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Transforming growth factor-β1 expression is up-regulated in maturation-stage enamel organ and may induce ameloblast apoptosis

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Transforming growth factor-β1 (TGF-β1) regulates a variety of cellular responses that are dependent on the developmental stage and on the origins of the cell or the tissue. In mature tissues, and especially in tissues of epithelial origin, TGF-β1 is generally considered to be a growth inhibitor that may also promote apoptosis. The ameloblast cells of the enamel organ epithelium are adjacent to and responsible for the developing enamel layer on unerupted teeth. Once the enamel layer reaches its full thickness, the tall columnar secretory-stage ameloblasts shorten, and a portion of these maturation-stage ameloblasts become apoptotic. Here we investigate whether TGF-β1 plays a role in apoptosis of the maturation-stage ameloblasts. We demonstrate *in vitro* that ameloblast lineage cells are highly susceptible to TGF-β1-mediated growth arrest and are prone to TGF-β1-mediated cell death/apoptosis. We also demonstrate *in vivo* that TGF-β1 is expressed in the maturation-stage enamel organ at significantly higher levels than in the earlier secretory-stage enamel organ. This increased expression of TGF-β1 correlates with an increase in expression of the enamel organ immediate-early stress-response gene and with a decrease in the anti-apoptotic Bcl2 : Bax expression ratio. We conclude that TGF-β1 may play an important role in ameloblast apoptosis during the maturation stage of enamel development.

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Enamel development progresses through defined stages that can be observed by the changing morphology of the enamel organ that covers the developing tooth. The ameloblasts of the enamel organ are adjacent to the developing enamel and are responsible for organizing and later degrading the extracellular organic enamel matrix so that the enamel can mature into its highly mineralized form. Mature enamel is the hardest substance in the body. Its hardness is intermediate between that of iron and carbon steel, but it has higher elasticity (1).

As ameloblasts proceed through the stages of enamel development, the enamel matures from a soft cheese-like substance into its final hardened form. Presecretory ameloblasts stop dividing and enlarge into tall columnar secretory ameloblasts that align more or less vertically to the adjacent enamel and are responsible for secreting enamel matrix proteins. During the maturation stage, the ameloblasts become shorter and assist in removal of proteins from the matrix as the enamel hardens (2, 3). Once the enamel is fully mature, ameloblasts regress and become part of the reduced enamel organ that covers and protects the completed enamel surface until the tooth erupts (4). It is during the transition stage, when the ameloblasts shorten from tall secretory cells to short maturation-stage cells, that the ameloblasts start to undergo apoptosis. Approximately 25% of ameloblasts are lost during the short transition stage and approximately another 25% of ameloblasts are lost throughout the remainder of the maturation stage (5).

Previously, expression of transforming growth factor-β1 (TGF-β1) and/or Smad was demonstrated in tissues responsible for tooth and dental enamel development (6–16). Active TGF-β1 has been characterized as a 25 kDa dimer in which the two polypeptides interact via a disulfide bond and hydrophobic interactions. Transforming growth factor-β1 initiates signaling by interacting with and complexing two receptor serine/threonine kinases, referred to as type I and type II receptors. This binding initiates sequential kinase activity that results in receptor-regulated Smad phosphorylation/activation. The activated Smad proteins may accumulate in the nucleus where they may collaborate with other transcription factors to regulate gene transcription, as reviewed previously 17, 18. Transforming growth factor-β1-mediated gene transcription is temporally and spatially regulated and is dependent on the stage of development and on the developmental origins of the affected cell type. Transcriptome analyses have

demonstrated that TGF-β1 may lead to the activation or repression of several hundred genes in a given cell type (19–24). A unifying factor is that, in general, TGF-β1 is considered to promote growth inhibition. However, genetic evidence from gene-ablation studies of TGF-β1 signaling components does not indicate a role for TGF-β1 as a growth inhibitor in early embryogenesis. It is usually later, after the tissues have matured, that TGF-β1 will induce growth arrest and/or cell death (18).

Although several studies have demonstrated TGF-β1 and/or Smad expression in enamel organ cells during dental enamel development, very few of these studies have examined TGF-β1 expression during the maturation stage of enamel formation. One recent study demonstrated that over-expression of Smad7, a potent TGF-β1 inhibitor, caused tooth and enamel malformations that appeared to occur during the latter stages of enamel development (13). Another study demonstrated that over-expression of TGF-β1 in mouse teeth caused ameloblasts to detach from forming enamel, resulting in the abnormal deposition of maturation-stage mineral as 'amorphous structures' (10). Even so, few studies have examined TGF-β1 expression at the later stages of enamel formation and no previous study has rigorously compared TGF-β1 expression levels between the secretory and maturation stages of enamel development.

