Published in final edited form as:

Matrix Biol. 2008 October ; 27(8): 682–692. doi:10.1016/j.matbio.2008.07.005.

MMP-1 (Collagenase-1) and MMP-13 (Collagenase-3) Differentially Regulate Markers of Osteoblastic Differentiation in Osteogenic Cells

Takayuki Hayami, Yvonne L. Kapila, and Sunil Kapila

The University of Michigan, 1011 North University Avenue, Ann Arbor, Michigan, 48109

Abstract

Previous studies have demonstrated an inverse relationship between constitutive or stimulated collagenase expression and osteoblastic phenotype of osteogenic cells. However, the direct effects of cell-secreted collagenases on osteoblastic differentiation, and the precise contributions of the key collagenolytic MMPs, MMP-1 and -13 to the modulation of specific osteoblastic markers have not been elucidated. Early passage osteogenic human periodontal ligament (PDL) cells were exposed to exogenous collagenase-1 in the presence and absence of dexamethasone. Alternatively, endogenous collagenases were modulated by transfecting the cells with cDNA or siRNA to MMP-1 and/or -13. Specific osteoblastic markers and collagenase expression and activity were then assayed. Increasing concentrations of exogenous collagenase or endogenous MMP-1 and MMP-13 produced a dose-dependent decrease in AP activity. Conversely, a dose-dependent increase in AP activity was observed with increasing concentrations of MMP-1 or MMP-13 siRNA. Overexpression of MMP-1 resulted in a significant decrease in Runx2, osteonectin (ON), osteopontin (OP), bone sialoprotein (BSP) and osteocalcin (OC), but an increase in osterix (Osx) mRNA levels. In contrast, knockdown of MMP-1 caused a significant increase in Runx2, ON, OP, BSP and OC levels and a decrease in Osx levels. MMP-13 overexpression resulted in diminished levels of Osx, OP and BSP, while its knockdown caused a significant increase in Osx and OP levels and a significant decrease in ON levels. The accretion of matrix molecules including collagen I(α 1) in cell-matrix extracts paralleled the changes in their respective mRNAs. Simultaneous suppression of both MMP-1 and MMP-13 resulted in significant increases in all osteoblastic markers assayed. MMP-1 and -13 differentially regulate osteoblastic markers and their combined suppression is important for the elaboration of an osteoblastic phenotype in PDL cells.

Keywords

Human periodontal ligament cells; MMP-1; MMP-13; osteoblastic differentiation

Address for correspondence: Sunil Kapila, PhD., 1011 North University Avenue, The University of Michigan, Ann Arbor, Michigan, 48109-1078, E-mail: E-mail: skapila@umich.edu, Tel: 734-647-8750.
e-mail: Takayuki Hayami: thayami@umich.edu, Yvonne L Kapila: ykapila@umich.edu, Sunil Kapila: skapila@umich.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The extracellular matrix (ECM) of bone contains important structural and soluble factors required for its development and morphogenesis. Type I collagen is the primary organic component of bone ECM and provides important cues necessary for the differentiation of osteoblasts (Andrianarivo et al., 1992; Franceschi and Iyer, 1992; Franceschi et al., 1994; Maehata et al., 2005; Mizuno et al., 2000). Thus, osteoblastic differentiation of the human osteosarcoma cell line, MG-63 is enhanced by seeding these cells on type I collagen-coated plates (Andrianarivo et al., 1992). Similarly, the interactions of collagen with integrins enhance the gene expression of the osteoblastic markers alkaline phosphatase (AP), osteocalcin (OC), and bone sialoprotein (BSP) in bone marrow cells (Mizuno et al., 2000). Finally, the ascorbic acid-mediated osteoblastic differentiation of MG-63 or MC3T3-E1 cells is blocked when these cells are cultured in the presence of inhibitors of collagen synthesis or triple-helix formation (Franceschi and Iyer, 1992; Franceschi et al., 1994; Maehata et al., 2005).

Since fibrillar collagens including type I collagen are primary substrates for the collagenase members of the matrix metalloproteinase (MMP) family of enzymes, it is plausible that these proteinases may modulate osteoblastic differentiation by degrading collagens thereby diminishing the stimulatory effect of collagens on preosteoblastic cells. Amongst the collagenases, MMP-1 (collagenase-1) and MMP-13 (collagenase-3) are the key enzymes responsible for degradation of type I collagen. These MMPs are known to be secreted by osteogenic cells (Partridge et al., 1996; Varghese and Canalis, 1997) and may prove important in regulating their osteoblastic differentiation. Indirect evidence for the contribution of collagenases to osteoblastic differentiation of osteogenic cells is provided by studies demonstrating that addition of bacterial collagenase (Franceschi et al., 1994) or induction of endogenous collagenase by exposure of osteogenic cells to IL-1 β (Chien et al., 1999), or transfection with a mutated type I collagen gene in which collagen turnover is increased (Wenstrup et al., 1996) diminishes osteoblastic differentiation of these cells. Additionally, bone morphogenetic protein-2 (BMP-2) (Takiguchi et al., 1998; Varghese and Canalis, 1997) stimulates the osteoblastic phenotype while concomitantly suppressing collagenase expression.

As with the studies cited above (Chien et al., 1999; Franceschi et al., 1994; Takiguchi et al., 1998; Varghese and Canalis, 1997), we have previously demonstrated a negative relationship between exogenously-mediated changes in collagenase activity and the expression of osteoblastic markers in human periodontal ligament (PDL) cells (Hayami et al., 2007; Shiga et al., 2003). PDL cells are a heterogeneous population, that include a critical reservoir of mineralized matrix forming cells (Hayami et al., 2007; Lekic et al., 2001; Nohutcu et al., 1997; Shiga et al., 2003), which contribute to the maintenance and repair of the bone surrounding teeth (Lekic et al., 2001; Lekic et al., 2001; Seo et al., 2004). Specifically, we found that dexamethasone's inhibition of collagenase expression and activity in PDL cells is accompanied by an increase in AP, Runt-related transcription factor 2 (Runx2), osteonectin (ON), osteopontin (OP), BSP and collagen I (α 1) expression (Hayami et al., 2007). Dexamethasone also produced a dose-dependent increase in AP that was paralleled by a dose-dependent decrease in collagenase activity. The findings that dexamethasone enhances specific markers of osteoblastic differentiation in PDL cells while concomitantly decreasing collagenase expression suggest that endogenous collagenase may regulate osteoblastic differentiation of these cells. However, as with all previous studies (Chien et al., 1999; Rydziel et al., 1997; Shiga et al., 2003; Takiguchi et al., 1998; Varghese and Canalis, 1997) the expression of collagenase was modulated by an exogenous agent that may have additional unknown effects in contributing to osteoblastic differentiation of these cells. Therefore, these studies do not provide conclusive evidence of a direct effect of collagenases on osteoblastic differentiation, nor do they identify the contributions that each of the key collagenolytic MMPs

MMP-1 and -13 make to the modulation of these osteoblastic markers. In this study we used siRNA-mediated knockdown of MMP-1 and/or -13 and transient overexpression of MMP-1 and MMP-13 to test the hypothesis that these MMPs are important regulators of osteoblastic differentiation in PDL cells.

