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

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ABSTRACT
Ascorbic acid (AA) enhances osteoblastic differentiation by increasing collagen accumulation, which in turn, results in increased alkaline phosphatase (AP) expression in some osteogenic cells. However, in other cells, including human periodontal ligament (PDL) cells, additional osteoinductive agents are required for this response. To understand the potential basis for the maintenance of the AP phenotype of PDL cells exposed to AA, we examined the modulation of the tissue-degrading matrix metalloproteinases (MMPs) and their inhibitors by AA in short-term cell cultures. Early passage PDL cells in serum-free medium were exposed to AA for 5 days. The samples were analyzed for MMPs and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), AP, collagen I(1), and osteocalcin. We found that AA dose-dependently increased the expression of collagenase-1, and minimally TIMP-1, but not stromelysin-1 or TIMP-2. Additionally, AA caused substantial increases in levels of type I collagen. AA was unable to increase AP activity or osteocalcin messenger RNA in PDL cells. However, the cells retained the ability to show a significantly greater AP expression in high- versus low-density cultures, and increased osteocalcin as well as AP levels when cultured in the presence of dexamethasone. Moreover, in cells exposed to dexamethasone, increases in AP and osteocalcin were accompanied by a repression of collagenase-1 expression. In contrast to PDL cells, AA did not induce collagenase but produced a significant increase in AP expression in MC3T3-E1 cells. These findings provide the first evidence that AA, by modulating both collagen and collagenase-1 expression in PDL cells, most likely contributes to a net matrix remodeling response in these cells. Furthermore, the relationship between changes in collagenase expression and alterations in AP activity in PDL and MC3T3-E1 cells suggests a potential role for collagenase in modulating the AP phenotype of cells with osteoblastic potential. (J Bone Miner Res 2003;18:67-77)

DISCUSSION ACKNOWLEDGMENTS REFERENCES
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INTRODUCTION

ASCORBIC ACID (AA) induces the synthesis of type I collagen in various connective tissue cells, including osteoblasts and chondrocytes. It enhances the accumulation of the extracellular matrices that in turn influence the differentiation of these cells. AA increases collagen matrix formation mainly by stimulating the proline hydroxylation pool, increasing the secretion and processing of type I procollagen components, stabilizing messenger RNA (mRNA), and slightly increasing procollagen synthesis and gene expression.((1-3)) In most osteogenic cells, the accumulation of an appropriate collagenous extracellular matrix in response to AA is a prerequisite for the osteoblastic differentiation of these cells. Thus, addition of AA to cultured mouse calvaria-derived MC3T3-E1 cells((2,4-6)) and primary rat calvarial osteoblast-like cells((7)) produces a temporal sequence of gene expression, with initial deposition of type I collagen matrix followed by the induction of genes specific to an osteoblastic phenotype, such as the bone/liver/kidney isozyme of alkaline phosphatase (AP) and osteocalcin.((2,4,8-10)) More evidence that the AA-mediated increase in collagen expression is required for the differentiation of MC3T3-E1 cells is provided by studies showing that inhibition of collagen synthesis blocks the induction of AP and osteocalcin.((2,4)) In vivo studies showing defective bone regeneration in AA-deficient rats provide further evidence of a relationship between AA, collagen synthesis, and bone repair.((11))

The fact that a stable collagenous matrix is necessary for osteoblastic differentiation is further highlighted by studies showing that the presence of exogenous bacterial collagenase((2)) or endogenous collagenase induced by interleukin (IL)-1((12)) inhibits osteoblastic differentiation. These findings suggest that the increased degradation of the collagen matrix may affect the accumulation of the appropriate matrices, which in turn limits the ability of the compromised matrix to induce osteoblastic differentiation. Interstitial collagenase, one of the

very few enzymes that degrades type I collagen, belongs to the matrix metalloproteinase (MMP) family of enzymes. MMPs constitute the major proteolytic enzyme group degrading extracellular matrix components. Currently this family of proteinases comprises more than 20 members, and is divided into five distinct groups: collagenases, stromelysins, gelatinases, membrane-type MMPs, and others.((13)) Although some variations exist among the individual members, the MMPs generally share several similar characteristics. Chief among these are their extracellular matrix substrate specificity, zinc-dependent activity, extracellular inhibition by tissue inhibitors of metalloproteinases (TIMPs), secretion as inactive proenzymes or zymogens, and sequence similarities.((14))

