2. Time course of the effect of AdOPN-AS infection in CFU-GM osteoclastogenesis cultures pre-exposed to sRANKL for 4 days. CFU-GM cultures treated with hM-CSF and sRANKL from day 0 were infected at day 4 with AdLacZ or AdOPN-AS. At the times shown, the dentine slices were processed for TRAP reaction to assess the number of osteoclasts (**A**. $TRAP^+$ cells with 2 or more nuclei) and their mean plan areas (**C**). The cells were then removed and slices sputter-coated with gold to assess percent dentine resorption (**B**). Quantification of these parameters was by transmission light microscopy and MCID software. Resorption per osteoclast (**D**) was calculated using the data of A and B. Results are expressed as mean ± SEM, n = 4–8 dentine slices per group. #, $P = 0.008$; *, $P = 0.000$; 2-sample *t*-test with Bonferoni correction for multiple comparisons (significance set at $P < 0.01$).

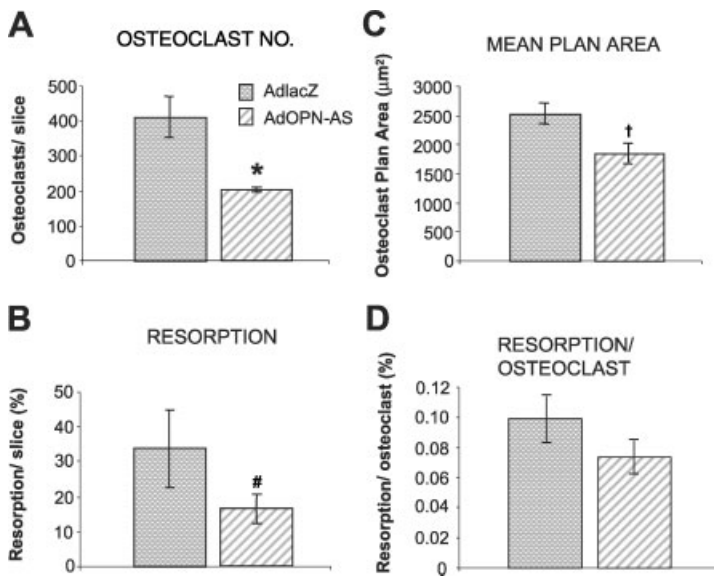

Fig. 3. Effect of AdOPN-AS infection in CFU-GM osteoclastogenesis cultures not pre-exposed to sRANKL. CFU-GM cultures were treated with hM-CSF from day 0. At day 4 co-treatment with sRANKL was commenced and the cultures were infected with AdLacZ or AdOPN-AS. At day 14 the parameters (**A–D**) were quantified as described in Figure 2. Results are pooled data from 3 separate experiments expressed as mean ± SEM, n = 6 dentine slices per group per experiment. *, $P = 0.000$; #, $P = 0.003$; †, $P = 0.012$; one-way ANOVA.

TABLE II. Effect of Down-Regulation of OPN in Human Osteoclasts Using AdOPN-AS on the Size of Resorption Pits and Tracks

	n	AdLacZ	AdOPN-AS	Significance
Pit diameter (μm)	100	31.1 ± 1.2	28.5 ± 1.1	$P = 0.122$
Track width (μm)	50	35.4 ± 1.8	35.0 ± 1.6	$P = 0.882$

Resorption pits and tracks were randomly selected on dentine slices from cultures infected with AdLacZ or AdOPN-AS. Diameters and widths were quantified using MCID software. Results expressed as mean ± SEM.

Tanaka et al. [1998] who over-expressed epidermal growth factor receptor in mature osteoclasts derived from human giant cell tumors and mouse bone marrow. Duong et al. [2001] transferred a protein-tyrosine kinase 2 (PYK2) antisense sequence into mouse osteoclast-like cells and showed that this reduced expression of PYK2 protein and altered the function of the cells. Adenoviral gene transfer has been successful in hemopoietic progenitor cells including $CD34^+$ cells derived from human bone marrow [Watanabe et al., 1996] and umbilical cord blood [Chatterjee et al., 1999].

In this study, we have shown that non-replicating adenovirus can be used to transfer constructs for β-galactasidase and osteopontin-antisense into human osteoclast precursors. The proportion of cells expressing β-galactasidase increased progressively when the cultures were infected at later time-points, presumably due to increasing expression of the adenovirus receptor $\alpha_v\beta_3$ integrin. Infection with AdOPN-AS inhibited osteoclast differentiation when infection occurred coincidentally with exposure of precursor cells to the critical osteoclast differentiation cytokine, sRANKL, but was much less effective if the cultures were previously exposed to sRANKL for 4 days. This result suggests that increased OPN expression is important in the early phase of osteoclast differentiation. Relevant to this, we found that although OPN secretion was increased in osteoclastogenic (i.e., sRANKL-treated) cultures during the first 8 days, macrophage and osteoclast cultures showed similar secretion after this time. Although down-regulation of OPN inhibited osteoclast formation, the ratio of resorption and osteoclast number was not significantly reduced, indicating that this does not substantially effect the ability of formed osteoclasts to resorb substrate, despite their slightly smaller size.

A potential weakness in this study is that OPN expression in the cultures was only reduced by about half and it is possible that a greater reduction in OPN may have resulted in significant inhibition of osteoclast resorption rate. Moreover, although we did not quantify the proportion of cells that were infected with AdOPN-AS, the results from the AdLacZ experiments suggest that only about 60% of the cells are likely to be infected and thus have down-regulated OPN expression. Furthermore, the dentine slices and FBS used in our experiments contain OPN and it is likely that contaminating macrophages secrete it. Therefore, the situation is significantly different to experiments with OPN-deficient mice when all cells would have absent OPN expression. In our experiments, some cells would have normal OPN expression and should differentiate normally. In this event, a bimodal distribution of osteoclast size might be expected but we did not observe this. However, OPN secreted by non-infected cells could have paracrine effects by "rescue" of AdOPN-AS-infected cells, analogous to the rescue seen with exogenous OPN addition [Asou et al., 2001; Chellaiah and Hruska, 2003]. Moreover, our finding that the osteoclasts formed in the AdOPN-AS-infected cultures and exhibited a similar resorption rate may be due to them not being infected with the adenovirus construct, or due to normalization of their function by extracellular OPN.

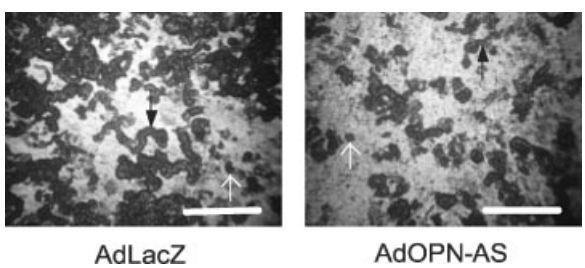

Fig. 4. Effect of AdOPN-AS infection on dentine resorption. Typical resorption pits indicated by white arrows and resorption tracks by black arrows. Bar = 200 μM.

In conclusion, we have shown that early down-regulation of OPN in human precursor cells substantially inhibits osteoclast differentiation in vitro, but appears to have little effect on their resorbing capacity. Our data suggest that up-regulation of OPN is an important component of the early differentiation events mediated by RANKL in human osteoclastogenesis. However, since OPN-deficient mice do not have reduced osteoclast numbers and have only a subtle increase in bone mass, alternative pathways must exist that allow osteoclastogenesis to proceed in vivo. Whether OPN-deficiency in man produces osteopetrosis is not known.

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