2. Results

2.1. Exogenous MMP-1 suppresses AP activity

To determine whether exogenous collagenase has specific effects on AP activity, PDL cells were treated with various concentrations of activated MMP-1 (0–200 ng/ml) with or without dexamethasone, which enhances osteoblastic differentiation of PDL cells (Hayami et al., 2007; Shiga et al., 2003). As expected, addition of exogenous MMP-1 caused a significant dose-dependent increase in collagenase-1 (Fig. 1A) and in collagenase activity (Fig. 1B). The MMP-1 levels and collagenase activity were reduced in the presence of dexamethasone presumably due to its downregulation of endogenous collagenase (Oishi et al., 2002; West-Mays et al., 1999), and confirms our previous observations (Hayami et al., 2007). Increasing concentrations of activated exogenous MMP-1 resulted in a corresponding dose-dependent decrease in AP activity, which was statistically significant relative to controls at 100 and 200 ng/ml of MMP-1 (Fig. 1C). AP activity was significantly greater in cells treated with dexamethasone relative to that in baseline controls and those treated with corresponding concentrations of MMP-1. However even in the presence of dexamethasone, addition of exogenous MMP-1 produced a dose dependent decrease in AP activity that was significant at 100 and 200 ng/ml of MMP-1. This finding indicates that collagenase is able to overcome the pro-osteogenic effects of dexamethasone on PDL cells. A strong negative correlation was observed between collagenase and AP activities ($p < 0.001$, $r = -0.90$). These results demonstrate the specific effects of exogenous collagenase in suppressing AP activity in PDL cells, as well as the ability of dexamethasone in partially alleviating decreases in AP activity produced in response to increased collagenase activity.

2.2. MMP-1 and MMP-13 overexpression increase collagenase activity and decrease AP activity

In order to confirm whether the findings on the effects of exogenous collagenase on AP phenotype of PDL cells can be replicated by modulation of endogenous collagenase and to determine whether the cells show varied responses to MMP-1 and -13, we next overexpressed each of these MMPs by transient cDNA transfections. Transfection of the MMP cDNAs had no effects on cell viability. Both Western blots and qRT-PCR demonstrated specific and significant upregulation of MMP-1 (Figs. 2A and C) or MMP-13 (Figs. 2B and D) mRNA and protein by the transfection of their respective cDNAs.

Assays specific for MMP-1 and -13 activities revealed that the baseline activity attributable to MMP-1 was two-fold greater ($0.06 \pm \text{SD } 0.05 \text{ ng}/\mu\text{g}$) than that due to MMP-13 ($0.03 \pm 0.01 \text{ ng}/\mu\text{g}$). The transfection of MMP-1 or -13 cDNA resulted in a statistically significant 2.8- and 1.4-fold increase in MMP-1 and MMP-13 activities over baseline levels, respectively (Fig. 2E and F). The total collagenase activity increased more than 2.3- and 1.7-fold over control levels following transfections with MMP-1 or -13 cDNA, respectively (Fig. 2G). The change in collagenase activity was accompanied by a significant 50% and 35% reduction in AP activity in response to MMP-1 and MMP-13 overexpression, respectively (Fig. 2H). Dose response studies with cDNA transfections also demonstrated a strong negative relationship between collagenase activity and AP phenotype of these cells ($p < 0.001$, $r = -0.95$).

2.3. Overexpression of MMP-1 and -13 results in differential modulation of osteoblastic markers in PDL cells

In order to determine whether overexpression of MMP-1 and -13 has a generalized effect on other markers osteoblastic differentiation, we next examined the contribution of each of these MMPs to the modulation of these markers and transcription factors. Since preliminary time-course studies showed that Runx2 and Osx are maximally modulated by day 3, while all of the other markers are modulated by day 5 of culture, these time-points were selected for subsequent assays for the respective markers.

Overexpression of MMP-1 and MMP-13 produced differential effects on the expression of the six osteoblastic markers assayed. Thus, MMP-1 overexpression resulted in a significant reduction in mRNA levels for Runx2, ON, OP, BSP and OC (Fig. 3A). The magnitude in inhibition of the levels of these genes varied from about 20% for Runx2 to as much as 80% for BSP. Unexpectedly, enhanced expression of MMP-1 was accompanied by increased Osx gene expression. In contrast to the relatively generalized effects of MMP-1 overexpression in suppressing osteoblastic markers, increase in MMP-13 caused decreases only in Osx (~25%), OP (~45%) and BSP (~20%), and no change in Runx2, ON and OC mRNA levels (Fig. 3B). Taken together, the overexpression of MMP-1 or MMP-13 significantly suppresses the mRNA expression levels of specific osteoblastic markers, with MMP-1 having greater effects than MMP-13 on the modulation of many of these markers.

2.4. siRNA inhibition of MMP-1 and MMP-13 suppresses collagenase activity and concomitantly stimulates AP activity in a dose-dependent manner

Given that MMP-1 and MMP-13 cDNA overexpression and addition of exogenous MMP-1 suppressed AP activity and various osteoblastic markers in PDL cells, we next used siRNA strategies to examine whether inhibition of MMP-1 and MMP-13 would conversely augment osteoblastic differentiation in this system. Transfection of MMP-1 or MMP-13 siRNA successfully and specifically inhibited mRNA and protein expression of the respective collagenases compared to the siRNA negative control (Figs. 4A to D) with no significant effects on cell viability. Quantitative RT-PCR demonstrated an almost 80% suppression of the MMP-1 and -13 by each of their siRNAs. Co-transfection with MMP-1 and MMP-13 siRNAs had the same effects on inhibition of mRNA and protein expression for MMP-1 and -13 as transfection with each of the MMPs alone, further underscoring the specificity of the MMP-1 and MMP-13 siRNAs for their target sequences. These decreases in MMP-1 and -13 expression by their respective siRNA were accompanied by a significant 87% and a 40% decrease in activities of these collagenases, respectively, compared to that with negative control siRNA (Figs. 4E and F). As with cells used in experiments on MMP-1 and -13 overexpression the control PDL cells had approximately two-fold greater MMP-1 ($0.055 \pm \text{SD } 0.05$ ng/mg) than MMP-13 activity ($0.025 \pm \text{SD } 0.01$ ng/mg) activity. Total collagenase activity decreased by significant 55% with by MMP-1 siRNA, and by 50% with MMP-13 siRNA compared to negative control siRNA (Fig. 4G). Co-transfection with MMP-1 and -13 siRNA produced an additional small decrease in both collagenase activity, which was significantly lower only relative to that produced with MMP-13 siRNA transfection, but not relative to MMP-1 siRNA transfection. AP activity was significantly increased concomitant with the reduction in collagenase expression and activity by each of these siRNAs (Fig. 4H). This increase in AP activity was significantly greater when siRNAs for both MMPs were co-transfected than with either MMP-1 or -13 siRNA transfections alone. Also, MMP-1 suppression had significantly greater effects in enhancing AP than did MMP-13 suppression. These results further confirm the role of MMP-1 and -13 in regulating AP activity in PDL cells.