Although the ability of AA to induce osteoblastic differentiation in several osteogenic cells is well established,((2,4,6,15)) some osteoprogenitor cells require the presence of other osteoinductive agents for increased AP expression and mineralized nodule formation. Among the latter group of cells are human periodontal ligament (PDL) cells((16-18)) that in culture are a heterogeneous population composed of fibroblastic and mineralized tissue-forming cells derived from fibrous and cellular connective tissues attaching teeth to bone.((19)) While a large percentage of PDL cells in mixed cell cultures are likely to be determined fibroblasts,((20,21)) a substantial proportion of the cells show an osteogenic response to appropriate stimulation.((12,17,22-28)) The mediators of osteogenic responses in these cells have been well characterized and include dexamethasone,((17,22,23)) dexamethasone and -glycerophosphate,((12,24,25)) -estradiol,((26)) 1,25 dihydroxyvitamin D3,((24,27)) and retinoic acid.((28)) These mediators enhance the expression of AP, type I collagen, osteocalcin, osteopontin, and bone sialoprotein, and increase the formation of mineralized nodules in PDL cells retrieved from humans and rats.((12,16-18,22-28)) However, no study has demonstrated that AA alone enhances an osteoblastic phenotype in PDL cells. Thus, it seems that, unlike MC3T3-E1 cells that undergo differentiation on exposure to AA alone, mixed PDL cell cultures require osteogenic stimuli in addition to AA for induction of an osteoblastic phenotype.((12)) The reason for this difference in the responsiveness to AA of cells with osteogenic potential remains unknown, but could potentially result from an increased degradation of collagen, particularly if AA itself causes a concomitant induction of collagenase in these cells. Thus, although it is presently accepted that AA produces a specific matrix anabolic response by inducing collagen expression, it is plausible that in PDL cells, this response is linked to a generalized increase in matrix remodeling activity resulting from AA's concomitant modulation of MMPs. AA's induction of collagenase in turn could limit its ability to enhance osteoblastic differentiation of PDL cellsa mechanism previously proposed to be responsible for inhibition of osteoblastic differentiation of these cells when exposed to appropriate exogenous agents in the presence of IL-1.((12))

Our primary goal in these studies was to characterize the effects of AA on the expression of specific MMPs and TIMPs in PDL cells. We also evaluated whether the AP phenotype of PDL cells remains consistent in the presence of AA, and if so, whether the cells retain the ability to show increased AP phenotype under appropriate culture conditions. To assess the potential relationship between collagenase expression and changes in AP phenotype in PDL cells, we determined whether dexamethasone's modulation of an osteoblastic phenotype in these cells is accompanied by downregulation of collagenase. We also studied AA's modulation of collagenase and AP expression in the osteoblast-like MC3T3-E1 cells that are known to undergo osteoblastic differentiation in the presence of AA. Because these studies were limited to short-term cultures, when modulation of an osteoblastic phenotype manifests primarily as changes in expression of type I collagen and AP,((2)) our studies focused on determining changes in the levels of these early markers of osteoblastic differentiation. Our findings show for the first time that AA induces collagenase-1 expression in PDL cells but not in MC3T3-E1 cells, and that the maintenance or decrease in collagenase expression is accompanied by increases in AP phenotype of PDL cells.

MATERIALS AND METHODS

PDL and MC3T3-E1 cell cultures

Human PDL cells, obtained from patients undergoing therapeutic third molar extractions or extraction of premolars for orthodontic reasons, were retrieved as described previously.((29)) Briefly, extracted teeth were washed twice with phosphate-buffered saline (PBS; 5 penicillin and streptomycin, and 1 fungizone). PDL tissue attached to the mid-third of the root was removed with a surgical scalpel. The PDL tissue was minced and placed in 35-mm tissue culture dishes. The explants were then covered with sterilized glass coverslips and kept in - minimum essential medium (MEM) with 10% fetal bovine serum (FBS) at 37°C in 5% CO2 in humidified air until cells grew out of the explants and reached confluency. Cells were then trypsinized and cells from passages one to five were used in subsequent experiments.

