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Dexamethasone's enhancement of osteoblastic markers in human periodontal ligament cells is associated with inhibition of collagenase expression

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Abstract

Although dexamethasone (Dex) substantially enhances the osteoblastic phenotype in osteogenic cells, including human periodontal ligament (PDL) cells, the basis for this response remains poorly understood. Since the accretion of a collagenous matrix is important for an osteoblastic response and dexamethasone is known to decrease collagenase expression, we examined whether osteoblastic differentiation mediated by Dex is linked to a decrease in collagenase expression in PDL cells. Early passage human PDL cells were exposed to Dex, or ascorbic acid (AA) or β-glycerophosphate (βGP) alone, or in various combinations in serum-free media for 3 or 5 days. Cells exposed to Dex alone or any combinations of treatments that included Dex demonstrated increased core binding factor alpha 1 (Cbfa1), alkaline phosphatase (AP), osteonectin (ON), osteopontin (OP), bone sialoprotein (BSP) and collagen I (α1) expression when compared to control cells or those exposed to AA or βGP. The induction of these osteoblastic markers was accompanied by a decrease in collagenase-1 expression. Collagenase activity showed a statistically significant strong negative relationship to Cbfa1 (Pearson's $r=-0.97$), AP ($r=-0.87$), OP ($r=-0.95$) and BSP ($r=-0.82$) in 5-day cultures, and moderately strong relationship to ON ($r=-0.74$) from 3 days culture. Dex also produced a dose-dependent increase in AP that was paralleled by a decrease in collagenase activity ($r=-0.98$). Addition of collagenase inhibitors increased AP expression while concomitantly suppressing collagenase activity. Conversely, addition of exogenous collagenase decreased the AP phenotype of the cells, which was more marked in the absence then in the presence of Dex. The findings indicate that Dex enhances specific markers of osteoblastic differentiation in PDL cells by decreasing collagenase expression, and suggest that endogenous collagenase may regulate osteoblastic differentiation of these cells.

Keywords: Human periodontal ligament cells; Dexamethasone; Collagenase-1; Osteoblastic markers

Introduction

The expression and accretion of collagen are important for osteoblastic differentiation of osteogenic cells. It has been shown that various factors, including ascorbic acid (AA) [1] and bone morphogenetic protein-2 [2], that enhance the accumulation of collagen stimulate an osteoblastic phenotype. AA, which increases collagen matrix formation by various mechanisms that include increasing procollagen synthesis and gene expression [3–5], is a potent mediator of osteoblast differentiation. Specifically, AA increases alkaline phosphatase (AP) and osteocalcin (OC) mRNAs in the MC3T3-E1 mouse calvarial-derived osteoblastic cell line [6], and this induction of osteoblastic differentiation is blocked by inhibitors of collagen-triple helix formation [7], bacterial collagenase [1] or by induction of endogenous collagenase by IL-1β [8]. Furthermore, the AA-mediated changes in osteoblast marker expression follow a clear and characteristic temporal sequence, where the earliest effects are on the stimulation of type I procollagen mRNA and collagen synthesis, followed by induction of AP and OC mRNAs [6,9]. While the mechanism by which collagen induces osteoblastic differentiation has not been determined, it has been postulated that collagen mediates this effect either

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through direct signaling to cells via receptors, or by presenting one or a combination of specific differentiation factors to cells in the appropriate context [10–19].

Unlike many types of primary or clonal calvarial cells that respond to AA by undergoing increased osteoblastic differentiation, osteogenic cells derived from the periodontal ligament (PDL) do not show similar responses to AA [20]. Cells from the PDL are heterogenous, containing several cell types including fibroblasts and mineralized tissue forming cells that show osteoblastic responses in vitro [21–24] and that contribute to bone repair in vivo [25]. These cells require the presence of dexamethasone (Dex), a member of the glucocorticoid class of hormones, for osteoblastic differentiation [26–29]. Dex also potentiates AA's effects on osteoblastic differentiation of other osteogenic cells, including rat calvarial cells [30–32]. In addition, in many osteogenic cells including osteosarcoma cells, calvarial-derived osteoblasts and bone marrow stromal cells from rats and humans, or in clonal cell lines, Dex enhances the expression of markers of osteoblast differentiation including AP [29,33,34], cAMP responses to parathyroid hormone [33,35], osteopontin (OP) [36], OC [28,36–39] and bone sialoprotein (BSP) [37–41].