In the present study we investigated whether maturation-stage ameloblasts, relative to secretory-stage ameloblasts, express increased levels of TGF-β1 that may mediate ameloblast apoptosis during the maturation stage of enamel development. Furthermore, we investigated whether TGF-β1 expression is associated with an increase in stress-response gene expression, which may play a role in apoptotic events associated with maturation-stage ameloblasts.

Material and methods

The Animal Care Committee of The Forsyth Institute approved the protocol for the handling, care, and usage of animals.

Cell growth assay

The mouse ameloblast-lineage cell line (ALC) was cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 50 units ml^{-1} of penicillin, and 50 μg ml^{-1} of streptomycin. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Sigma, St Louis, MO, USA) were performed to assess cell proliferation. MTT is a tetrazolium salt that is turned into a colored dye by mitochondrial dehydrogenase enzymes. The amount of colored dye produced is proportional to the number of living cells. ALC cells were plated in 96-well plates for 18 h. Transforming growth factor-β1 (0.00, 0.10, 0.50, 1.00, or 5.00 ng ml^{-1}) was then added to the respective wells. After 24 h, cell growth was determined by measuring MTT reductase activity. Briefly, MTT (0.5 mg ml^{-1} final concentration) was added and the resulting absorbance was measured, after 3.5 h, at a wavelength of 550 nm using a microplate reader (POLARstar Optima; BMG Labtech, Durham, NC, USA). Six wells were analyzed and the mean value was calculated for each TGF-β1 concentration. Experiments were performed in triplicate and were repeated three times.

Cell survival assay

ALC cells in DMEM containing 2% FBS were plated at a density of 1,000 cells in 25 cm^2 flasks for 18 h and were exposed to concentrations of TGF-β1 from 0 to 1 ng ml^{-1} for a period of 24 h. Cells were washed with phosphate-buffered saline (PBS) and allowed to grow in medium containing 2% FBS for approximately 12–14 d, with a change of medium every 3 d. The resulting colonies were stained with 0.5% methylene blue in 50% methanol and counted. Percentage cell survival was then calculated (25, 26).

Immunohistochemistry

Immunohistochemical methods were used to identify TGF-β1, Smad2/3, and phosphorylated c-Jun (p-c-Jun) in ameloblasts of the continuously erupting mouse incisor enamel organ. Mouse incisors were formalin-fixed, decalcified in a solution of 20% sodium citrate/4% formic acid, paraffin-embedded, and sectioned. Sections were incubated for 1 h in blocking agent (10% goat serum in PBS), then overnight in antiserum that detects TGF-β1 (LifeSpan Biosciences, Seattle, WA, USA), Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or p-c-Jun (Santa Cruz), in peroxidase-conjugated antibody (Vectastain Elite Reagent; Vector Laboratories, Burlingame, CA, USA). Staining was visualized by incubation in Sigma Fast DAB substrate with counterstaining in 0.1% FastGreen for 2 min. Sections were then examined by light microscopy for the presence of each protein.

Quantitative real-time polymerase chain reaction

Enamel organs were dissected from the mandibular first molars of 4-d-old mice (secretory stage) or from the mandibular first molars of 11-d-old mice (maturation stage). Six or seven mice were used per stage per experiment. Total enamel organ RNA was extracted using Trizol (Invitrogen) and converted to cDNA using the superscript III first-strand synthesis system (Invitrogen) for quantitative real-time PCR (qPCR) analysis. The primer sequences used in expression analyses are shown in Table 1. The PCR temperature profile was 3 min at 95°C for the initial melt; 20 s at 95°C, 30 s at 60–66°C for 45 cycles, then 30 s at 95°C for 1 cycle; and 1 min at 55°C, followed by stepwise temperature increases from 55 to 95°C to generate the melt curve. Standard curves were generated with each primer set by use of untreated control cDNA preparations and a 10-fold dilution series ranging from 100 ng ml^{-1} to 100 pg ml^{-1}. Polymerase chain reaction efficiencies and relative expression levels of the selected genes as a function of the internal reference control gene (eEF1α1) expression were calculated as previously described (27).