In contrast to many previous studies, all our experiments were performed in serum-free conditions with a previously defined supplement((29)) to eliminate the complex effects of serum on the responses of the cells. For the first set of studies to determine the modulation of MMPs by AA, two different cell isolates from two subjects were plated at 3.0 104 cells/cm2 in six-well plates in MEM with 10% FBS. After 24-48 h, the cells were washed with PBS, and the medium was replaced with serum-free medium (MEM plus 0.2% lactalbumin hydrolysate [LAH]). Cells were rinsed again after 6 h, and fresh serum-free medium, with or without 50 µg/ml AA, was added. The medium was replaced with fresh medium without or with AA every 24 h for 5 days. A 5-day time

period was selected because previous studies((2)) show that AA causes initial deposition of type I collagen, which continues to increase for up to 3 days, followed by the induction of AP, which is first detected at about day 3 in osteogenic cells.((2)) After 5 days of culture, the cell-conditioned medium was collected and stored at -70°C until further analysis. Cells were washed in PBS, lysed in distilled water, scraped, and the lysate was assayed for AP and total protein. For AA dose-response experiments, PDL cells were cultured at 3.0 104 cells/cm2 as described above and exposed to 0, 5, 10, 25, 50, and 100 μg/ml of AA for 5 days. All other procedures were performed as described above. Three wells were used for each cell isolate, and subsequent assays were performed in triplicate.

For studies to determine changes in collagen I(1) and collagenase-1 mRNA expression, two PDL cell isolates were cultured and exposed to AA for 5 days as described above. The cells were washed twice in cold Ca2+- and Mg2+-free PBS, and total RNA was extracted.

To determine whether the PDL cells retain the ability to show an increased AP phenotype, we compared the AP expression in high-density versus low-density cultures; the former conditions having previously been shown to enhance the expression of osteoblastic markers.((30,31)) In these experiments, PDL cells from four independent cell isolates (including one from the previous experiment) from different subjects were seeded in MEM containing 10% FBS at an initial density of 6.0 104 cells/cm2 (high density) or at 1.5 104 cells/cm2 (low density). After 24 h the cells were washed with PBS, and subsequent procedures were performed as described above. For all experiments, three wells were used for each cell isolate, and assays for total protein, MMPs, and AP were performed in triplicate.

For experiments testing the ability of dexamethasone to modulate markers of osteoblastic phenotype in PDL cells, the cells were plated at 3 104 cells/cm2 in medium containing 10% FBS. After 24 h the cells were washed and serum-free medium was added. Cells were rinsed again after 6 h, and fresh serum-free medium, without or with 50 μg/ml AA, or with 50 μg/ml AA plus 10 nM of dexamethasone was added. The medium was replaced with fresh medium without or with AA or AA and dexamethasone every 24 h for 5 days. Subsequent procedures were as described above for the first experiment. Additionally, total RNA was extracted as described below for osteocalcin RT-PCR. For all experiments, three wells were used for one cell isolate, and assays for total protein, collagenase-1, and AP were performed in triplicate.

To determine that the PDL cells used in our experiments indeed have the ability to form mineralized nodules, long-term experiments were performed in the presence of serum-containing medium. Briefly, cells were plated in -MEM with 10% FBS alone or with 50 μg/ml of AA, or AA plus 10 nM dexamethasone or AA plus dexamethasone plus10 mM -glycerophosphate. The latter two mediators served as positive controls for osteoblastic differentiation and mineralized nodule formation as demonstrated previously.((12,16,25)) The medium was changed every 3 days and the experiments terminated at day 27 of culture. Mineralized nodules were visualized by von Kossa stain.((12)) The cells were fixed in 10% formaldehyde, washed, incubated in 5% silver nitrate for 5 minutes under sunlight, washed, and incubated for 2 minutes in 5% sodium thiosulfate. The mineralized nodules per well were counted and imaged.

Because the response of MC3T3-E1 cells to AA, including the induction of type I collagen, AP, and other osteoblastic markers, has been well characterized,((2,4,15)) we used these cells as positive controls to show AA's ability to enhance an osteoblastic phenotype. MC3T3-E1 cells were plated at a density of 3.0 104 cells/cm2 in MEM containing 10% FBS for 2 days in the presence or absence of 50 μg/ml AA. The cells were washed twice with PBS (Ca2-and Mg2+-free) and the medium changed to MEM supplemented with 0.1% bovine serum albumin (BSA; Fisher Chemical, Fair Lawn, NJ, USA), with or without AA. Six hours later, the cells were again washed twice with PBS (Ca2+- and Mg2+-free) and fresh MEM (0.1% BSA), with or without AA, was added. The medium was replaced with fresh medium without or with AA every 24 h. BSA was used as a serum-free supplement in these experiments instead of 0.2% LAH because MC3T3-E1 cells were not viable over extended periods of time in medium with LAH, and because BSA-containing medium is compatible with viability and normal function of MC3T3-E1 cells. Because a different medium supplement was used with MC3T3-E1 cells than used in the previous experiments with PDL cells, we performed concurrent experiments with PDL cells using the 0.1% BSA media supplement. The experiments were terminated at day 5 of culture. The cell-conditioned medium was collected and stored at -70°C for future protein and proteinase assays. The cells were washed three times with PBS, lysed in deionized water, and stored at -70°C for future assays for AP. All experiments were performed in triplicate wells and repeated three times.

