Protein Extracts of Dentin Affect Proliferation and Differentiation of Osteoprogenitor Cells In Vitro

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PROTEINS ASSOCIATED WITH THE MINERAL PHASE of dentin are considered to have the potential to alter cell function within the local environment, during development and regeneration of tooth/periodontal tissues. Cells that may be altered include osteoblasts, ameloblasts, periodontal ligament cells, odontoblasts, and cementoblasts. However, specific factors within dentin controlling cell activity have not been elucidated. To investigate further the role of dentin proteins in regulating cell behavior, MC3T3-E1 cells, a mouse osteoprogenitor cell line, were exposed to guanidine/EDTA extracts of dentin (G/E-D) prepared from bovine teeth. Cells, with or without G/E-D (2 to 50 μg/ml), were evaluated for proliferative activity and for mRNA expression of bone-associated genes. Results indicated that G/E-D suppressed cell proliferation and caused striking morphological changes, including the conversion of cuboidal cells into fibroblastic, spindle-shaped cells. Markers of osteoblast differentiation, osteocalcin and bone sialoprotein mRNA were decreased, while osteopontin mRNA was enhanced in cells exposed to G/E-D. Since transforming growth factor beta ($TGF\beta_1$) has been reported to influence cells in a similar fashion, G/E-D were examined for the presence of and concentration of TGFβ using slot blot analysis and enzyme immunoassay (ELISA), respectively. These analyses demonstrated that G/E-D contained 6.6 ng/mg of $TGF\beta_1$. Next, cells were exposed to G/E-D in conjunction with anti-$TGF\beta_{1,2,3}$ antibody. When cells were exposed to antibody, G/E-D-mediated changes in morphology and gene expression were blocked. These results suggest that $TGF\beta_1$ and perhaps other factors in dentin can regulate cell behavior and, therefore, can influence development, remodeling, and regeneration of mineralized tissues. *J Periodontol 1998;69:1247–1255.*

Key Words: Cell activity; cell cycle proteins; dentin; cell growth inhibition; bone regeneration; bone mineralization; bone remodeling.

Appropriate cell-matrix interactions are necessary for development and for regeneration of tissues, including mineralized tissues present in the oral cavity; i.e., bone, cementum, enamel, and dentin. Although significant progress has been made toward understanding the properties and functions of various molecules associated with these mineralized tissues during development, maintenance, and regeneration of tooth associated structures, the role of dentin matrix in regulating cells within the local environment remains unclear. Such cells would include osteoblasts, ameloblasts, periodontal ligament cells, odontoblasts, and cementoblasts. Researchers have demonstrated that demineralized dentin matrix induces bone formation in vivo[1–3] and cartilage formation in vitro.[4,5] Additional studies support a role for the dentin matrix in regulating formation of cementum and bone.[6–8] However, precise cellular and molecular mechanisms through which dentin matrix may influence cementoblast and osteoblast activity have not been elucidated.

Clues as to the potential role of dentin matrix in controlling cellular activity come from studies focused on understanding the composition of the dentin matrix. Dentin is composed of 70% mineral matrix and 20% organic matrix by weight, with the remaining portion being water

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(see references 9–11 for review). Non-collagenous proteins of dentin include those common to cementum and bone, such as osteocalcin, bone sialoprotein, and osteopontin. Several growth factors associated with bone and cementum have been identified in dentin/odontoblasts and include those belonging to the transforming growth factor superfamily (bone morphogenetic proteins [BMPs] and TGFβs) and insulin-like growth factors.[12,13] Furthermore, 3 phosphorylated non-collagenous proteins, phosphophoryn (dentin phosphoprotein), dentin matrix protein-1, and dentin sialoprotein are reported to be selective to dentin and are considered to be involved in conversion of predentin to dentin.[11,14] Recent data indicate that dentin sialoprotein and dentin phosphoprotein are expressed as a single cDNA transcript which codes for a protein that is cleaved into 2 smaller polypeptides, each having unique properties.[15]

Previously, we reported that gingival fibroblasts exhibit increased protein production when exposed to guanidine/EDTA extracts of dentin (G/E-D), but not to guanidine extracts of dentin.[16] The guanidine/EDTA extracts of mineralized tissue contain collagen, as well as non-collagenous proteins that bind strongly to the mineral phase and are extractable only after the addition of EDTA. Minimal information exists as to the effects of mineral bound dentin proteins on proliferation and differentiation of osteoblast-like cells. In the investigations described here MC3T3-E1 cells, a mouse osteoprogenitor cell line, were used to examine the effects of dentin matrix extracts on these cells in vitro. This cell line represents a well-characterized model for examining gene expression during osteoblast differentiation.[17] In the present study, the ability of G/E-D to affect proliferation and differentiation of an osteoblast-like cell line, MC3T3-E1 cells, was determined. We report that G/E-D inhibit cell proliferation and suppress differentiation of MC3T3-E1 cells. Furthermore, antibodies to TGFβ were able to block G/E-D mediated changes in MC3T3-E1 cells.