Statistical analyses

Statistical analyses were performed using PRISM software (GraphPad prism version 5.0; GraphPad Software, La Jolla, CA, USA). Statistical significance was evaluated by

Table 1

Quantitative polymerase chain reaction (qPCR) primers for gene expression analysis

Primer	GenBank acc. no.	Sequence (5′–3′)
Bax	NM_007527.3	F: CCGGCGAATTGGAGATGAACTG R: AGCTGCCACCCGGAAGAAGACCT
Bcl2	NM_009741.3	F: TGTCACAGAGGGGCTACGAGT R: TCAGGCTGGAAGGAGAAGATG
eEF1α1	NM_010106.2	F: ATTCCGGCAAGTCCACCACAA R: CATCTCAGCAGCCTCCTTCTCAAAC
Egr-1	NM_007913.5	F: AGGGGAGCCGAGCGAACA R: GAGAAGCGGCCAGTATAGGTGATG
c-Fos	NM_010234.2	F: AGCAAAAACAAAACAAAACAAAACAAAA R: CTAAGGAGAAAGAGAAAAGAGACACAGACC
c-Jun	NM_010591.2	F: CGACGTCGGGCTGCTCAAG R: GGTGGGGGTCGGTGTAGTGGT
c-Myc	NM_010849.4	F: AGACACCGCCCACCACCAG R: AGCCCGACTCCGACCTCTTG
TGF-β1	NM_011577.1	F: AGGACCTGGGTTGGAAGTGGAT R: AAGCGCCCGGGTTGTGTT

F, forward; R, reverse; TGF- β1, transforming growth factor-β1.

performing a non-parametric analysis of variance (ANOVA) followed by Bonferroni's post test.

Results

ALC growth arrest and cell death after treatment with TGF-β1

We initially used an *in vitro* experimental model to determine if ameloblasts are sensitive to the effects of TGF-β1. The ALC line is a spontaneously immortalized epithelial cell line from mouse enamel organ that was previously demonstrated to have alkaline phosphatase activity and was shown to form calcified nodules in long-term culture (28). It therefore possesses several properties present in normal ameloblast cells. We utilized ALC cells to determine whether they were sensitive to treatment with TGF-β1.

ALC cells were treated in triplicate with incremental doses of TGF-β1 for 24 h and were then assessed for proliferation by use of the MTT assay. As shown in Fig. 1A, ALC cells were very sensitive to TGF-β1-mediated growth arrest. Cell proliferation was significantly inhibited by treatment with as little as 0.05 ng ml^{-1} of TGF-β1 and doses above that level resulted in proportionately less cell growth (Fig. 1A). We then investigated whether treatment with TGF-β1 caused ALC cell death. For these experiments, ALC cells were again treated with incremental doses of TGF-β1 for 24 h and were then plated at limiting dilutions into small culture flasks. After 12–14 d, the number of colonies that grew in each flask was quantified. A decrease in the number of TGF-β1-treated cell colonies relative to the untreated control served as an indication of cell death. Figure 1B shows that treatment with TGF-β1 caused a significant amount of ALC cell death, even at the lowest dose tested (0.01 ng ml^{-1}), with higher doses eliciting progressively increased cell death levels.

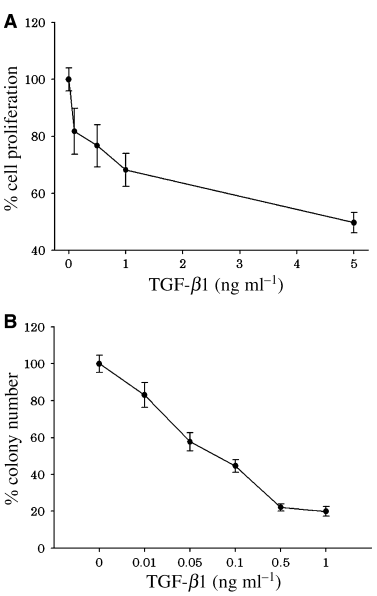

Fig. 1. Antiproliferative and toxic effects of transforming growth factor-β (TGF-β1) on ameloblast-lineage cell line (ALC) cells. (A) To assess cell proliferation, ALC cells were seeded into 96-well plates and treated for 24 h with the indicated concentrations of TGF-β1. Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan dye by mitochondrial enzymes was quantified for each well by measurements of the absorbance (A) at 550 nm and the results were used to calculate percentage cell proliferation (treated A_{550}/untreated A_{550}) × 100. Error bars represent the standard error of the mean. (B) To assess cell survival, limiting dilutions of ALC cells were seeded into culture flasks, allowed to adhere for 18 h, and then treated for 24 h with the indicated concentrations of TGF-β1. After 12–14 d, the resulting colonies were stained, counted, and percentage cell survival was calculated (number of treated colonies /number of untreated colonies) × 100. Error bars represent the standard error of the mean.