Total protein

Total protein in cell lysates and cell-conditioned medium was assayed for standardization of assays using the Bradford microassay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Substrate zymograms and reverse zymograms

Gelatin substrate zymography was used to detect and characterize proteinase activity in PDL and MC3T3-E1 cell-conditioned medium. The conditioned medium, standardized by total protein, was mixed with 4 sample buffer and electrophoresed in 10% SDS-PAGE gels containing 2 mg/ml of gelatin as described previously.((32)) After, SDS was removed by washing the gels in 2.5% Triton X-100. The gels were placed in incubation buffer at 37°C for 24 h and stained with 0.5% Coomassie blue and destained until proteinase bands were clearly visible.

Proteinase bands were further characterized by incubating zymograms in incubation buffer containing 0.3 mM 1,10-phenanthroline (Sigma, St. Louis, MO, USA), a zinc chelator, and metalloproteinase inhibitor.

Reverse zymograms were used to detect proteinase inhibitors in PDL cell-conditioned medium by using 18% SDS-PAGE as described above, except that after being washed with Triton X-100, the gels were incubated for 15 minutes with 4-aminophenylmercuric acetate-activated rabbit skin-conditioned medium. The gels were then incubated in incubation buffer for 18 h, stained, and destained as described above.

Images of the substrate zymograms and reverse zymograms were video-digitized by a CCD camera and analyzed with image software (Image Version 1.42; National Institutes of Health, Bethesda, MD, USA). We standardized the gel imaging by capturing the zymograms at the same focal length and exposure and quantified the intensity and area of proteolytic and inhibitor activities by video densitometry.

Western blots

Western blots were used to identify collagenase-1, stromelysin-1, TIMP-1, and TIMP-2, and to validate the findings of the substrate and reverse zymograms. After electrophoretic resolution of the conditioned medium using 10-18% SDS-PAGE gels, the proteins were transferred to nitrocellulose membranes. Nonspecific binding was blocked with 3% dry low-fat milk in tris-buffered saline (TBS) for 1 h, and membranes were washed twice with TBS and incubated for 1 h either with rabbit anti-human collagenase-1 (MMP-1) antibody (Chemicon International, Temecula, CA, USA), mouse anti-human stromelysin-1 (MMP-3) antibody (Oncogene Science, Cambridge, MA, USA), or rabbit anti-human TIMP-1 or TIMP-2 antibodies (Triple Point Biologics, Forest Grove, OR, USA). After further washes with TBS or TBS with 0.1% Tween, the membranes were incubated with a 1:1000 dilution of peroxidase-conjugated goat anti-mouse (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) or goat anti-rabbit (Pierce, Rockford, IL, USA) secondary antibody in TBS for 1 h. The membranes were washed again and the bands were visualized by incubating the blot in 20 μg/ml of NBT/BCIP solution (Bio-Rad).

AP activity assay

AP activity was assayed in the cell lysates by enzymatic conversion of p-nitrophenylphosphate substrate to p-nitrophenol. The amount of p-nitrophenol produced was measured spectrophotometrically at a wavelength of 410 nm and quantified against a standard curve in nanomolar per microgram of protein per minute.

Determination of collagenase-1, type I collagen, and osteocalcin mRNA levels

Total RNA was extracted for Northern blots by lysing, scraping, and homogenizing cells in 1 ml of lysis buffer (Ultraspec RNA Isolation System; BIOTECX, Houston, TX, USA). The RNA was precipitated and extracted by chloroform and isopropanol using standard procedures.((33)) The yield and purity of RNA was determined by ultraviolet (UV) spectroscopy.