We also performed dose-response experiments to examine whether the suppression of each of the two collagenases has specific effects on AP activity. Transfection of MMP-1 or MMP-13

siRNA produced a dose-dependent decrease in collagenase activity (Fig. 5A) and a concomitant dose-dependent increase in AP activity (Fig. 5B). The increase in AP activity was more marked with the inhibition of MMP-1 (180% at 200 pmol/well of siRNA) than with suppression of MMP-13 (135%), confirming observations from our previous experiments. The induction of AP activity had a strong negative correlation both to the inhibition of MMP-1 ($r = -0.95$, $P < 0.0001$) and MMP-13 ($r = -0.96$, $P < 0.0001$) expression.

2.5. Suppression of MMP-1 and MMP-13 differentially regulate osteoblast-specific transcription factors and markers in PDL cells

We also examined the effects of MMP-1 and/or MMP-13 suppression on mRNA levels of select osteoblastic markers previously assayed in the MMP overexpression experiments. As with MMP-1 and -13 overexpression studies, suppression of each MMP modulated different osteoblastic markers and also produced different magnitude of changes in levels of individual osteoblastic genes compared to siRNA negative controls (Figs. 6A to F). Overall, suppression of MMP-1 alone resulted in a small but statistically significant increase in ON (Fig. 6C), a moderate 30 to 50% increase in Runx2 (Fig. 6A) and BSP (Fig. 6E), and a large increase of over 250% in OP (Fig. 6D) and OC (Fig. 6F) mRNAs. Inhibition of this MMP caused a significant decrease in mRNA levels of Osx (Fig. 6B).

Inhibition of MMP-13 produced an approximately 2-fold increase in expression of Osx mRNA (Fig. 6B) and OP (Fig. 6D), no change in levels of Runx2, BSP and OC (Figs. 6A, E and F), and a decrease in ON mRNA (Figs. 6C). Concurrent suppression of both MMP-1 and MMP-13 resulted in a significant increase in mRNA levels for all markers assayed, ranging from a small increase for ON (Fig. 6C), a moderate 30 to 40% increase for Runx2 and Osx (Figs. 6A and B), and relatively large increases of 200% to 600% for OP, BSP and OC (Figs. 6D to F). The combined suppression of both MMPs overcame the effects that suppression of MMP-1 or MMP-13 alone had on the downregulation of Osx and of ON, respectively. In general, MMP-1 suppression had greater effects than MMP-13 suppression on the upregulation of most of the osteoblastic genes.

2.6. Modulation of endogenous MMP-1 and -13 affects the accretion of type I collagen, OP and OC in cell-matrix extracts

Finally, to determine whether type I collagen, OC and OP accretion was modulated by changes in MMP-1 and -13 levels, we performed western blots for these proteins in cell-matrix extracts. Type I collagen, OP and OC were decreased in the cell-matrix fraction by overexpression of MMP-1 and -13 (Fig. 7), and increased by suppression of these MMPs (Fig. 8). Densitometric analyses of the protein levels for the Western blots confirmed these results (Fig. 7B–D and Fig. 8B–D). The changes in the accretion of these matrix molecules paralleled changes in their respective mRNAs, and likely result from both their altered synthesis and degradation.

3. Discussion

In these studies we show for the first time the specific effects of endogenous collagenases in regulating osteoblastic differentiation of PDL cells. In general, decreased collagenase activity through suppression of MMP-1 and/or MMP-13 resulted in an increase in levels of the osteoblastic markers assayed, while the reverse was true when the expression of these MMPs was enhanced. Interestingly, our findings also demonstrate distinct differences in the effects of MMP-1 and MMP-13 in modulating the expression of specific osteoblastic markers in these cells. Thus, with the exception of Osx, the modulation of MMP-1 appears to affect the expression of all markers of differentiation assayed, whereas the effects of MMP-13 modulation are more selective in affecting the gene levels for mid stage markers of differentiation (Osx, AP, OP), with Runx2 and OC being non-responsive to any modulation

of MMP-13 expression. The reasons for the differences in osteogenic responses of these cells to MMP-1 and -13 are not clear. However, our results showing that the baseline activity attributable to MMP-1 is two-times greater than that due to MMP-13, which would also be reflected in the changes in these MMPs by cDNA and siRNA transfections, may provide a partial explanation for the observed differences. Furthermore, it is well established that MMP-1 is more potent than MMP-13 in degrading type I collagen (Knauper et al., 1996). This suggests that MMP-1 may more readily degrade and diminish the accumulation of collagen required for osteoblastic differentiation than does MMP-13, thereby contributing to its greater effects in modulating osteoblast differentiation. However, it should be noted that the observations on the differences in modulation of osteoblast markers by MMP-1 and -13 are made in short-term cultures, and it is plausible that the two MMPs may elicit similar effects in longer-term experiments.

While the mechanisms for the modulation of osteoblast phenotype by collagenases are not understood, on the basis of previous findings it can be postulated that various mechanisms either in isolation or together may be pertinent. These include the MMP-mediated changes to the stable collagenous matrix (Andrianarivo et al., 1992; Chien et al., 1999; Franceschi and Iyer, 1992; Franceschi et al., 1994; Hayami et al., 2007; Maehata et al., 2005; Mizuno et al., 2000; Rydziel et al., 1997; Shiga et al., 2003; Takiguchi et al., 1998; Varghese and Canalis, 1997; Wenstrup et al., 1996), which may modify the cues provided to the cells including those from altered collagen-integrin interactions (Cheng et al., 2001; Jikko et al., 1999; Mizuno et al., 2000; Schneider et al., 2001; Xiao et al., 1998; Xiao et al., 2002), or MMP-modulated changes in bioavailability of bioactive agents through the release of matrix bound growth factors into solution or the activation or inactivation of latent bioactive agents (Karsdal et al., 2002; Lee et al., 2005; Nakamura et al., 2005). There is ample evidence for the direct effects of collagenous matrices and matrix-integrin signaling in osteoblastic differentiation. Thus, it has been shown that osteoblast-specific gene expression requires ascorbic acid-dependent assembly of a collagenous ECM (Franceschi and Iyer, 1992; Franceschi et al., 1994). Furthermore, the inhibition of collagen synthesis attenuates the stimulative effect of Vitamin D3 and ascorbic acid on the AP activity in MC3T3-E1 and human MG63 osteoblastic cell lines (Franceschi and Iyer, 1992; Maehata et al., 2005). Finally, evidence that the collagen-initiated signals responsible for osteoblast differentiation are mediated via integrins is provided by studies showing that disruption of $\alpha 2$ -integrin- and/or $\beta 1$ -integrin-type I collagen interactions inhibit osteoblast differentiation (Jikko et al., 1999; Schneider et al., 2001; Xiao et al., 1998). On the basis of these findings and our findings on the changes in MMP-mediated accretion of collagen in cell-matrix extracts, it is conceivable that modulation of collagen stability by the up- or down-regulation of MMP-1 and/or MMP-13 influences this collagen-integrin signaling and the subsequent downstream regulation of osteoblast differentiation.