MATERIALS AND METHODS

Preparation of Guanidine/EDTA Extracts of Dentin

The methods for obtaining extracts of bovine dentin were similar to those described previously.[16] Briefly, teeth and periodontal tissue were removed from jaws of 2-year old cows and placed immediately in 0.02 M phosphate buffered saline (PBS), pH 7.4, containing protease inhibitors (0.05 M 6-amino-n-hexanoic acid, 0.005 M benzamidine HCl, 0.001 M phenylmethysulfonyl fluoride) at 4°C. Adherent soft tissue and cementum were removed from the roots with scalers. Next, crowns were separated from roots and pulpal tissues were carefully removed. Root dentin was cut into small pieces and then sequentially extracted in 50 mM Tris HCl buffer (pH 7.4; 50 ml/g wet weight) containing 4 M guanidine HCl at 4°C for 1 week, followed by 4 M guanidine HCl with 0.5M EDTA at 4°C, until the dentin was demineralized, usually 10 days with one change of buffer solution at day 5. Extracts were concentrated by ultrafiltration,[§] dialyzed extensively against distilled water and lyophilized. Lyophilized extracts were dissolved in sterile deionized water at various concentrations for use in assays.

Cell Culture

MC3T3-E1 cells were maintained in α-MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics, and were used between passage 10 to 15. For morphological observations and cell growth analysis, cells were plated into 24-well plates at an initial density of 5,000 cells per well. For northern blot analysis of osteoblast differentiation, cells were seeded in T-75 tissue culture flasks[‖] at an initial density of 50,000 cells/cm^2. After incubation for 24 hours in α-MEM with 10% FBS, medium was changed to α-MEM with 2% FBS containing 50 μg/ml ascorbic acid to induce osteoblastic differentiation.[17] Cells were treated with vehicle or G/E-D at 2.5, 25, and 50 μg/ml. Controls included cells cultured without ascorbic acid. Media were changed every third day for the duration of the experiment.

Cell Growth Analysis

At days 2, 3, 5, and 7 cells were harvested, in triplicate wells, by incubating in 0.05% trypsin and 0.01% EDTA, and cell number determined by Coulter counter.

Northern Blot Analysis

On day 8, total cellular RNA was extracted using a modified guanidine thiocyanate procedure.[18] Total RNA (20 μg) was denatured, fractionated on a 6% formaldehyde-1.2% agarose gel, transferred onto a nylon membrane,[¶] and immobilized using a UV crosslinker.[¶] The blots were then hybridized with radiolabeled probes generated from randomly primed cDNA for bone sialoprotein (BSP), osteocalcin (OCN) and osteopontin (OPN) and exposed to film[#] with intensifying screens at −70°C for 24 to 72 hours. To evaluate relative loading of RNA samples, photographs were taken of ethidium bromide stained gels prior to probing for specific markers.

Probes

Mouse BSP[19] and human OPN cDNA[20] probes were generous gifts from Drs. Larry Fisher and Marion Young (NIDR, NIH). Human osteocalcin cDNA probe[21] was obtained from Dr. John Wozney, Genetics Institute, Cambridge, MA.

[§]Amicon YM-10, W.R. Grace & Co., Danvers, MA.
[‖]Falcon, Becton Dickinson, Franklin Lakes, NJ.
[¶]Duralon-UV, Stratagene, Inc., La Jolla, CA.
[#] Kodak X OMAT, Eastman Kodak, Rochester, NY.

Western/Slot Blot Analysis

To determine the presence of TGFβ in G/E-D, slot blot analysis was used. G/E-D, prepared as a stock solution in water, were added to slots (200 μl of G/E-D per slot) at a concentration range from 1 to 100 μg/ml. After washing with Tris buffered saline (TBS) containing 0.05% Tween-20, followed by blocking non-specific staining with TBS-Tween-20 buffer containing 0.5% bovine serum albumin (BSA) for 1 hour, the nitrocellulose blot** was incubated with a mouse anti-bovine $TGFβ_{1,2,3}$ monoclonal antibody†† at a concentration of 1 μg/ml in TBS-Tween-20 with 0.5% BSA for 1 hour. The blot was washed in TBS-tween and then incubated with a goat anti-mouse IgG monoclonal antibody conjugated with horseradish peroxidase at 400 pg/ml in TBS-Tween-20 with 0.5% BSA for 1 hour. After washing the blot, bound secondary antibody was visualized by enhanced chemiluminescence‡‡ (ECL) and the image was captured on film. Recombinant human $TGFβ_1$, 10 ng, and BSA, 10 μg, were used as positive and negative controls, respectively.