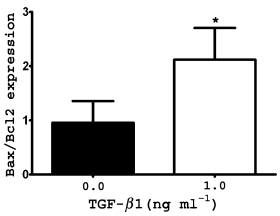

Fig. 2. Quantification, by quantitative real-time polymerase chain reaction (qPCR) analysis, of Bax : Bcl2 expression ratio in ameloblast-lineage cell line (ALC) cells treated with transforming growth factor-β1 (TGF-β1) for 24 h. Bax or Bcl2 expression was determined and each were normalized to the expression levels of the internal reference control gene (eEF1α1). The bar charts show the Bax : Bcl2 expression ratio, with and without treatment for 24 h with 1 ng ml^{-1} of TGF-β1. Six wells were assayed for each experimental treatment and error bars represent the standard error. *Statistical significance ($P < 0.01$).

Bax : Bcl2 ratios in TGF-β1-treated ALC cells

To determine if the TGF-β1-mediated cell death response in ALC cells was caused by apoptosis, we quantified the level of the pro-apoptotic Bax transcripts vs. the anti-apoptotic Bcl2 transcripts before and after treatment (for 24 h) with TGF-β1 (1 ng ml^{-1}). Quantitative PCR was performed, with eEF1α1 as the internal reference control mRNA, to generate the results presented in Fig. 2. Treatment with TGF-β1 significantly ($P < 0.01$) increased the Bax : Bcl2 ratio, indicating that the TGF-β1-mediated ALC cell death was caused by apoptotic events (Fig. 2). As ALC cells are a spontaneously immortalized cell line, results generated with these cells are not necessarily representative of an *in vivo* ameloblast response. We therefore characterized TGF-β1 and the expression of stress-response genes in mouse secretory-stage and maturation-stage enamel organs.

Immunohistochemical assessment of TGF-β1 expression in mouse incisors

Because it was demonstrated previously that ameloblasts undergo apoptosis during transition through maturation stages of enamel development, we wanted to confirm that TGF-β1 is expressed during these developmental stages and we also investigated whether higher levels of expression of TGF-β1 were present at these stages relative to the earlier secretory stage of enamel development. We performed immunohistochemical experiments on adult demineralized mouse incisors to identify the location of the TGF-β1 protein (Fig. 3). In general, the tooth tissues displayed diffuse staining. However, it did appear that a greater amount of staining for TGF-β1 was present within the cytoplasm of the maturation-stage ameloblasts (Fig. 3D) than was present within the cytoplasm of secretory-stage ameloblasts (Fig. 3C).