For collagenase-1 and type I collagen Northern blots, the RNA was standardized by total RNA concentrations and loaded on a 1% agarose-formaldehyde gel. After electrophoresis, the RNA was transferred to nylon membranes through passive transfer and crosslinked by ultraviolet transillumination. Plasmids pUC and SP64 containing complementary DNA (cDNA) inserts for human collagen I(1)((34)) or collagenase-1((35)) were amplified in E. coli, the DNA was isolated, and the cDNA insert was retrieved by restriction digests and separation on 1% low-melting agarose gel.((33)) This cDNA template was used to synthesize a [32P]deoxycytidine triphosphate-labeled probe using a random priming kit (Amersham, Arlington Heights, IL, USA) according to the manufacturer's instructions. The probe was purified and hybridized to the nylon membrane for 1 h at 65°C. After washes with 2 SSC buffer containing 0.1% SDS, the signal was detected by autoradiography on Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY, USA). The message was quantitated by video-densitometry using glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) as an internal standard to normalize the densitometric data for total RNA loaded. The fold-induction of collagenase-1 and collagen I(1) mRNA by AA relative to baseline levels was determined.

For osteocalcin reverse transcriptase-polymerase chain reaction (RT-PCR), 0.1 μg total RNA was used in the initial reaction mix. The human osteocalcin primer sequences used were as described previously,((36)) yielding a 297-bp product. Reverse transcription and amplification reactions with MuLV reverse transcriptase and AmpliTaq DNA polymerase, respectively, were performed according to the manufacturer's instructions (GeneAmp, RNA PCR kit; Applied Biosystems, Foster City, CA, USA). All amplifications were performed for 40 cycles with the annealing temperature set at 55°C. For each primer pair, parallel reactions with water to replace template were run to control for template contamination.

Statistical analysis

The effects of ascorbic acid and plating density on the expression of AP, collagenase-1, and mineralized nodule formation was determined by a two-way analysis of variance (ANOVA), and the intergroup differences were determined by Scheffé multiple comparisons test with the level of significance set at $p < 0.05$. The statistical significance of the fold-induction for collagenase-1, stromelysin-1, and TIMPs mediated by AA was analyzed by paired t-test.

RESULTS

AA dose-dependently and specifically induces collagenase-1 but not stromelysin-1 and minimally modulates TIMPs in PDL cells

Using two cell isolates from two subjects, we first determined by substrate zymography, Western blot, and Northern blots whether AA mediated any changes in the expression of collagenase. PDL cells in the absence or presence of AA expressed three gelatin-degrading activities at 72, 53/58, and 43/48 kDa (Fig. 1A). All these proteinases were inhibited by 1,10-phenanthroline (Fig. 1A, lane 5), a metalloproteinase inhibitor, and therefore are likely to be the MMPs 72-kDa gelatinase (MMP-2), and inactive (proMMP-1) and active collagenase (MMP-1), respectively. The 53/58-kDa gelatinolytic proteinase was identified as procollagenase-1 by Western blot (Fig. 1B). The 43/48-kDa proteinase, which is likely active collagenase, was observed in the gelatin zymogram but not in the Western blot because of the relatively greater sensitivity of the former assay. AA produced substantial increases in levels of procollagenase and active collagenase in both cell isolates. The increases in expression of collagenase-1 protein were paralleled by changes in mRNA levels for this enzyme (Figs. 1C and 1D). AA's modulation of collagenase expression was further determined with dose-response experiments. Cells exposed to increasing concentrations of AA demonstrated a dose-dependent increase in expression of gelatin-degrading proteinase at 53/58 kDa (procollagenase) that was paralleled by increases in its activated 43/48-kDa enzyme (collagenase; Fig. 1E).

Because the expression of stromelysin is often coordinately regulated with that of collagenase,((37)) we also determined whether AA induced stromelysin-1 in parallel with collagenase-1. Western blots showed that in contrast to AA's upregulation of collagenase-1, it produced minimal changes in the expression of stromelysin-1 (data not shown). These findings were further confirmed by dose-response experiments that demonstrated minimal changes in expression of stromelysin-1 with increasing concentrations of AA (data not shown). These findings suggest that in PDL cells, AA's regulation of collagenase-1 is uncoupled from that of stromelysin-1.

To determine whether the induction of collagenase-1 is accompanied by changes in TIMPs, which could potentially negate any increase in matrix-degradative activity, we evaluated the effects of AA on expression of TIMPs. PDL cells produced basal levels of two proteinase inhibitors of approximately 30 and 20 kDa (Fig. 2A, lanes 1 and 3), corresponding to TIMP-1 and 2, respectively. Exposure of the cells to AA produced a slight increase in expression of the 30-kDa inhibitor, whereas the levels of the 20-kDa inhibitor decreased slightly (Fig. 2A, lanes 2 and 4). Western blots identified these proteinase inhibitors as TIMP-1 (Fig. 2B) and TIMP-2, respectively (Fig. 2C), and also confirmed the slight modulation of these inhibitors by AA. Using a bigger sample size of four cell isolates from four subjects cultured at low density, we found the AA-mediated changes in TIMP-1 and TIMP-2 expression were not statistically significant ($p > 0.05$; Fig. 2D).