Quantitative Immunoassay for $TGFβ_1$ in G/E-D (ELISA)

Quantitative sandwich enzyme immunoassay technique was employed to determine $TGFβ_1$ concentration in G/ED using a $TGFβ_1$ immunoassay kit§§ (sensitivity, 5.0 pg/ml; range, 31.2 to 200 pg/ml). Briefly, after activation of latent $TGFβ_1$ to immunoreactive $TGFβ_1$ with 1 N HCl and neutralization of the sample by 1.2 N NaOH and 0.5 M HEPES, 200 μl of sample or appropriate standard were added to each well of a 96 well-microtiter plate coated with TGFβ soluble receptor type II and incubated for 3 hours at room temperature. Following a wash to remove unbound proteins, 200 μl of a horseradish peroxidase conjugated polyclonal antibody against human $TGFβ_1$ was added to the wells and incubated for 1 hour at room temperature. After washing, 200 μl of a substrate solution was added to the well and incubated for 20 minutes at room temperature. The color development was stopped with 50 μl of 2 N sulfuric acid and the intensity of the color was measured spectrophotometrically, at 450 nm. The concentration of $TGFβ_1$ in G/E-D was determined by comparing the optical density of the sample to a standard curve.

Effects of Anti-TGFβ Antibody on Response of MC3T3-E1 Cells to G/E-D

For these experiments MC3T3-E1 cells were cultured in α-MEM, 2% FCS, containing 50 μg/ml G/E-D, plus or minus a murine monoclonal anti-bovine $TGFβ_{1,2,3}$ IgG_1 antibody†† for 8 days with media changes every third day. Thirty μg/ml antibody was used to neutralized 0.5 ng/ml of TGFβ in G/E-D, where the concentration of TGFβ in G/E-D was determined by ELISA, as discussed above. Controls included cells exposed to G/E-D along with mouse IgG_1 (MOPC 21).‖‖

Antibodies

Antibodies to $TGFβ_1$ from a bovine source were not available. Since TGFβ demonstrates cross-species reactivity, we were able to use human $TGFβ_1$ antibodies. Nevertheless, results most likely reflect relative levels of $TGFβ_1$ in G/E-D extracts and not exact concentrations since the antibodies used may recognize TGFβs from various species differently.

Statistics

For proliferation assays, data were analyzed using computer software¶¶ to perform ANOVA and Tukey-Kramer multiple comparison test.

RESULTS

Effect of G/E-D on Morphology of MC3T3-E1 Cells

Under basal conditions, MC3T3-E1 cells in culture, with or without ascorbic acid, exhibited a cobblestone appearance. Upon exposure to G/E-D their appearance changed with time to a more spindle shaped-fibroblastic morphology (Fig. 1). Such changes were noted as early as 24 hours after addition of extracts. Morphological changes were dose-dependent, with greater spindle shaped appearance in cells exposed to higher concentrations of extracts. These changes were noted in extract-exposed cells grown in medium, with or without ascorbic acid; however, in the presence of ascorbic acid more pronounced morphological changes were noted.

Effect of G/E-D on Proliferation of MC3T3-E1 Cells

Over the time period examined, the presence of G/E-D suppressed proliferation of MC3T3-E1 cells in a dose-dependent manner. This growth inhibition was noted as early as day 2 with marked decrease of cell proliferation at all doses from 2.5 μg/ml to 100 μg/ml noted at day 7 (Fig. 2).

Effect of G/E-D on OCN, BSP and OPN mRNA Expression in MC3T3-E1 Cells

As previously established,[17] MC3T3-E1 cells, in the presence of ascorbic acid, differentiated into mature osteoblasts and expressed mRNA for OPN, OCN, and BSP (Fig. 3). Furthermore, steady state levels for OCN and BSP mRNA were substantially lower in cells cultured in

**Protran, Schleicher & Schuell, Keene, NH.
††Genzyme, Cambridge, MA.
‡‡Amersham, Piscataway, NJ.
§§Quantikine, R&D Systems, Minneapolis, MN.
‖‖Sigma Chemical Co., St. Louis, MO.
¶¶Instat, GraphPad Software, San Diego, CA.

the absence versus the presence of ascorbic acid (Fig. 3; 2% FBS versus 2% FBS+AA). Normally ascorbic acid stimulates expression of OCN and BSP mRNA, however when G/E-D is added to the media, expression of both OCN and BSP mRNA was inhibited. Inhibition of OCN mRNA expression was noted at an extract concentration as low as 2.5 μg/ml when compared to 2% FBS+AA (control). To achieve a reduction in BSP mRNA, 50 μg/ml of extract was required (Fig. 3; 2% FBS+AA versus 50 μg/ml G/E-D+AA). In contrast to inhibition of OCN and BSP mRNA expression in cells exposed to G/E-D, mRNA for OPN was enhanced in a dose-dependent fashion, independent of the presence of ascorbic acid.