A possible mechanism of action for Dex in promoting an osteoblastic phenotype may be through its inhibition of collagenase expression that would in turn limit collagen breakdown, and thereby enhance its accumulation. Indeed, Dex is known to inhibit collagenase-1 expression in various cell types [42–44] potentially through indirect mechanisms that include suppression of pro-inflammatory and matrix metalloproteinase (MMP)-inductive cytokines [45,46], IL-1β [44,47] and TNF-α [48]. Collagenases are a member of the MMP family of enzymes that are primarily responsible for the initial degradation of collagens. Of these proteinases, collagenase-1 (MMP-1) is the key enzyme responsible for degradation of type I collagen, which is the predominant collagen expressed by osteoblastic cells [49,50]. Since the degradation of the collagenous matrices by induction of endogenous collagenase [8] or addition of exogenous collagenase [6] has been shown to inhibit differentiation of osteogenic cells, the suppression of collagenase expression by Dex may be a potential mechanism by which Dex helps promote osteoblast differentiation. However, this novel mechanism of action of Dex remains to be examined. The objective of this study was to test the hypothesis that Dex mediates osteoblastic differentiation of human PDL cells, at least in part, by inhibition collagenase expression and activity. Specifically, we determined the relationship between the Dex-mediated changes in collagenase and early- and mid-phase osteoblastic markers, core binding factor alpha 1 (Cbfa1), AP, collagen I (α1), OP, BSP, OC and osteonectin (ON). We also determined whether addition of exogenous collagenase-1 inhibits the Dex-induced AP phenotype in these cells and conversely whether MMP inhibitors can increase the AP phenotype in these cells. Our findings demonstrate that a substantial portion of Dex's modulation of the osteoblast phenotype in PDL cells is likely mediated by its inhibition of collagenase-1 expression and activity.

Materials and methods

PDL cell cultures

Primary human PDL cells, obtained from patients undergoing therapeutic third molar extractions or extraction of premolars for orthodontic reasons, were retrieved as described previously [51]. The use of human PDL cells for these studies was approved by the Institutional Review Board. 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 covered with sterile glass coverslips and kept in α-minimum essential medium (αMEM) with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 and antibiotics (1× penicillin and streptomycin) in humidified air until cells grew out of the explants and reached confluency. Cells were trypsinized and used at passages one to five in subsequent experiments.

In contrast to many previous studies, all our experiments were performed in serum-free conditions with a previously defined supplement in order to eliminate the complex effects of serum on the responses of the cells. For these studies, a minimum of three different cell isolates from three subjects were plated at 3.0×10^4 cells/cm^2 in six-well plates in αMEM with 10% FBS. After 24 to 48 h, the cells were washed with PBS and the medium was replaced with serum-free medium (αMEM plus 0.2% lactalbumin hydrolysate, LAH) and antibiotics (1× penicillin and streptomycin). Cells were rinsed again after 6 h, and either fresh serum-free medium alone, or medium with 10^{-7} M Dex or 50 μg/ml AA or 10 mM β-glycerophosphate (βGP), or AA+βGP or AA+Dex or βGP+Dex or AA+βGP+Dex was added. The medium was replaced with fresh medium every 24 to 48 h for a total of 3 to 5 days. A 3- or 5-day time period was selected on the basis of previous studies showing the temporal modulation of osteoblast makers including the early induction of ON followed by type I collagen, and then by AP and OP, which occur between days 2 to 6 in osteogenic cells [6,9]. In order to determine the optimal time at which various markers are upregulated by Dex in our serum-free conditions, we first performed time-course experiments. Our findings showed that Dex caused a significant induction of ON at day 3, while that for Cbfa1, collagen I (α1), AP, OP, OC and BSP occurred at day 5 of culture (data not shown). Therefore, experiments for determination of the effects of Dex and collagenase activity on ON were performed on 3-day cultures, while its effects on the other six markers were determined at 5 days of culture.

After 3 or 5 days of culture, the cell-conditioned medium was collected and stored at −70°C until further analysis for collagenase activity and for collagenase-1 or tissue inhibitor of metalloproteinase-1 (TIMP-1) levels. Cells were washed in PBS, trypsinized, counted, suspended and lysed in distilled water, and assayed for AP levels or total RNA was extracted for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses for collagenase-1, Cbfa1, collagen I (α1), ON, OP, BSP and OC.

For Dex dose–response experiments, PDL cells were cultured at 3.0×10^4 cells/cm^2 as described and exposed to varying concentrations of Dex ranging between 10^{-8} and 5×10^{-7} M. The medium was changed every 24 to 48 h. After 5 days of culture, the cell-conditioned medium was collected and stored for collagenase assays and the cells were counted and lysed for AP assays.

The association between collagenase activity and AP phenotype was examined by adding TIMP-1 (Chemicon International, Inc; Temecula, CA, USA) or a metalloproteinase inhibitor (GM6001; Chemicon International, Inc.). PDL cells were cultured at 3.0×10^4 cells/cm^2 as described and exposed to 50 ng/ml TIMP-1 or 10 nM GM6001 or its analog. The media were changed every 24 to 48 h. After 5 days of culture, the cell-conditioned medium was collected and stored and the cells were counted and lysed for analysis as described above.

The effects of exogenous collagenase on AP phenotype were determined by adding activated collagenase-1 (Calbiochem, San Diego, CA, USA) at concentrations ranging from 100 ng/ml in the absence or presence of 10^{-7} M Dex to cell cultures. A collagenase concentration of 100 ng/ml was selected on the basis of our findings, which showed that the baseline levels of collagenase in these cells ranged from 50 to 400 ng/ml. Collagenase-1 was activated by 2.5 mM *p*-aminophenylmercuric acetate according to manufacturer's instructions, and then dialyzed (TUBE-O-DIALYZER kit; Calbiochem). Briefly cells