AA increases type I collagen mRNA but not AP expression in PDL cells

We next determined whether AA has the ability to modulate the expression of type I collagen and AP in PDL cells. Northern blot analysis revealed that AA produced a 3- and 7.5-fold increase in type I(1) collagen mRNA in two cell isolates (Figs. 3A and 3B). In contrast, neither of the two isolates showed any increases in AP expression when exposed to AA (Fig. 3C). These findings suggest that AA's induction of type I collagen is not related to its modulation of AP in short-term PDL cell cultures.

PDL cells retain the ability to show increased AP expression and mineralized nodule formation under appropriate conditions

To determine whether the PDL cells do indeed retain the ability to show an increased AP phenotype under appropriate conditions, we next assessed the AP levels in these cells when grown in low-density and high-density cultures; the latter conditions were previously shown to mediate osteoblastic differentiation.((30,31)) We also evaluated whether the manipulation of AP status impacted on modulation of collagenase-1 expression by AA.

These experiments were performed on one cell isolate used in the previous experiments and three additional cell isolates from four subjects. The four cell isolates demonstrated substantial heterogeneity in their basal levels of AP (data not shown), as reported previously.((22,38)) All four cell isolates demonstrated significantly greater ($p < 0.05$) AP expression when plated at high versus low density (Fig. 4A). However, as observed in our previous experiments, AP expression remained relatively unchanged on exposure of both low- and high-density cell cultures to AA. On the average, high-density cultures had approximately 1.5-fold greater levels of AP than low-density cultures both in the absence or presence of AA. These findings reflect significant effects of plating density ($p = 0.006$), but not of AA ($p = 0.8$), on fold-increase in AP expression in PDL cells.

Irrespective of the plating density and AP phenotype, exposure of all four cell isolates to AA produced a significant (ANOVA $p < 0.001$) 2- and 3-fold increase in collagenase-1 in low- and high-density cultures, respectively (Figs. 4B and 4C). The lack of modulation of stromelysin expression by AA in both low-($p = 0.19$) and high-density ($p = 0.48$) cultures (data not shown) confirmed our previous observations. Similarly, TIMP-1

was also not significantly modulated by AA in either low-density ($p = 0.12$) or high-density ($p = 0.70$) cultures (data not shown).

To determine that the PDL cells used in the short-term serum-free experiments have the ability to generate mineralized nodules, we performed long-term experiments in serum-containing medium. We found that cells cultured under control conditions or in the presence of AA alone showed minimal numbers and very small mineralized nodules (Figs. 5A, 5B, and 5E). Addition of dexamethasone and AA caused a statistically insignificant increase (Scheffé $p > 0.05$) in mineralized nodules (Figs. 5C and 5E) relative to control and AA-treated cells. Mineralized nodule formation was significantly enhanced (ANOVA $p < 0.0001$) in cells incubated in the presence of AA, dexamethasone, and -glycerophosphate relative to control (Scheffé $p < 0.0003$), AA-treated ($p < 0.0004$), and AA plus dexamethasone-treated ($p < 0.01$) cells (Figs. 5D and 5E).

Dexamethasone's repression of collagenase-1 is accompanied by an increase in AP and osteocalcin expression in PDL cells

Further evidence that PDL cells have the ability for increased expression of osteoblastic markers with appropriate stimulation was provided by studies in which the cells were cultured in the presence of dexamethasone and AA, which have previously been shown to enhance the osteogenic responses in these cells.((12,24,25)) These experiments demonstrated that cells exposed to dexamethasone and AA had significantly increased AP activity ($p < 0.05$) and substantially greater osteocalcin mRNA expression then control cells or those cultured in the presence of AA alone (Figs. 6A and 6B, respectively). We also determined whether the induction of AP activity and osteocalcin mRNA expression by dexamethasone was accompanied by changes in collagenase-1 expression in PDL cells. As observed previously, AA increased the expression of collagenase-1 relative to control baseline levels (Fig. 6C, lanes 1 and 2). However, the expression of collagenase-1 was completely repressed in cells exposed to dexamethasone and AA. These findings show that dexamethasone's inhibition of constitutively expressed and AA-induced collagenase-1 is accompanied by an induction of AP and osteocalcin in PDL cells.