Western/Slot Blot Analysis of G/E-D for Presence of TGFβ Using Anti-TGFβ Antibody and ELISA to Determine TGFβ Concentration in G/E-D

Next, since TGFβ has been shown to exhibit similar effects on osteoblasts as those noted for G/E-D and the presence of TGFβ has been reported in dentin extracts,[13,22–27] studies were directed at determining whether extracts had TGFβ. G/E-D, 20 μg showed a positive reaction with anti-TGFβ antibody, by slot blot analysis. The antibody was sensitive to concentrations of rhTGFβ1 as low as 10 ng. No positive reaction was observed in slots containing 10 μg BSA, or in slots where primary antibody was eliminated (Fig. 4). ELISA indicated that the concentration of TGFβ in G/E-D was approximately 6.6 ng/mg dry weight.

Effects of Anti-TGFβ Antibody on OCN, OPN and BSP mRNA Expression and Morphology in MC3T3-E1 Cells Treated With G/E-D

Based on the above results, studies were pursued to determine whether TGFβ was responsible for the striking morphological changes and the decreased expression of BSP and OCN mRNA observed in MC3T3-E1 cells with G/E-D. For these experiments MC3T3-E1 cells were exposed to 50 μg/ml G/E-D in the presence of ascorbic acid and 2% FBS, with or without the addition of anti-TGFβ antibody (30 μg/ml). As represented in Figure 1, MC3T3-E1 cells, which normally exhibit a cobblestone appearance in culture, exhibit a spindle-shaped morphology when cultured with G/E-D. This change in morphology was prevented with the addition of anti-TGFβ antibody in the culture medium (Fig. 5), while IgG controls had no effect on the morphological changes induced by G/E-D. In conjunction with the ability of anti-TGFβ antibody to block G/E-D mediated effects on cell morphology, the antibody prevented the down regulation of BSP mRNA (Fig. 6; G/E-D+AA+Ab versus G/E-D +AA+IgG control); however the antibody did not completely eliminate the negative effects of G/E-D on BSP mRNA expression. The antibody also increased OCN mRNA expression in cells exposed to G/E-D (Fig. 6; G/E-D+AA+Ab versus G/E-D+AA+IgG/PBS); however this increase was minimal. In addition, the antibody decreased G/E-D observed enhancement of OPN mRNA in MC3T3-E1 cells.