Since increases in *Osx* often occur downstream of *Runx2* during osteoblast differentiation (Nakashima et al., 2002), it would be expected that collagenase-mediated changes in *Runx2* would be paralleled by changes in *Osx*. However, we found that the modulation of MMP-1 produced opposing effects on *Runx2* and *Osx* expression levels. Similarly, modulation of MMP-13 resulted in the expected changes in *Osx* levels, however, without the concomitant changes in *Runx2* expression. This lack of coordinated regulation of *Runx2* and *Osx* can be attributed to the fact that in addition to being modulated by *Runx2*, *Osx* is also downstream of other transcription factors involved in differentiation of mineralized tissue forming cells (Lee et al., 2003). Thus, for example BMP-2 induces *Osx* expression in *Runx2* null cells, which can be abrogated by antisense blocking of *Dlx5* suggesting *Osx* is induced downstream of *Dlx5* rather than *Runx2* in this system. Our findings on the modulation of *Osx* and the downstream marker OP, but not OC and ON by MMP-13 are also consistent with previous findings showing that overexpression of *Osx* can induce OP without affecting levels of OC and ON (Kim et al., 2006). These observations taken together with other findings (D'Alonzo et al., 2002) suggest

that MMP-13 modulates *Osx* and *OP* levels in a *Runx2* independent or *Dlx5* dependent pathway, and emphasize that the hierarchical regulatory relationships between *Runx2* and *Osx* may not apply in all situations because of the presence of alternate regulatory cascades.

PDL cells are comprised of a heterogeneous cell population that include mineralized tissue forming progenitor cells, stem cells, fibroblasts, osteoblasts and cementoblasts (Lekic et al., 1996; Lekic et al., 2001; Nohutcu et al., 1997; Seo et al., 2004; Shiga et al., 2003). These mixed PDL cell populations have the ability to express osteoblastic markers and form mineralized nodules *in vitro* (Hayami et al., 2007; Lekic et al., 1996; Lekic et al., 2001; Nohutcu et al., 1997; Shiga et al., 2003) and cells transplanted into wounded periodontal areas have the ability to repair alveolar bone *in vivo* (Lekic et al., 2001; Lekic et al., 2001; Seo et al., 2004). Also, early passage PDL cells populations as used in our studies contain cells that show an osteogenic response to appropriate stimulation (Kuru et al., 1999; Liu et al., 1997; Nohutcu et al., 1997; San Miguel et al., 1998). As with osteoblastic cells, mixed PDL cell populations respond to dexamethasone (Basdra and Komposch, 1997; Chien et al., 1999; Kuru et al., 1999; Liu et al., 1997; Nohutcu et al., 1997; Ogata et al., 1995), β -estradiol (Morishita et al., 1999) and 1,25 dihydroxy-vitamin D₃ (Basdra and Komposch, 1997; Piche et al., 1989) by the induction of AP, type I collagen, OC, OP and BSP, and formation of mineralized nodules. Additionally, human PDL cells exposed to dexamethasone show a dose-dependent c-AMP response to PTH indicating the presence of osteoblast-like PDL cell populations *in vitro* (Nohutcu et al., 1995). Although, the current experiments in serum-free medium necessitate short-term studies and do not permit the assessment of mineralized nodule formation, our previous findings in longer-term cultures in which serum supplements were used do indeed show that inhibition of collagenase by dexamethasone enhances mineralized nodule formation in PDL cells (Shiga et al., 2003). On the basis of these studies and our findings, which show that PDL cells respond to modulation of MMP-1 and -13 by changes in expression of osteoblast-specific markers, it is likely that PDL cells, like those used in our studies, contain cells with osteoblastic characteristics.

Besides the relevance of these findings to normal bone development and repair, they may also be pertinent to various pathologies including periodontal and other osteolytic diseases. During periodontal disease, for example, there is breakdown and loss of the ECM that is largely mediated by MMPs expressed by inflammatory and resident PDL cells (Reynolds et al., 1994). Following disease, the residual PDL cells are critical for the regeneration of lost periodontal and mineralized tissues- a repair that results in part due to the differentiation of precursor cells into osteoblasts. Our findings suggest that in the presence of elevated levels of MMP-1 and/or -13 this differentiation may be inhibited thereby diminishing the pool of osteoblasts available to repair and replace the bone. The contribution of a mechanism that involves MMP-1 and -13-mediated inhibition of osteoblastogenesis in limiting bone repair following disease needs to be validated through further *in vivo* studies. However, support for the likely *in vivo* relevance of our studies is provided by observations in various MMP knockout mouse models including those of the collagenases MMP-13 and MMP-14 (Membrane type I-MMP) as well as MMP-2 (72-kDa gelatinase) that exhibit bone phenotypes consistent with cartilage and osteoblast defects (Kosaki et al., 2007; Mosig et al., 2007; Stickens et al., 2004; Zhou et al., 2000). These *in vivo* observations highlighting the importance of MMPs in osteoblast function are complemented by *in vitro* studies such as ours, which provide an additional important dimension in deciphering the role of MMPs in osteoblast differentiation.

4. Experimental procedures

4.1. Retrieval and isolation of PDL cells

Primary human PDL cells, obtained from patients undergoing therapeutic third molar extractions or extraction of premolars for orthodontic reasons, were retrieved as described

previously (Shiga et al., 2003). The use of human PDL cells for these studies was approved by the University of Michigan Institutional Review Board. Briefly, extracted teeth were washed twice with phosphate-buffered saline (PBS) containing 5x penicillin and streptomycin and 1x fungizone. PDL tissue attached to the mid-third of the root was then removed with a surgical scalpel. The PDL tissue was minced and placed in 35-mm tissue culture dishes. The explants were covered with sterilized glass coverslips and kept in α -minimum essential medium (α MEM) with 10% fetal bovine serum (FBS) and at 37°C in 5% CO₂ and antibiotics (1x penicillin and streptomycin) in humidified air until cells grew out of the explants and reached confluency. Cells were trypsinized and cells from passages one to five were used in subsequent experiments. To eliminate the complex effects of various factors in serum on cells, all experiments were performed in serum-free conditions with a previously defined supplement, lactalbumin hydrolysate (LAH) (Shiga et al., 2003). For this reason the experiments were limited to a maximum of 5 days of culture. To assay for markers that are regulated at different time-points of osteoblastic differentiation samples were retrieved at days 3 and 5 of culture.