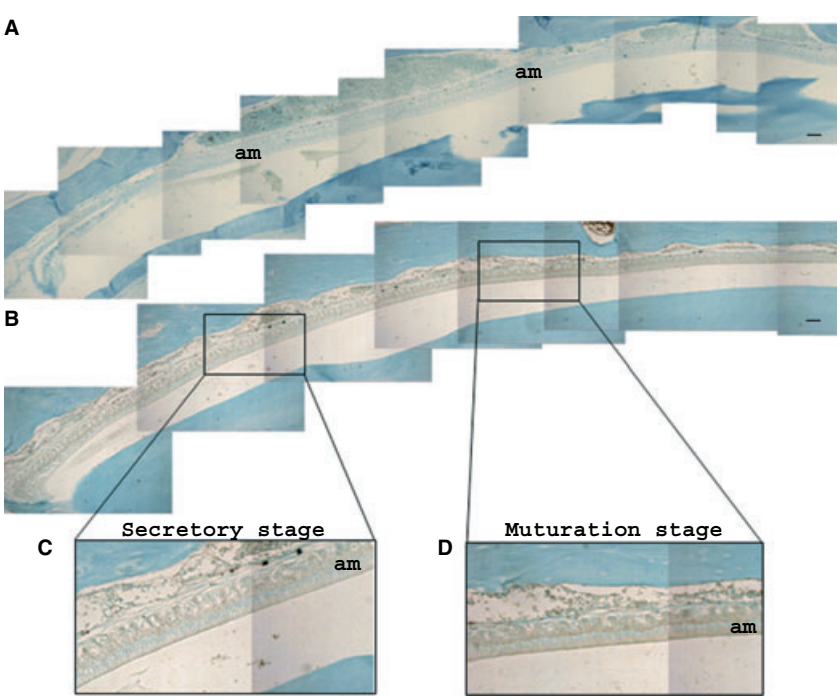

Fig. 3. Identification of transforming growth factor-β1 (TGF-β1) in ameloblast cells of the enamel organ as determined using immunohistochemistry analyses. (A) Negative control that lacked treatment with primary antisera. Note that some background staining was observed above the ameloblasts (am). (B) Staining for TGF-β1 in an adult mouse mandibular incisor (20× magnification). (C) Magnified image of the indicated secretory-stage ameloblasts from panel B. (D) Magnified image of the indicated maturation-stage ameloblasts from panel B. Note that staining appeared to be enhanced for TGF-β1 in the ameloblast cytoplasm at the maturation stage of enamel development compared with the secretory stage of enamel development. Scale bars represent 120 μm.

Quantification of TGF-β1 expression in mouse enamel organs as a function of developmental stage

To quantify any difference in TGF-β1 abundance, we performed qPCR analyses for TGF-β1 on mouse enamel organ mRNA that was isolated from the first molars of 4-d-old mice (predominately secretory stage) and 11-d-old mice (predominately maturation stage). Quantitative PCR of TGF-β1 mRNA isolated from kidney epithelia served as the negative control. The amount of TGF-β1 transcripts in the 11-d-old maturation-stage enamel organ was almost double ($P < 0.01$) that observed in the 4-d-old secretory-stage enamel organ (Fig. 4A). By contrast, no significant difference in TGF-β1 expression was observed between the 4-d-old kidney epithelia and the 11-d-old kidney epithelia (Fig. 4B). Therefore, the ameloblast apoptosis observed during the maturation stage of enamel development is consistent with increased levels of TGF-β1 expression.

Expression of immediate-early stress-response genes in mouse enamel organs as a function of developmental stage

Because TGF-β1 expression was increased during the maturation stage and because the ameloblasts undergo apoptosis during this stage, we investigated whether immediate-early stress-response genes were also up-regulated from the secretory to maturation stages of development. Quantitative PCR expression analysis demonstrated that c-Jun (Fig. 5A), c-Fos (Fig. 5B), and Egr-1 (Fig. 5C), but not c-Myc (Fig. 5D), were expressed at significantly higher levels ($P < 0.01$) during the maturation stage (i.e. in 11-d-old enamel organ) when compared with the secretory stage (i.e. in 4-d-old enamel organ) of enamel development. This suggests that maturation-stage ameloblasts are under an increased level of stress as the enamel matures.

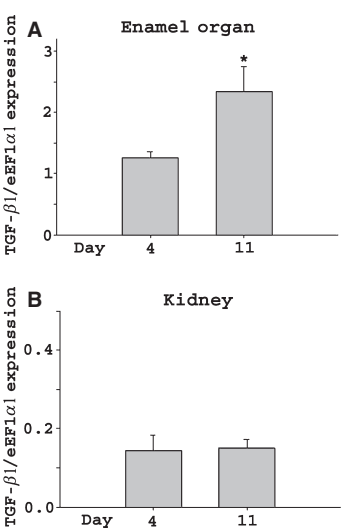

Fig. 4. Quantification, by quantitative real-time polymerase chain reaction (qPCR) analysis, of transforming growth factor-β1 (TGF-β1) expression in mouse enamel organ during the secretory and maturation stages of enamel development. (A) Total RNA from first molar enamel organs was extracted from 4-d-old mice (secretory stage) or from 11-d-old mice (maturation stage) and was assessed for TGF-β1 expression levels. (B) Total RNA was also extracted from the kidney of 4- and 11-d-old mice. The kidney served as an epithelial-derived negative control for the enamel organ results. Each expression-analysis result was normalized to the expression level of the internal reference control gene (eEF1α1). Six different samples were assayed for each tissue, and bars representing the standard error are shown. *Statistical significance ($P < 0.01$).