MC3T3-E1 cells in short-term cultures show no change in collagenase levels but increased AP expression on exposure to AA

Finally, we sought further evidence of a potential link between the induction of collagenase by AA and the modulation of the AP phenotype, by evaluating whether MC3T3-E1 cells which undergo osteoblastic differentiation in the presence of AA alone, show any changes in collagenase expression on exposure to AA. In contrast to our findings with PDL cells, AA did not cause any modulation of the constitutively expressed 53/58-kDa gelatinolytic proteinase (procollagenase) in short-term MC3T3-E1 cell cultures (Fig. 7A). Additionally, as opposed to the lack of an AP response to AA in PDL cells, MC3T3-E1 cells showed significant ($p < 0.001$) increases in AP expression on exposure to AA (Fig. 7B). Although it is helpful to compare changes in AP expression between PDL cells and MC3T3-E1 cells, it should be noted that MC3T3-E1 cells have substantially higher basal levels of AP expression than PDL cells. Therefore, PDL cells should not be equated directly with clonal osteoblastic cells such as the MC3T3-E1 cells.

DISCUSSION

Our studies show that besides having the ability to increase the expression of type I collagen, AA has a collagenase-1-inductive effect in PDL cells. Among the MMP family, and that of its inhibitors, the TIMPs, this induction was specific for collagenase-1, but was not seen for stromelysin-1, 72-kDa gelatinase, or TIMP-2. AA caused a small but statistically insignificant increase in TIMP-1. Our studies also show that although PDL cells retain their ability for increased AP expression under high-density culture conditions, and in the presence of dexamethasone, AA alone did not increase the expression of AP in these cells. We also noted that dexamethasone's induction of AP and osteocalcin was accompanied by a repression in collagenase-1 expression in PDL cells. In contrast to PDL cells, AA did not induce collagenase expression in short-term MC3T3-E1 cell cultures, but was capable of increasing their AP phenotype.

While there is some discussion on the predominant phenotype and origin of various cell types in the periodontal ligament,((20,21)) early passage PDL cell populations as used in our studies usually contain cells that show an osteogenic response to appropriate stimulation.((17,22,25,28)) The percentage of cell cultures that show some sort of osteoblastic response range from 40% of cloned PDL cells((22)) to 100% of mixed PDL cell populations.((17,25,28)) As with osteoblastic cells, mixed PDL cell populations respond to dexamethasone,((12,17,22-25)) -estradiol,((26)) and 1,25 dihydroxyvitamin D3((24,27)) by the induction of AP, type I collagen, osteocalcin, osteopontin, and bone sialoprotein, and formation of mineralized nodules. Additionally, human PDL cells exposed to dexamethasone show a dose-dependent cAMP response to PTH, indicating the presence of osteoblast-like PDL cell populations in vitro.((39)) Based on these studies and our findings, it is very likely that the responses to dexamethasone and differences in AP phenotype between high-

and low-density cultures of PDL cells used in our studies indicate the presence of cells with osteoblastic characteristics within our cultures.

Several studies in which collagen synthesis is perturbed((4,8)) or its degradation enhanced((2,12)) indicate that, in cells with osteogenic potential, the deposition of type I collagen is a prerequisite for increased AP expression, followed by induction of osteocalcin((4,8)) and subsequent mineralized nodule formation in long-term cultures.((8,12)) In contrast to these previous long-term studies, the limitation on cell viability imposed by serum-free culture conditions used in our investigation necessitates short-term studies such that subsequent markers of osteoblastic differentiation including mineralized nodule formation cannot be readily monitored. Because in our short-term studies the PDL cells may not be receptive to the downstream effects of AA, the findings may not necessarily mimic in vivo responses or those observed in long-term in vitro studies. However, the findings of studies using serum-free medium enables one to obtain insights into likely mechanisms of cellular responses to specific mediators in the absence of the numerous agents present in serum. Nevertheless, our current findings and those of others((25,26,40)) on long-term serum-containing cultures of human PDL cells have demonstrated that these cells have the ability to form mineralized nodules in the presence of dexamethasone and -glycerophosphate. As such, our study suggests a potential relationship between collagenase expression and changes in AP and osteocalcin expression that may be difficult to discern in the presence of serum.