4.2. Addition of exogenous collagenase to PDL cells

In the first experiment we examined the effects of exogenous MMP-1 in the absence or presence of dexamethasone on osteoblastic differentiation of PDL cells. Recombinant human MMP-1 (Calbiochem, San Diego, CA, USA) was activated with 2.5 mM p-aminophenylmercuric acetate according to the manufacturer's recommendations, and then dialyzed against PBS using a membrane with nominal molecular weight limit of 10,000 Daltons (Amicon Ultra-15, Millipore, Bradford, MA, USA). PDL cells were cultured at 3.0×10^4 cells/cm² in α MEM containing 10% FBS. After 24 hours, the cells were washed with PBS and medium was replaced with serum-free α MEM with 0.2% LAH. After 6 hours, the cells were rinsed again with PBS and incubated in serum-free medium containing varying concentrations of activated MMP-1 (0 ng/ml to 200 ng/ml) with or without dexamethasone (10^{-8} M). The medium was changed after 2 days. After 5 days of treatment, the cell-conditioned medium was collected and stored at -80°C for MMP assays. Cells were washed in PBS, trypsinized, counted, suspended, lysed in distilled water, and assayed for AP levels.

4.3. MMP overexpression with cDNA transfection

Early passage cells were plated at 3.0×10^4 cells/cm² in serum-containing medium without antibiotics for 24 hours before transfections. Plasmids containing MMP-1 cDNA (kindly provided by Dr. Gregory I. Goldberg (Goldberg et al., 1986) of Washington University in St. Louis), or MMP-13 cDNA (kindly provided by Dr. Carlos Lopez-Otin (Freije et al., 1994) of Universidad de Oviedo in Oviedo, Spain) or empty plasmid (pBluescript KS containing the T7 and T3 promoters, Stratagene, La Jolla, CA, USA) was transfected into the cells using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Since preliminary dose response studies showed that 2 μ g/well of MMP cDNA produced optimal overexpression of MMP-1 or -13 and repression of AP activity, all transfections were done with these concentrations of cDNA. After 24 hours, the cells were washed with PBS, and the medium was replaced with serum-free medium. The cell vitality was assayed with trypan blue staining and cell-conditioned-medium, cell lysates was retrieved after 5 days for assays. Total RNA was also extracted with RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions after 3 or 5 days for qRT-PCR assays of MMP-1, MMP-13, Runx2, Osx, ON, OP, BSP and OC.

4.4. Suppression of MMP-1 and -13 with siRNA transfection

To suppress collagenase expression, Stealth™ Select 3 RNAi Set (Invitrogen Corp.) was used to select three target sites on MMP-1 or MMP-13 mRNA as follows:

1. MMP-1 siRNA1 UCAACCACUGGGCCACUAUUUCUCC

2. MMP-1 siRNA2 AUCAAUGUCAUCCUGAGCUAGCUGA
3. MMP-1 siRNA3 CCCGGAAGUUGAGCUCAAUUUCAUU
4. MMP-13 siRNA1 AACAGCUGCACUUAUCUUCUUAACU
5. MMP-13 siRNA2 AUUUCUCGGAGCCUCUCAGUCAUGG
6. MMP-13 siRNA3 GGUUCCUGAUGUGGGUGAAUACAAU

Each site was checked to ensure it had no homology to any other known human gene using a nucleotide BLAST search. A Stealth™ Negative Control siRNA (Invitrogen Corp.) was used as a negative control. The specificity of these siRNAs to suppress the respective MMPs was confirmed by Western blots to MMP-1 and -13. Cells were plated as described above for MMP cDNA experiments and 250 pmol/well of MMP-1 siRNA and/or MMP-13 siRNA or control siRNA were transfected into the cells using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen Corp.) for 24 hours. The cells were washed and cultured in serum-free medium for 3 or 5 days with one change of medium at 2 days. The cell vitality was assayed with trypan blue staining and samples were retrieved after 3 or 5 days for assays.

Next, we determined the specificity of the AP response to MMP inhibition by performing siRNA dose-response experiments. Briefly, cells were plated as described above and 0 to 200 pmol/well of MMP-1 siRNA or MMP-13 siRNA or control siRNA were transfected with Lipofectamine 2000 for 24 hours. The cells were washed and cultured as described above and the samples collected after 3 or 5 days for analysis.

4.5. Total protein

For standardization of subsequent assays total protein from the cell-conditioned medium or cell lysates was measured by a Bradford microassay (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

4.6. Collagenase activity assays

The level of total active collagenase in unactivated conditioned medium that contains active and inactive MMPs was assessed by a FITC-collagen degradation assay. A 96-well plate was coated with FITC-collagen (15 µg/well) (Chondrex, Redmond, WA, USA) overnight at 4°C and washed twice with PBS. Cell-conditioned medium (100 µl) was added to the wells, and the plate was incubated at 35°C for 1 hour. As a reference, 100 µl of control medium containing 3000 ng of bacterial collagenase was added to one set of wells for complete digestion of FITC-collagen. After incubation, 90 µl from each well was transferred to another 96-well plate, and the fluorescence intensity of degraded FITC-collagen products was determined with a microplate spectrofluorometer (Spectramax M2, Molecular Devices, Sunnyvale, CA, USA) with excitation at 494 nm and emission at 518 nm. The data were converted to relative fluorescence units (RFU) of collagenase activity as described by the manufacturer and standardized to the total protein in the medium.

The level of active MMP-1 and MMP-13 in the cell-conditioned medium was assessed by Fluorokine® E enzyme activity assay kits (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Briefly, 200 µl of samples or standards were incubated for 3 hrs in 96-well plates coated with MMP-1 or -13 monoclonal antibodies. The wells were washed, and the fluorogenic substrate linked to a quencher molecule was added and incubated for 20 hours at 37 °C. The monoclonal antibody bound MMP cleaves the substrate between the fluorophore and quencher and the fluorescence was read at an excitation wavelength of 320 nm and emission wavelength of 405 nm using a microplate spectrofluorometer (Spectramax M2). The RFU for each sample was determined and converted to ng of MMP-1 or -13 using the standard curve, and the amount of MMP was standardized by total protein in the medium.

4.7. Western blots

Western blots were used to identify and compare the changes in MMP-1 and -13 expression in conditioned medium and in type I collagen, OP and OC in cell-matrix extracts. The conditioned medium or cell-matrix extracts, standardized by total protein, were electrophoretically resolved on 10% SDS-PAGE gels, and the proteins transferred to PVDF membranes. Non-specific binding was blocked with 2.5% dry non-fat milk in tris-buffered saline with 0.1% Tween 20 (TBST) overnight, and membranes were washed twice with TBST and incubated for 2 hours with rabbit anti-human MMP-1 or -13 antibodies (Chemicon International, Inc., Temecula, CA, USA) or with rabbit antihuman OP (Sigma-Aldrich), OC (Abcam Inc., Cambridge, MA, USA) or type I collagen (Calbiochem) primary antibodies. After further washes with TBST, the membranes were incubated with a 1:1000 dilution of a peroxidase-conjugated goat anti-rabbit antibody (Sigma-Aldrich) in TBST for 1 hour. The membranes were washed again and the bands were visualized by enhanced chemiluminescence (SuperSignal West Pico, Pierce).