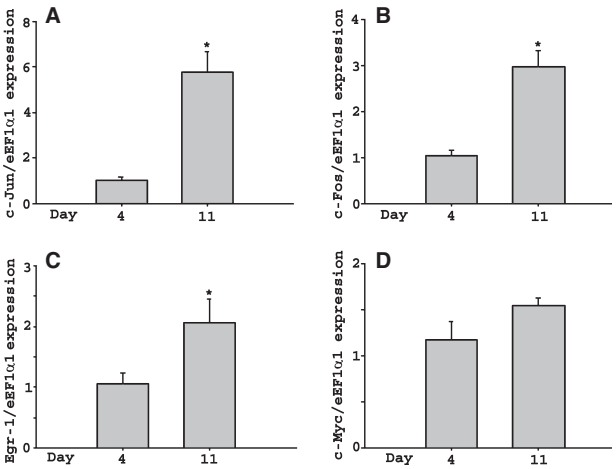

Fig. 5. Quantification, by quantitative real-time polymerase chain reaction (qPCR) analysis, of immediate-early stress-response gene expression in mouse enamel organ during the secretory and maturation stages of enamel development. Total RNA extracted from first molar enamel organs from 4-d-old mice (secretory stage) and 11-d-old mice (maturation stage) was assessed for gene expression. (A) c-Jun, (B) c-Fos, (C) Egr-1, and (D) c-Myc. Each expression-analysis result was normalized to the expression level of the internal reference control gene (eEF1α1). Six samples were assayed for each tissue for each gene and error bars show the standard error. Note that c-Jun expression increased at the maturation stage to almost six times over that observed in the secretory stage of enamel development. *Statistical significance ($P < 0.01$).

Increased levels of p-Smad2/3 and p-c-Jun in maturation-stage ameloblasts

In this part of the study we investigated whether TGF-β1 signals through the TGF-β/activin receptor pathway in the mouse enamel organ. We performed immunohistochemistry to determine if increased levels of Smad2 or Smad3 were present in maturation-stage ameloblasts. Ameloblast staining dramatically increased in the maturation stage when compared with the staining observed in ameloblasts of the secretory stage, suggesting that TGF-β1 does signal through this receptor-mediated pathway (Fig. 6).

Smad3 was previously demonstrated to synergize with c-Jun at AP-1 transcription factor binding sites within gene promoters to initiate transcriptional activation by TGF-β1 (29). Because c-Jun is an immediate-early response gene that can play a role in several stress-response pathways, we sought to confirm that active p-c-Jun was present within maturation-stage ameloblasts. Phosphorylated c-Jun did locate primarily to the

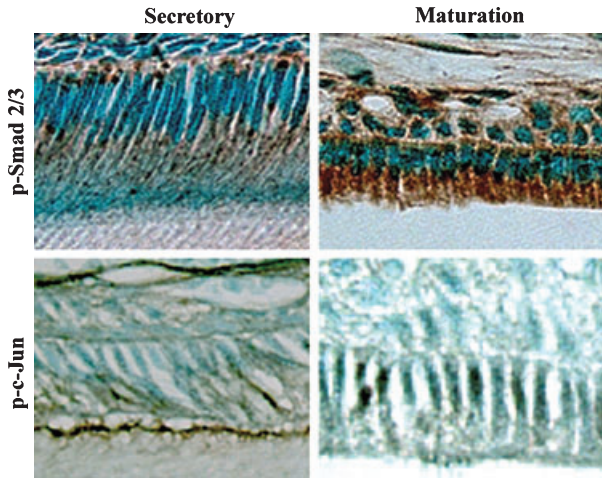

Fig. 6. Identification of p-Smad2/3 and phosphorylated c-Jun (p-c-Jun) in ameloblast cells of the enamel organ, as determined using immunohistochemistry analyses. (Top panels) Staining for p-Smad2/3 in an adult mouse mandibular incisor (40× magnification). (Bottom panels) Staining for p-c-Jun in an adult mouse mandibular incisor (40× magnification). Note that staining for Smad2/3 was dramatically increased in the maturation-stage ameloblasts and that increased p-c-Jun nuclear staining was present in the maturation-stage ameloblasts when compared with secretory-stage ameloblasts.