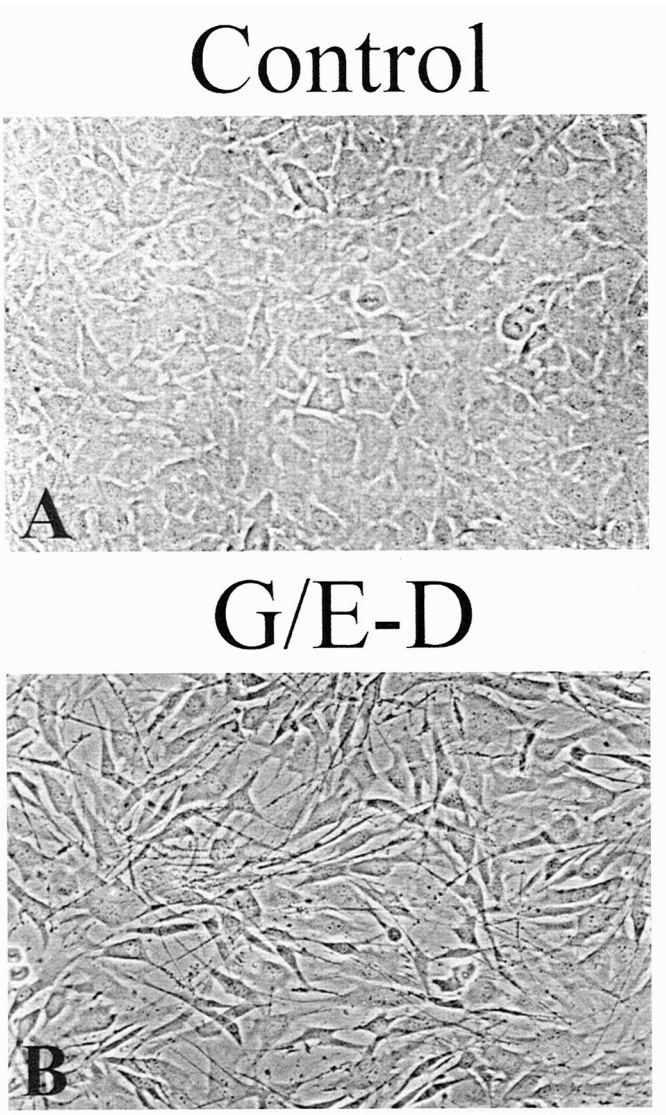
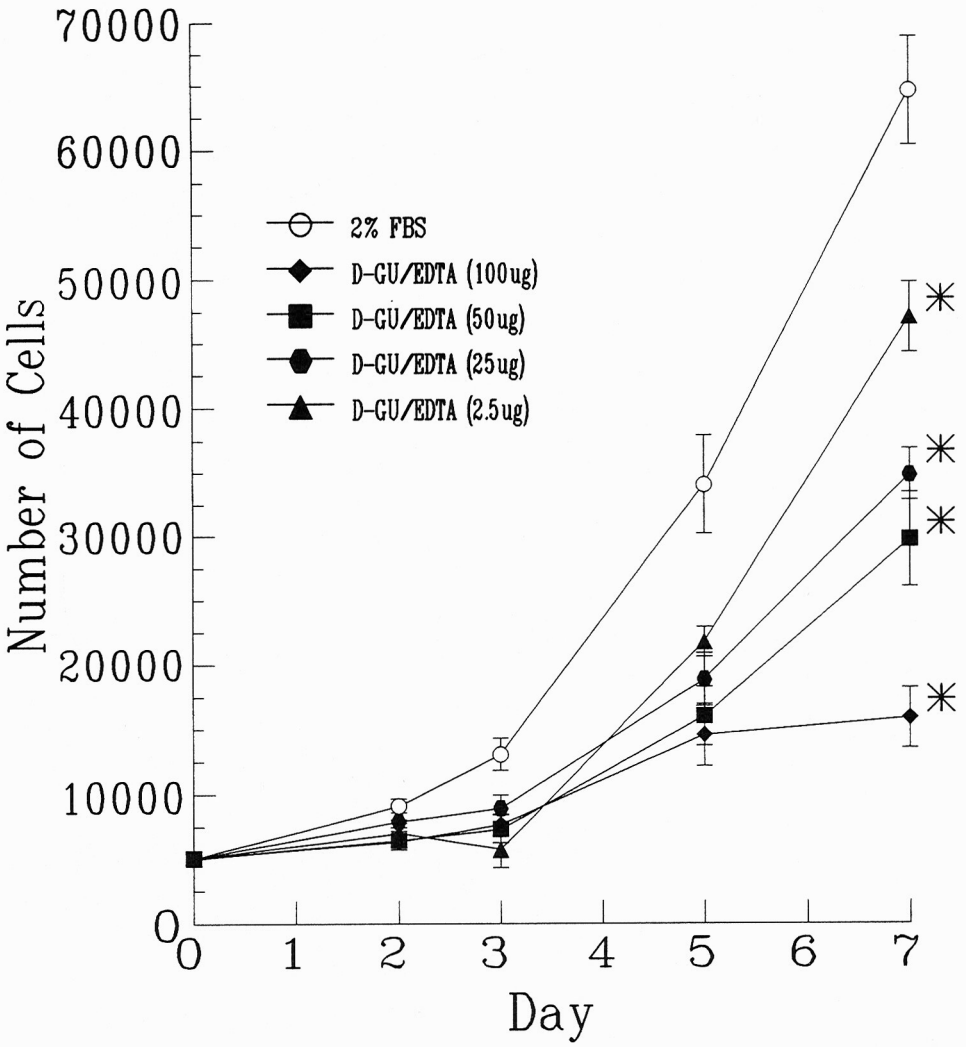


Figure 1. Effect of G/E-D on morphology of MC3T3-E1 cells. ***A.*** *Control: MC3T3 cells, media plus 50 μg/ml ascorbic acid/2% fetal calf serum (control media). Note cuboidal appearance.* ***B.*** *G/E-D: Cells exposed to media as in A with 50 μg/ml G/E-D. Note fibroblastic appearance. Photographs were taken at day 6 (original magnification ×100).*

DISCUSSION

Non-collagenous proteins present in the mineral phase of hard tissues are considered to have the capacity to regulate a variety of cellular functions and activities including migration, attachment, differentiation, proliferation, and expression and secretion of specific proteins. Indeed, demineralized dentin matrix has been reported to unmask the osteoinductive potential of sequestered proteins and therefore have the capacity to promote mineral tissue formation in vivo,[1–3,28] induce cartilage formation in vitro,[4,5]



P Values for Cell Proliferation Versus 2% FBS Control

	Day			
Dose G/E-D	2	3	5	7
2.5 μg	0.010	0.001	0.010	0.001
25 μg	0.05	0.010	0.001	0.001
50 μg	0.001	0.001	0.001	0.001
100 μg	0.001	0.010	0.001	0.001

Figure 2. Effect of G/E-D on proliferation of MC3T3-E1 cells. Cells were seeded at 5,000 cells/well and allowed to attach overnight. Day 0 = initial number of cells attached prior to addition of agents. Media with ascorbic acid, 2% FBS alone or with G/E-D at 2.5, 25 or 50 μg/ml were added to dishes and cells counted by Coulter counter on day 2, 3, 5 and 7. Results were reproduced in two separate experiments.

and elicit increases in total protein production and collagen production by human gingival fibroblasts, in vitro.[16] However, the specific factors promoting cell activity have not been fully explored. In these studies, we focused on determining whether or not dentin proteins affect differentiation of osteoprogenitor cells. Based on results of those investigations, studies were initiated to dissect out the influence of specific molecules within dentin on osteoblast activity.