4.8. Alkaline phosphatase assay

AP activity was assayed in the cell lysates from 5 day cultures by enzymatic conversion of p-nitrophenylphosphate (p-NT) to p-nitrophenol (Sigma-Aldrich) as described previously (Shiga et al., 2003). The amount of p-nitrophenol produced was measured spectrophotometrically at 410 nm, quantified against a standard curve in nM, and the results were standardized by cell number per minute.

4.9. Quantitative reverse transcriptase-polymerase chain reaction

MMP-1, MMP-13, Runx2, Osx, ON, OP, BSP, OC and GAPDH mRNA levels were assayed by qRT-PCR. Reverse transcription was performed using 50 ng of total RNA in the initial mix with M-MLV reverse transcriptase according to the manufacturer's instructions (SuperScript^{TM} III, Invitrogen). All primers and probes (GAPDH Cat # Hs99999905-m1; MMP-1 Cat # Hs00233958-m1; MMP-13 Cat # Hs00233922-m1; Runx2 Cat # Hs00231692-m1; Osx Cat # Hs01866874-m1; ON Cat # Hs00277762-m1; OC Cat # Hs00609452-m1; BSP Cat # Hs00173720-m1) were obtained commercially and are proprietary, thus sequences are not available (TaqMan® Gene Expression Assay, Applied Biosystems, Foster City, CA, USA) and amplified using a kit per the manufacturer's instructions (TaqMan® Universal PCR Master Mix, Applied Biosystems). Amplification was done under the following conditions: 50°C, 2 min; 95°C, 10 min; followed by 40 cycles of 94°C, 15 s and 60°C, 1 min. Data were analyzed using the ABI Prism 7500 SDS 1.2 Software (Applied Biosystems).

4.10. Statistical analysis

All experiments were performed in triplicate wells for a minimum of three cell isolates from different subjects, and subsequent assays were performed in triplicate. Because of inherent variations in data from different cell isolates, all quantitative data were derived as fold changes relative to baseline control levels. The effects of MMP-1 and -13 siRNA or cDNA transfections, or of exogenous MMP-1 on the expression of each of the osteoblastic markers were determined by a two-way ANOVA and the intergroup differences were determined by Fisher's PLSD test with the level of significance set at $P < 0.05$. The relationship between the fold change in AP activity and collagenase activity for MMP-1 dose response experiments was determined by a Pearson's correlation analysis.

Acknowledgements

We wish to thank to Dr. Gregory I. Goldberg of Washington University in St. Louis for providing MMP-1 cDNA, and Dr. Carlos Lopez-Otin of Universidad de Oviedo in Oviedo, Spain for providing MMP-13 cDNA. This study was supported by NIH R01 DE16671.

References

- Andrianarivo AG, Robinson JA, Mann KG, Tracy RP. Growth on type I collagen promotes expression of the osteoblastic phenotype in human osteosarcoma MG-63 cells. *J Cell Physiol* 1992;153:256–265. [PubMed: 1429847]
- Basdra EK, Komposch G. Osteoblast-like properties of human periodontal ligament cells: an in vitro analysis. *Eur J Orthod* 1997;19:615–621. [PubMed: 9458594]
- Cheng SL, Lai CF, Blystone SD, Avioli LV. Bone mineralization and osteoblast differentiation are negatively modulated by integrin $\alpha(v)\beta 3$. *J Bone Miner Res* 2001;16:277–288. [PubMed: 11204428]
- Chien HH, Lin WL, Cho MI. Interleukin-1 β -induced release of matrix proteins into culture media causes inhibition of mineralization of nodules formed by periodontal ligament cells in vitro. *Calcif Tissue Int* 1999;64:402–413. [PubMed: 10203417]
- D'Alonzo RC, Kowalski AJ, Denhardt DT, Nickols GA, Partridge NC. Regulation of collagenase-3 and osteocalcin gene expression by collagen and osteopontin in differentiating MC3T3-E1 cells. *J Biol Chem* 2002;277(27):24788–24798. [PubMed: 11964409]
- Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res* 1992;7:235–246. [PubMed: 1373931]
- Franceschi RT, Iyer BS, Cui Y. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Miner Res* 1994;9:843–854. [PubMed: 8079660]
- Freije JM, Diez-Itza I, Balbin M, Sanchez LM, Blasco R, Tolivia J, Lopez-Otin C. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J Biol Chem* 1994;269:16766–16773. [PubMed: 8207000]
- Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA, Eisen AZ. Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. *J Biol Chem* 1986;261:6600–6605. [PubMed: 3009463]
- Hayami T, Zhang Q, Kapila Y, Kapila S. Dexamethasone's enhancement of osteoblastic markers in human periodontal ligament cells is associated with inhibition of collagenase expression. *Bone* 2007;40:93–104. [PubMed: 16934542]
- Jikko A, Harris SE, Chen D, Mendrick DL, Damsky CH. Collagen integrin receptors regulate early osteoblast differentiation induced by BMP-2. *J Bone Miner Res* 1999;14:1075–1083. [PubMed: 10404007]
- Karsdal MA, Larsen L, Engsig MT, Lou H, Ferreras M, Lochter A, Delaisse JM, Foged NT. Matrix metalloproteinase-dependent activation of latent transforming growth factor- β controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis. *J Biol Chem* 2002;277:44061–44067. [PubMed: 12226090]
- Kim YJ, Kim HN, Park EK, Lee BH, Ryoo HM, Kim SY, Kim IS, Stein JL, Lian JB, Stein GS, van Wijnen AJ, Choi JY. The bone-related Zn finger transcription factor Osterix promotes proliferation of mesenchymal cells. *Gene* 2006;366:145–151. [PubMed: 16314050]
- Knauper V, Lopez-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. *J Biol Chem* 1996;271:1544–1550. [PubMed: 8576151]
- Kosaki N, Takaishi H, Kamekura S, Kimura T, Okada Y, Minqi L, Amizuka N, Chung UI, Nakamura K, Kawaguchi H, Toyama Y, D'Armiento J. Impaired bone fracture healing in matrix metalloproteinase-13 deficient mice. *Biochem Biophys Res Commun* 2007;354:846–51. [PubMed: 17275784]
- Kuru L, Griffiths GS, Petrie A, Olsen I. Alkaline phosphatase activity is upregulated in regenerating human periodontal cells. *J Periodontol Res* 1999;34:123–127. [PubMed: 10207841]
- Lee MH, Kwon TG, Park HS, Wozney JM, Ryoo HM. BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem Biophys Res Commun* 2003;309:689–694. [PubMed: 12963046]
- Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol* 2005;169:681–691. [PubMed: 15911882]