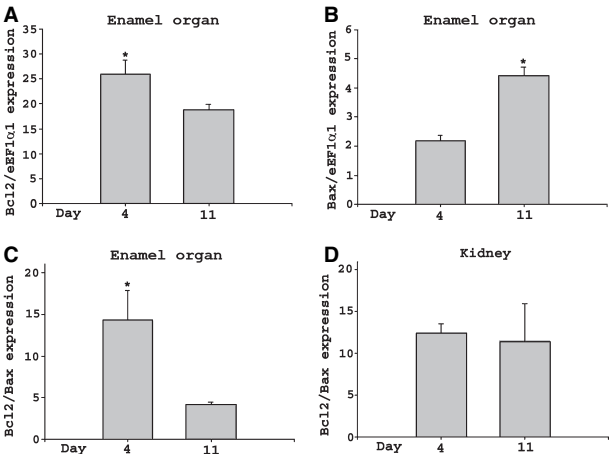

Fig. 7. Quantification, by quantitative real-time polymerase chain reaction (qPCR) analysis, of Bcl2 and Bax gene expression in mouse enamel organ during the secretory and maturation stages of enamel development. Total RNA extracted from first molar enamel organs from 4-d-old mice (secretory stage) and 11-d-old mice (maturation stage) was assessed for gene expression. (A) Bcl2, (B) Bax, and (C) Bcl2 : Bax ratio. (D) The kidney served as an epithelial-derived negative control for the enamel-organ results for Bcl2 : Bax. Each expression analysis result was normalized to the expression level of the internal reference control gene (eEF1α1). Six samples were assayed for each tissue for each gene and error bars indicate the standard error. Note that that the greatly decreased ratio of Bcl2 expression to Bax expression in the maturation-stage enamel organ favors apoptosis. *Statistical significance ($P < 0.01$).

nucleus of maturation-stage ameloblasts (Fig. 6) and therefore may synergize with Smad3 to activate the transcription of selected genes, including stress-response genes.

Bcl2 : Bax ratios in mouse enamel organs as a function of developmental stage

Finally, we investigated whether TGF-β1 expression in the mouse enamel organ correlates positively with pro-apoptotic Bax expression and negatively with anti-apoptotic Bcl2 expression, as occurred for the ALC cells (Fig. 2). The expression of Bcl2 was reduced significantly ($P < 0.01$) in the 11-d-old enamel organ compared with the 4-d-old enamel organ (Fig. 7A), whereas the expression level of Bax was significantly increased (Fig. 7B). The ratio of Bcl2 : Bax expression in enamel organ also displayed a highly significant reduction in 11-d-old mice compared with 4-day-old mice (Fig. 7C), but no significant difference was observed between these age groups in the kidney (Fig. 7D).

Taken together, the results are consistent with a role for TGF-β1 in signaling through the TGF-β/activin pathway to induce immediate-early stress-response genes that lead to a reduction in Bcl2 expression and an induction of Bax expression. Thus, TGF-β1 may be responsible for the observed increase in apoptosis of ameloblasts during the maturation stage of enamel development.

Discussion

Transforming growth factor-β regulates a wide variety of cellular processes, including cell growth, growth arrest, apoptosis, differentiation, migration, and extracellular matrix production, as reviewed previously 18, 30. In the present study we demonstrated that treatment of cells of the ameloblast lineage cell line with TGF-β1 significantly reduced cell growth and cell survival in a dose-dependent manner (Fig. 1). Furthermore, we showed that TGF-β1 increases the Bax : Bcl2 ratio to favor apoptosis in ALC cells. Members of the Bcl2 family bind to Bax to prevent its activation and subsequent cell apoptosis. Bax normally resides in an inactive state within the cytoplasm, but when activated it inserts into the mitochondrial outer membrane and forms pores that release cytochrome *c* and other pro-apoptotic factors into the cytoplasm. Therefore, the Bcl2 : Bax ratio in a cell may determine its susceptibility to survival or apoptosis 17. Previously, it was demonstrated that a decrease in Bcl2 levels was observed in TGF-β1-mediated apoptosis in a variety of cell types, including human lens epithelial cells (31), multipotent hematopoietic cell lines (32), and thecal/interstitial cells (33). In addition, TGF-β1-mediated induction of Bax was demonstrated in bovine epithelial BME-UV1 cells (34). Our results demonstrate that, in addition to the above listed cell types, TGF-β1-treated ameloblast-lineage cells also display decreased Bcl2 expression and increased Bax expression.