Results indicated that guanidine/EDTA extracts of dentin have the capacity to alter osteoblast cell behavior. Guanidine/EDTA extracts were able to suppress cell proliferation in a dose-dependent manner. The anti-proliferative effect of the extract was noted after 2 days exposure, and by 7 days G/E-D inhibited cell proliferation significantly, $P < 0.01$, at all doses from 2.5 μg/ml to 100 μg/ml (Fig. 2). At a morphological level, this extract altered the appearance of MC3T3-E1 cells, where they change

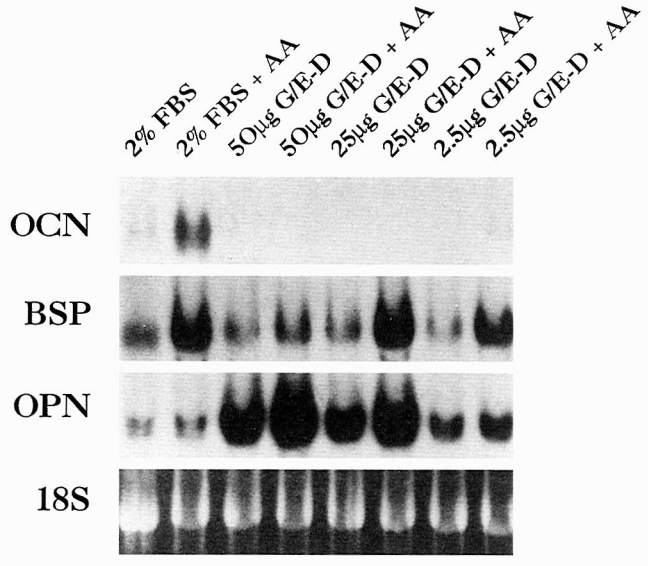


Figure 3. Effect of G/E-D on bone sialoprotein, osteocalcin, and osteopontin mRNA expression in MC3T3-E1 cells. Cells were cultured in media containing 2% FBS, ± ascorbic acid (AA), and G/E-D (2.5, 25, 50 μg/ml) for 8 days and then RNA extracted from cells. mRNA expression of proteins was analyzed by northern analysis. 18s ethidium stained gel shown as evidence of comparable loading. BSP = bone sialoprotein; OCN = osteocalcin; OPN = osteopontin.

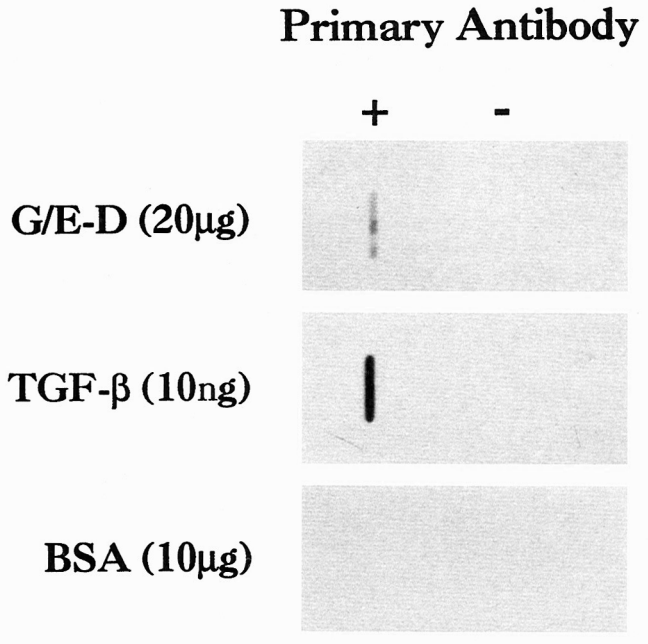


Figure 4. Slot blot analyses for TGF-$_\beta$ in G/E-D. Twenty μg G/E-D, 10 ng TGF-$_\beta$ and 10μg BSA were applied to nitrocellulose using a slot blotter apparatus and western analysis used to determine presence of TGF-$_\beta$ (+ lane), Ig control (− lane).