- Lekic P, Sodek J, McCulloch CA. Relationship of cellular proliferation to expression of osteopontin and bone sialoprotein in regenerating rat periodontium. *Cell Tissue Res* 1996;285:491–500. [PubMed: 8772163]
- Lekic P, Rojas J, Birek C, Tenenbaum H, McCulloch CA. Phenotypic comparison of periodontal ligament cells in vivo and in vitro. *J Periodontal Res* 2001;36:71–79. [PubMed: 11327081]
- Lekic PC, Rajshankar D, Chen H, Tenenbaum H, McCulloch CA. Transplantation of labeled periodontal ligament cells promotes regeneration of alveolar bone. *Anat Rec* 2001;262:193–202. [PubMed: 11169914]
- Liu HW, Yacobi R, Savion N, Narayanan AS, Pitaru S. A collagenous cementum-derived attachment protein is a marker for progenitors of the mineralized tissue-forming cell lineage of the periodontal ligament. *J Bone Miner Res* 1997;12:1691–1699. [PubMed: 9333130]
- Maehata Y, Takamizawa S, Ozawa S, Kato Y, Sato S, Kubota E, Hata RI. Both direct and collagen-mediated signals are required for active vitamin D(3)-elicited differentiation of human osteoblastic cells: Roles of osterix, an osteoblast-related transcription factor. *Matrix Biol* 2005;30:30.
- Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen- α 2 β 1 integrin interaction. *J Cell Physiol* 2000;184:207–213. [PubMed: 10867645]
- Morishita M, Yamamura T, Shimazu A, Bachchu AH, Iwamoto Y. Estradiol enhances the production of mineralized nodules by human periodontal ligament cells. *J Clin Periodontol* 1999;26:748–751. [PubMed: 10589811]
- Mosig RA, Dowling O, DiFeo A, Ramirez MC, Parker IC, Abe E, Diouri J, Aqeel AA, Wylie JD, Oblander SA, Madri J, Bianco P, Apte SS, Zaidi M, Doty SB, Majeska RJ, Schaffler MB, Martignetti JA. Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth. *Hum Mol Genet* 2007;16:1113–23. [PubMed: 17400654]
- Nakamura M, Miyamoto S, Maeda H, Ishii G, Hasebe T, Chiba T, Asaka M, Ochiai A. Matrix metalloproteinase-7 degrades all insulin-like growth factor binding proteins and facilitates insulin-like growth factor bioavailability. *Biochem Biophys Res Commun* 2005;333:1011–1016. [PubMed: 15964556]
- Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrughe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 2002;108:17–29. [PubMed: 11792318]
- Nohutcu RM, Somerman MJ, McCauley LK. Dexamethasone enhances the effects of parathyroid hormone on human periodontal ligament cells in vitro. *Calcif Tissue Int* 1995;56:571–577. [PubMed: 7648489]
- Nohutcu RM, McCauley LK, Koh AJ, Somerman MJ. Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization in vitro. *J Periodontol* 1997;68:320–7. [PubMed: 9150036]
- Ogata Y, Niisato N, Sakurai T, Furuyama S, Sugiya H. Comparison of the characteristics of human gingival fibroblasts and periodontal ligament cells. *J Periodontol* 1995;66:1025–1031. [PubMed: 8683414]
- Oishi Y, Fu ZW, Ohnuki Y, Kato H, Noguchi T. Molecular basis of the alteration in skin collagen metabolism in response to in vivo dexamethasone treatment: effects on the synthesis of collagen type I and III, collagenase, and tissue inhibitors of metalloproteinases. *Br J Dermatol* 2002;147:859–868. [PubMed: 12410694]
- Partridge NC, Walling HW, Bloch SR, Omura TH, Chan PT, Pearman AT, Chou WY. The regulation and regulatory role of collagenase in bone. *Crit Rev Eukaryot Gene Expr* 1996;6:15–27. [PubMed: 8882305]
- Piche JE, Carnes DL Jr, Graves DT. Initial characterization of cells derived from human periodontia. *J Dent Res* 1989;68:761–767. [PubMed: 2541186]
- Reynolds JJ, Hembry RM, Meikle MC. Connective tissue degradation in health and periodontal disease and the roles of matrix metalloproteinases and their natural inhibitors. *Adv Dent Res* 1994;8:312–319. [PubMed: 7865092]

- Rydziel S, Varghese S, Canalis E. Transforming growth factor beta1 inhibits collagenase 3 expression by transcriptional and post-transcriptional mechanisms in osteoblast cultures. *J Cell Physiol* 1997;170:145–152. [PubMed: 9009143]
- San Miguel SM, Goseki-Sone M, Sugiyama E, Watanabe H, Yanagishita M, Ishikawa I. The effects of retinoic acid on alkaline phosphatase activity and tissue-non-specific alkaline phosphatase gene expression in human periodontal ligament cells and gingival fibroblasts. *J Periodontal Res* 1998;33:428–433. [PubMed: 9842508]
- Schneider GB, Zaharias R, Stanford C. Osteoblast integrin adhesion and signaling regulate mineralization. *J Dent Res* 2001;80:1540–1544. [PubMed: 11499509]
- Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahimi J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;364:149–155. [PubMed: 15246727]
- Shiga M, Kapila YL, Zhang Q, Hayami T, Kapila S. Ascorbic acid induces collagenase-1 in human periodontal ligament cells but not in MC3T3-E1 osteoblast-like cells: potential association between collagenase expression and changes in alkaline phosphatase phenotype. *J Bone Miner Res* 2003;18:67–77. [PubMed: 12510807]
- Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, Werb Z. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* 2004;131:5883–5895. [PubMed: 15539485]
- Takiguchi T, Kobayashi M, Suzuki R, Yamaguchi A, Isatsu K, Nishihara T, Nagumo M, Hasegawa K. Recombinant human bone morphogenetic protein-2 stimulates osteoblast differentiation and suppresses matrix metalloproteinase-1 production in human bone cells isolated from mandibulae. *J Periodontal Res* 1998;33:476–485. [PubMed: 9879521]
- Varghese S, Canalis E. Regulation of collagenase-3 by bone morphogenetic protein-2 in bone cell cultures. *Endocrinology* 1997;138:1035–1040. [PubMed: 9048606]
- Wenstrup RJ, Fowlkes JL, Witte DP, Florer JB. Discordant expression of osteoblast markers in MC3T3-E1 cells that synthesize a high turnover matrix. *J Biol Chem* 1996;271:10271–10276. [PubMed: 8626594]
- West-Mays JA, Cook JR, Sadow PM, Mullady DK, Bargagna-Mohan P, Strissel KJ, Fini ME. Differential inhibition of collagenase and interleukin-1alpha gene expression in cultured corneal fibroblasts by TGF-beta, dexamethasone, and retinoic acid. *Invest Ophthalmol Vis Sci* 1999;40:887–896. [PubMed: 10102285]
- Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. *J Biol Chem* 1998;273:32988–32994. [PubMed: 9830051]
- Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res* 2002;17:101–110. [PubMed: 11771655]
- Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase 1. *Proc Natl Acad Sci U S A* 2000;97:4052–7. [PubMed: 10737763]

Fig. 1.