Evidence that a stable collagenous matrix is important for the progression of osteoblastic differentiation, particularly during the preosteoblastic phase of development, is provided by studies showing that inhibition of collagen expression((4,8)) or its increased degradation((2,12)) leads to a decrease or delay in the expression of markers of osteoblastic differentiation. Thus, when MC3T3-E1 cells are exposed to AA, they show increased expression of AP that is partially inhibited when the cells are grown in the presence of bacterial collagenase.((2)) A further indication that collagen remodeling affects the differentiation of MC3T3-E1 cells is provided by studies((6)) in which cells stably transfected with a plasmid expressing high-turnover type I collagen chains produced abnormal collagen fibrils, resulting in a delayed and attenuated increase in AP. Finally, when PDL cells are cultured in the presence of medium containing dexamethasone and AA, which supports osteoblastic differentiation, and exposed to IL-1, they undergo a significant increase in collagenase mRNA expression that is associated with an inability to form mineralized nodules.((12)) Although the cells exposed to IL-1 do not form mineralized nodules, they retain the ability to synthesize collagen, suggesting that IL-1 inhibits mineralized nodule formation through increased collagen-degradative activity. These studies, together with our findings, provide evidence on the important role of collagenase in the regulation of osteoblastic differentiation. However, our findings are unique in showing that AA, which is traditionally thought to induce collagen leading to osteoblastic differentiation, may not produce this response in PDL cells because it concurrently increases the expression of collagenase. These findings suggest that, despite AA's induction of collagen in PDL cells, its concomitant increase in collagenase expression may lead to enhanced collagen turnover that in turn counteracts AA's expected stimulation of osteoblastic differentiation of these cells.

Because the PDL is comprised of a heterogenous pool of cells with differing physiologic functions, it is likely that the various cellular phenotypes show varied responses to AA. Thus, for example, as observed with established osteoblastic cells,((2,4,8-10)) bone-forming PDL cells lining the lamina dura may undergo further differentiation in the presence of AA. In contrast, specific pools of PDL cells that maintain the fibrous composition of the PDL may respond to AA by increasing both collagen and collagenase-1 expression, thereby maintaining a high state of matrix turnover necessary for an actively remodeling tissue like the PDL. AA's increased turnover of collagen may also limit the signals mediated by the matrix that are necessary to increase cellular AP and subsequent osteoblastic differentiation of this pool of PDL cells, further aiding in the maintenance of fibrous tissues and preventing ankylosis. This postulate on the role of AA in the matrix remodeling processes in vivo remains to be tested.

In contrast to MC3T3-E1 cells, which undergo osteoblastic differentiation when exposed to AA alone,((2,4,6,15)) human PDL cells require dexamethasone for the upregulation of AP expression as shown in our and previous studies.((12,16-18)) AA alone is not sufficient for modulating the AP phenotype of PDL cells, but dexamethasone when used on its own stimulates AP expression in these cells.((17)) Although the mechanisms by which dexamethasone increases AP expression in PDL cells remain poorly understood, it is plausible that it modulates osteoblastic differentiation by downregulating collagenase expression in PDL cells, thereby counteracting the induction of collagenase by AA. Dexamethasone's repression of both constitutively expressed collagenase or collagenase induced by interleukins or other stimulatory agents in fibroblastic cells is well documented.((41-43)) Our studies show that dexamethasone has the ability to repress both constitutive and AA-induced collagenase-1 expression in PDL cells, and this is accompanied by an increase in AP phenotype and osteocalcin mRNA levels. Because the net accretion of collagen is dependent on its synthesis and degradation, it is plausible that the AA-enhanced secretion of collagen and the dexamethasone-mediated decrease in collagenase expression together potentiate the accumulation of an appropriate collagenous matrix by PDL cells, thereby providing an environment conducive to their differentiation.

Because the mean concentration of AA in gingival crevicular fluid from healthy volunteers is 207 ± 81.8 μM or 36.46 ± 14.40 μg/ml,((44)) our findings on the response of PDL cells to AA at concentrations of 50 μg/ml may have some physiological relevance. Based on our findings on the effects of AA in the induction of

collagenase, it is plausible that AA at physiologic concentrations may participate in the normal turnover of the PDL or could possibly prevent osteoblastic differentiation of PDL cells by enhancing collagen degradation through induction of collagenase. Further in vivo studies are recommended to determine the contribution of AA in oral fluids including gingival crevicular fluid to the physiology of the healthy PDL.

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