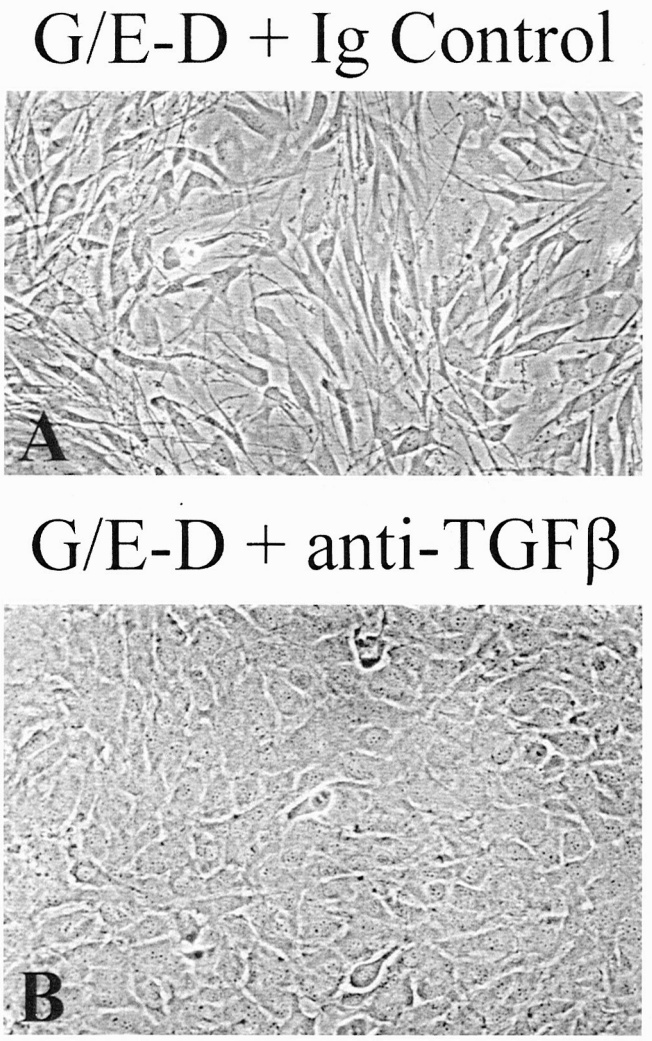


*Figure 5. Effect of TGFβ antibody on G/E-D induced morphological changes. **A.** Ig control: cells cultured with media containing 50 μg/ml ascorbic acid/2% fetal calf serum. 50 μg/ml G/E-D and Ig (control). Note there is no change in spindle shaped morphology seen in cells exposed to G/E-D alone (see Fig. 1B). **B.** TGFβ antibody. Cells cultured as in A above with anti-TGFβ antibody (30 μg). Note cuboidal appearance (see Fig. 1A). Photographs were taken at day 6 (original magnification ×100).*

from a cubodial shape to a more spindle shape, suggestive of a shift in phenotype to a more fibroblast-like cell phenotype versus osteoblast-like cell phenotype. This was confirmed by northern analysis, which demonstrated that MC3T3-E1 cells cultured in the presence of G/E-D exhibited a decrease in mRNA levels for proteins associated with differentiation of these cells along the osteoblast lineage; i.e., BSP and OCN.[17,29] At this point, we had established that G/E-D extracts inhibited osteoblast differentiation and thus experiments were designed to identify factors within the extract responsible for this effect. Studies by McCauley and colleagues[25] indicating that TGFβ inhibited osteoblastic maturation of MC3T3-E1 cells, coupled with the recognition that dentin contains TGFβ,[13] prompted us to investigate whether TGFβ was present in the G/E-D extract. By ELISA, we determined that G/E-D preparations contained 6.6 ng TGFβ per mg dry dentin weight. Results from other groups have shown that the concentration at which TGFβ affects a variety of cell

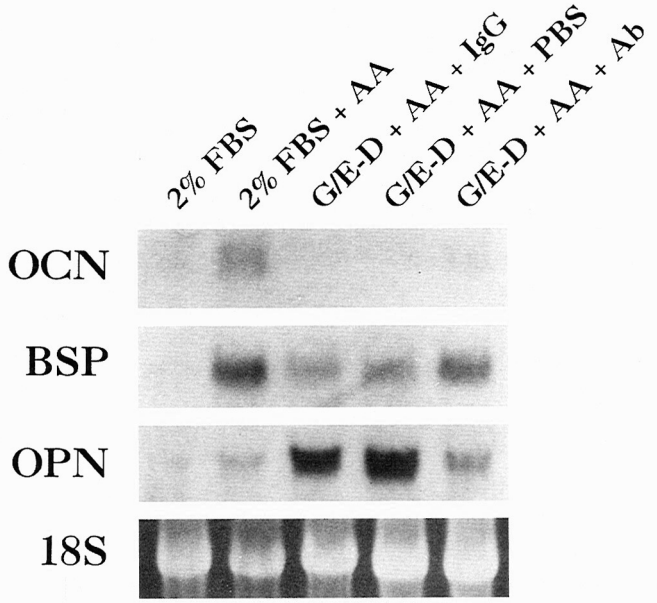


Figure 6. Effect of TGFβ antibody on osteocalcin, osteopontin, and bone sialoprotein mRNA expression in MC3T3 cells. Cells were treated with media containing 2% FBS ± ascorbic acid (AA) or 2% FBS, ascorbic acid and G/E-D (50 μg/ml) + PBS or IgG or TGFβ antibody. RNA was extracted from cells after 8 days of culture and northern analysis used to determine mRNA expression of specific proteins.

types, including osteoblasts, is normally in the range from 0.001 to 4 ng/ml.[22-25,27,30,31] Thus, the concentration of G/E-D used in our experiments contains TGFβ (0.33 ng/ml) within the range described above. Next, to determine whether TGFβ was responsible for observed effects, the ability of anti-TGFβ antibody to block the inhibitory effects of G/E-D on differentiation of MC3T3-E1 cells was tested. These studies demonstrated that the inhibitory effects of G/E-D on osteoblast maturation could be prevented if antibody to TGFβ was present, thus suggesting that TGFβ or TGFβ-like molecules were responsible for G/E-D's inhibitory activity.