Exogenous MMP-1 dose dependently increases collagenase activity and concomitantly suppresses AP activity in the absence and presence of pro-osteogenic dexamethasone (Dex). PDL cells were cultured in serum-free medium and exposed to increasing concentrations of activated MMP-1 in the absence or presence of Dex for 5 days. The cell-conditioned medium was retrieved and assayed by Western blot (A) or FITC-collagen degradation assay (B), and the cell-matrix extract was assayed for AP activity (C). Addition of exogenous MMP-1 caused a dose-dependent increase in collagenase levels (A) and activity (B), which was diminished by Dex that is known to downregulate collagenase. (C) Increasing concentrations of MMP-1 produced a concomitant dose-dependent decrease in AP activity both in the absence or presence of the pro-osteogenic Dex. (Data represent mean $\pm$ SD of three experiments each performed in triplicate. P * < 0.05, ** < 0.01, *** < 0.001 relative to corresponding baseline and Dex-treated controls).

Fig. 2.

Overexpression of endogenous MMP-1 or MMP-13 suppresses AP activity in parallel with increased collagenase expression and activity. Early passage PDL cells were transiently transfected with empty plasmid or plasmid containing MMP-1 cDNA or MMP-13 cDNA. After 5 days of culture the RNA was retrieved for qRT-PCR for MMP-1 (A) and MMP-13 (B). The cell-conditioned medium was assayed by Western blots for MMP-1 (C) and -13 (D), for MMP-1 (E) and MMP-13 (F) activity, and for total collagenase activity with an FITC-collagen degradation assay (G). Cell lysates were collected and assayed for AP activity (H). Transfection of MMP-1 specifically increased the mRNA, protein levels of collagenase-1 and activity of MMP-1 (A, C and E, respectively), while MMP-13 transfection similarly upregulated collagenase-3 mRNA (B), protein (D) and its activity (F). The overexpression of both MMP-1 and MMP-13 were accompanied by almost similar increases in total collagenase degradative activities (G) and a concomitant decrease in AP activity (H). (Data represent mean $\pm$ SD of three experiments each performed in triplicate. $P < 0.05$, $** < 0.01$, $*** < 0.001$, relative to controls).

Fig. 3.

Osteoblast markers are differentially modulated by overexpression of MMP-1 and -13. Early passage PDL cells were transiently transfected with empty plasmid or plasmids containing MMP-1 cDNA or MMP-13 cDNA. After 3 or 5 days, the RNA was retrieved and subjected to qRT-PCR for Runx2, Osx, ON, OP, BSP and OC. Relative to control baseline levels (dashed line), MMP-1 overexpression produced statistically significant decrease in mRNA levels of Runx2, ON, OP, BSP and OC and an upregulation of Osx (A). Overexpression of MMP-13 resulted in a significant decrease in Osx, OP and BSP mRNA levels only. (Data represent mean $\pm$ SD of three experiments each performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ relative to controls).

Fig. 4.

Suppression of endogenous MMP-1 and/or -13 upregulates AP activity. Early passage PDL cells were transfected with control siRNA or siRNA to MMP-1 or MMP-13 or both MMPs together. After 5 days of culture the RNA was retrieved for qRT-PCR for MMP-1 (A) and MMP-13 (B). The cell-conditioned medium was assayed by Western blots for MMP-1 (C) and -13 (D) and for active collagenase activity with an FITC-collagen degradation assay (E). Cell lysates were collected and assayed for AP activity (F). siRNA to MMP-1 specifically inhibited MMP-1 mRNA (A), protein expression (C) and its activity (E). Similarly, siRNA to MMP-13 specifically downregulated MMP-13 mRNA (B), protein levels (D) and its activity (F). Suppression of MMP-1 or MMP-13 expression alone or together was accompanied by a significant decrease in total collagenase activity (G), and a concomitant increase in AP activity (H). (Data represent mean $\pm$ SD of three experiments each performed in triplicate. Statistically significant differences are represented as follows: a = relative to controls; b = relative to MMP-1 siRNA transfection; c = relative to MMP-13 siRNA transfection).

Fig. 5.

Suppression of collagenase activity with increasing concentrations of MMP-1 or -13 siRNAs is accompanied by a dose-dependent increase in AP activity. The experiments were performed as described in figure 4, with the exception that the cells were transfected with increasing concentrations of MMP-1 or -13 siRNAs or with control siRNA. The cell-conditioned medium was assayed for total collagenase activity (A) and the cell lysates subjected to AP activity assay. (Data represent mean $\pm$ SD of three experiments each performed in triplicate. $P^* < 0.05$, $** < 0.01$, $*** < 0.001$ relative to siRNA negative controls).

Fig. 6.

Osteoblast markers are differentially modulated by suppression of MMP-1 and/or MMP-13. Early passage PDL cells were transiently transfected with control siRNA or siRNA to MMP-1 and/or MMP-13. After 3 or 5 days, the RNA was retrieved and subjected to qRT-PCR for Runx2 (A), Osx (B), ON (C), OP (D), BSP (E) and OC (F). Relative to control baseline levels, MMP-1 suppression produced statistically significant increases in mRNA levels of Runx2, ON, OP, BSP and OC and a downregulation of Osx. Suppression of MMP-13 resulted in a significant upregulation of Osx and OP, no change in Runx2, BSP and OC and a decrease in ON mRNA levels. Downregulation of both MMP-1 and -13 was accompanied by an increase in mRNA levels of all the osteoblast markers assayed. (Data represent mean $\pm$ SD of three experiments each performed in triplicate. Statistically significant differences are represented as follows: a = relative to controls; b = relative to MMP-1 siRNA transfection; c = relative to MMP-13 siRNA transfection; d = relative to MMP-1 and -13 transfection).

Figure 7.

Enhancement of MMP-1 or -13 expression contributes to inverse changes in collagen I, OP and OC accumulation in cell-matrix extracts. (A) PDL cells were transfected with cDNA to MMP-1 or -13, washed and incubated in serum-free medium for 3 to 5 days. The cell-matrix extract was retrieved and subjected to Western blot analysis for collagen I ($\alpha 1$), OP, OC and actin. Densitometric analyses were performed for the protein bands for type I collagen (B), OP (C), and OC (D), standardized to the corresponding actin band, and plotted as fold change relative to control baseline levels of the protein. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 8. Suppression of MMP-1 and/or -13 expression contributes to inverse changes in collagen I, OP and OC accumulation in cell-matrix extracts. (A) PDL cells were transfected with siRNA to MMP-1 or -13 or both, washed and incubated in serum-free medium for 3 to 5 days. The cell-matrix extract was retrieved and subjected to Western blot analysis for collagen I ($\alpha 1$), OP, OC and actin. Densitometric analyses were performed for the protein bands for type I collagen (B), OP (C), and OC (D), standardized to the corresponding actin band, and plotted as fold change relative to control baseline levels of the protein. (Statistically significant differences at $p < 0.05$ or more are represented as follows: a = relative to controls; c = relative to MMP-13 siRNA transfection; d = relative to MMP-1 and -13 transfection).