To ascertain the relevance of the results generated *in vitro* to the situation *in vivo*, we performed TGF-β1 immunohistochemistry on mouse incisors (Fig. 3) and qPCR analysis for gene expression on mouse first molar enamel organs that were either predominantly in the

secretory (4-d-old) or in the maturation (11-d-old) stages of enamel development (Figs 4, 5, and 7). Because a proportion of ameloblasts undergo apoptosis during the maturation stage (5, 35, 36), we investigated whether TGF-β1 expression was up-regulated during this stage. The immunohistochemical results suggested that TGF-β1 was up-regulated and the qPCR analysis definitively demonstrated that the maturation-stage enamel organ expressed more TGF-β1 than did the secretory-stage enamel organ (Fig. 4). Previous studies have demonstrated that TGF-β is expressed in the murine enamel organ (6, 13, 14, 16) and a recent study demonstrated that over-expression in epithelial tissues of Smad7, which negatively regulates TGF-β1 signaling, resulted in the development of malformed teeth and enamel (13). Our results are the first to use qPCR analysis to show that TGF-β1 expression becomes up-regulated during the maturation stage of enamel development when a proportion of ameloblasts become apoptotic.

Because previous studies have shown that exposure to TGF-β1 may induce the expression of immediate-early stress-response genes (20–22, 24, 34, 37–39), we sought to determine whether selected immediate early genes were induced in the maturation-stage enamel organ when compared with the expression levels observed in the secretory-stage enamel organ. We found that the expression levels of c-Jun, c-Fos, and Egr-1 were significantly higher in the maturation-stage enamel. However, there was no significant difference in c-Myc expression (Fig. 5). c-Fos and c-Jun are members of the AP-1 transcription factor family. They bind to AP-1 sites and may also interact with Smads to activate transcription in response to TGF-β signaling (39). c-Jun was previously shown, using immunohistochemical methods, to become more prevalent in rat maturation-stage ameloblast nuclei when compared with secretory-stage ameloblasts (40), and analysis of Smad3 null mice found that Smad3 is required for enamel biomineralization (16). Thus, our data support and confirm these findings. Egr-1 is a zinc finger transcription factor early growth response gene. Transforming growth factor-β1 was demonstrated to induce both protein and mRNA expression of Erg-1 in human skin fibroblasts (37). Interestingly, c-Myc is a proto-oncogene that promotes the cell cycle and simultaneously primes activation of the mitochondria-mediated apoptosis pathway (41). In general, TGF-β1 is an inhibitor of c-Myc (42–44). However, under specific instances, such as in dermal fibroblasts, TGF-β1 will induce c-Myc expression (24). Although we found a trend for increased expression of c-Myc in maturation-stage enamel organ vs. secretory-stage enamel organ, the trend was not significant. Taken together, the expression level of the assayed immediate-early response genes is consistent with the increased expression of TGF-β1 in the maturation-stage enamel organ. This suggests that c-Jun, c-Fos, and Egr-1, and perhaps c-Myc through its apoptosis activity, may play a role in the downstream events leading to the apoptosis of maturation-stage ameloblasts.

Finally, we examined Bcl2 and Bax expression in mouse first molar secretory-stage enamel organ and in mouse first molar maturation-stage enamel organ. As for the *in vitro* TGF-β1-treated ALC cells (Fig. 2), we found that *in vivo*, the maturation stage, with the up-regulated TGF-β1 expression levels (Fig. 4), also had increased expression of Bax and decreased expression of Bcl2 (Fig. 7). This is consistent with the observed increased levels of apoptosis in the maturation stage of enamel development (5, 35, 36). One previous study showed that Bax and Bcl2 are expressed in the developing human enamel organ (45). This study demonstrated that during the early stages of enamel development, Bcl2 levels predominated over Bax. However, the study did not assess Bax : Bcl2 levels in maturation-stage enamel organ. Another study on developing rat enamel organs demonstrated, using TdT-mediated biotin–dUTP nick-end labelling (TUNEL) assays and immunohistochemistry, a positive correlation between Bax : Bcl2 abundance and ameloblast apoptosis (35). When Bax abundance was elevated relative to Bcl2, greater amounts of ameloblast apoptosis were observed. These data support our conclusion that TGF-β1 signaling, perhaps through Smad3 and immediate-early stress-response genes, increases the ratio of Bax to Bcl2 and plays a role in initiating apoptosis in maturation-stage ameloblasts.

In conclusion, we have demonstrated by both *in vitro* and, more importantly, *in vivo* experimental models that TGF-β1 probably plays a role in ameloblast apoptosis during the maturation stage of enamel development.

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