While data from some laboratories support this finding, it is important to recognize that in vitro studies report diverse findings on the effect of TGFβ on osteoblastic cell proliferation and differentiation.[22-27,31-39] For example, McCauley et al.[25] demonstrated that exposure of MC3T3-E1 cells to TGFβ1, at 3 ng/ml, blocked both osteoblast maturation as measured by decreased mRNA for OCN and PTH receptor, and inhibited mineral nodule formation as measured by von Kossa technique. However, the ability of $TGF\beta_1$ to inhibit osteoblast maturation was differentiation stage dependent, with minimal effects noted when it was introduced at later times in culture.

Despite the fact that TGFβ has been reported to be present in dentin, the ability of TGFβ present in dentin extracts to induce changes in osteoblastic proliferation and differentiation has not been investigated. Similar to our findings with G/E-D, dramatic morphological changes of MC3T3-E1 cells from osteoblastic to fibroblastic appearance have been reported in cells exposed to TGFβ.[25,30,31,36,38,39] Antibody to TGFβ inhibited the G/E-D morphologic conversion (Fig. 5). Thus, the results here suggest that TGFβ or comparable molecules present in G/E-D blocked expression of an osteoblast-like phenotype as measured by decreased OCN and BSP mRNA in cells exposed to the extracts. It is important to mention that while reversal of morphological conversion was complete using the antibody to TGFβ, the ability to reverse the effects of G/E-D on expression of OCN and BSP mRNA was not complete. This suggests that other factors present in the matrix extract may contribute to the ability of G/E-D to inhibit osteoblast differentiation.

In contrast to the ability of G/E-D to suppress expression of BSP and OCN in MC3T3-E1, this extract enhanced OPN expression. This response also can be attributed to TGFβ since this effect is also attenuated by the addition of antibody (Fig. 6). TGFβ has been reported to enhance OPN mRNA levels in a variety of cell types, including osteoblasts.[30,35] Although OPN is expressed in several cell types, its exact role in different tissues remains to be established.[40] In the case of mineralized tissues, current hypotheses as to function include acting as a regulator of osteoclast migration and attachment at resorption fronts,[41] and as a regulator of crystal growth.[42-45]

As discussed above, BSP and OCN expression was decreased in response to G/E-D extracts, in contrast to increased OPN mRNA in comparably treated cells. Evidence to date indicates that both BSP and OCN are more selective to mineralized tissues than OPN. The down-regulation of BSP and OCN genes by G/E-D extracts is another indication, in addition to the morphological changes, that G/E-D extracts inhibit the differentiation of MC3T3-E1 cells toward a mature osteoblast. BSP has been suggested to function as a cell adhesive molecule[46] and also as an initiator of crystal formation.[44] Recent OCN knock-out experiments suggest that OCN may function to regulate the degree of mineral formation. Osteocalcin-deficient mice exhibited increased bone mass when compared with their wild type littermate controls.[47]

In summary, the results of the studies presented here indicate that TGFβ and possibly other proteins associated with dentin can inhibit osteoblast maturation, in vitro. Future directions would include size fractionation of the G/E-D in an attempt to determine which factors present have inhibitory as well as stimulatory effects on osteoblast-like cells, as well as other cell types. The inhibitory activity in dentin extracts may be important for regulation of mineral formation both during development and during regeneration of the periodontium. For example, while highly speculative, it is possible that proteins within dentin have the capacity to regulate cementum formation, allowing for development of a functional periodontal ligament with insertion of fibers between surrounding alve-

olar bone and tooth versus a fusion of surrounding bone with root cementum; i.e., ankylosis. Currently, the mechanisms and cells regulating cementum formation are not known, but 2 major theories are 1) that the epithelial root sheath cells and related products control cementum formation; i.e., epithelial-mesenchymal interactions; and 2) that the dentin extracellular matrix can regulate cementum formation. In the mature periodontium, dentin proteins may have a similar influence on PDL cells. In support of this, in vitro studies indicate that TGFβ can stimulate proliferation of PDL cells to a significantly greater extent than proliferation of gingival fibroblasts, and can enhance the proliferative response of PDL cells to platelet derived growth factor.[48,49]

Predictable restoration of periodontal tissues is the ultimate goal of periodontal regenerative therapies. Continued studies on the influence of molecules derived from bone, cementum, dentin, or enamel on cells associated with these tissues during development, maintenance, and regeneration should provide information needed to develop improved treatment modalities for promoting regenerating of oral tissues.

Acknowledgments

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