Insulin-like Growth Factor 1 and Transforming Growth Factor-β Stimulate Cystine/Glutamate Exchange Activity in Dental Pulp Cells

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Abstract

Introduction: The growth factors insulin-like growth factor (IGF-1) and transforming growth factor-β (TGF-β) are protective to dental pulp cells in culture against the toxicity of the composite materials Durafill VS and Flow Line (Henry Schein Inc, New York, NY). Because the toxicity of these materials is mediated by oxidative stress, it seemed possible that the protective effects of IGF-1 and TGF-β were through the enhancement of an endogenous antioxidant mechanism. **Methods:** We used cultured dental pulp cells to determine the mechanism of the protective effects of IGF-1 and TGF-β, focusing on the glutathione system and the role of cystine/glutamate exchange (system xc-). **Results:** We found that the toxicity of Durafill VS and Flow Line was attenuated by the addition of glutathione monoethylester, suggesting a specific role for the cellular antioxidant glutathione. Supporting this hypothesis, we found that IGF-1 and TGF-β were protective against the toxicity of the glutathione synthesis inhibitor buthionine sulfoximine. Because levels of cellular cystine are the limiting factor in the production of glutathione, we tested the effects of IGF-1 and TGF-β on cystine uptake. Both growth factors stimulated system xc–mediated cystine uptake. Furthermore, they attenuated the glutathione depletion induced by Durafill VS and Flow Line. **Conclusions:** The results suggest that IGF-1 and TGF-β are protective through the stimulation of system xc–mediated cystine uptake, leading to maintenance of cellular glutathione. This novel action of growth factors on dental pulp cells has implications not only for preventing toxicity of dental materials but also for the general function of these cells. *(J Endod 2011;37:943–947)*

Key Words

Cystine, dental pulp, glutathione, system xc-, toxicity

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Death of dental pulp cells after exposure to the environment and how it may be prevented are of great interest. Trauma, rapidly progressing caries, or overly aggressive restorative procedures can cause exposure of dental pulp often resulting in death of the pulp (1). The damaged dental pulp must then be removed by a root canal procedure. Treatment options to attempt to save the dental pulp after exposure involve pulp-capping therapies (2). The goal of these procedures is to use a pulp-capping material to stimulate the formation of a dentin bridge over the exposed pulp followed by the application of a restoration. Calcium hydroxide ($Ca[OH]_2$)-containing materials are widely used for pulp capping because of their ability to stimulate reparative dentin formation (3). However, $Ca(OH)_2$ has been found to be toxic to dental pulp cells (4). Reports of the success rate of pulp-capping treatments vary greatly (5), and the effectiveness of such treatment has been questioned (1). Clearly, better treatments are required. The use of mineral trioxide aggregate (MTA), originally developed as a root-end filling material, is gaining popularity as a pulp capping material (6). However, it has a long setting time (approximately 4 hours) that may limit its usefulness as a pulp-capping agent (7), and its long-term effects still need to be fully analyzed. A potential alternative or adjunct therapy is the use of growth factors in pulp-capping procedures. Growth factors are naturally occurring proteins that can alter cell proliferation, differentiation, maturation, and survival. Interestingly, they have been shown to stimulate odontoblast differentiation and dentin formation (8, 9). A potential advantage of growth factors over $Ca(OH)_2$ is that they stimulate the formation of reparative dentin that is primarily superficial to the pulp tissue, whereas $Ca(OH)_2$ effects are often at the expense of the pulp tissue (10). We have shown previously that a number of growth factors can alter pulp cell differentiation and make them resistant to the toxicity of restoration materials (11, 12).

In the current study, we set out to determine the mechanism of the protective effects of two growth factors: insulin-like growth factor (IGF-1) and transforming growth factor-β (TGF-β). We chose these growth factors because of their known effects on dental pulp cells. TGF-β stimulates primary odontoblasts (13), increases dentin formation (14), is expressed in developing teeth (15), and increases alkaline phosphatase activity and formation of mineralization nodules (16). IGF-1 has been shown to increase alkaline phosphatase activity in cultured dental pulp cells (17) and can enhance reparative dentin formation *in vivo* (18). Determining the mechanism by which growth factors are protective against dental material toxicity is important for understanding which growth factors should be used in conjunction with each dental material. The commonly used composite materials Durafill VS and Flow Line (Henry Schein Inc, New York, NY) are known to induce oxidative stress mediated death of pulp cells (12), and this served as the basis to explore the mechanism of protective effects of the growth factors. We focused on the role of the cystine/glutamate exchanger (system xc-), which regulates the influx of cystine, which is the rate limiting factor in the production of the main intracellular free radical scavenger, glutathione. The purpose of the current studies is to determine whether growth factors make dental pulp cells resistant to cell death by up-regulating system xc-.

Materials and Methods

Materials

Serum was obtained from Atlanta Biologicals (Atlanta, GA). Growth factors were obtained from ProSpec-Tany Technogene (Rehorot, Israel). All other chemicals were obtained from Sigma (St Louis, MO).

Subjects and Human Dental Pulp Cell Cultures

Normal human impacted third molars were collected from adults at the Marquette University School of Dentistry Surgical Services Department with informed consent under a protocol approved by the Institutional Review Board at Marquette University. Ten third molars from six patients were used in the current students. Tooth surfaces were cleaned and cut around the cementum-enamel junction by using sterilized diamond stones to access the pulp chamber. The pulp tissue was separated from the tooth and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 hour at 37°C (11, 19). The cells were plated onto 24-well plates coated with poly-D-lysine and laminin in Eagle medium supplemented with 20% fetal calf serum/100 μmol/L L-ascorbic acid 2-phosphate/2 mmol/L L-glutamine/100 U/mL penicillin/100 μg/mL streptomycin, and then incubated at 37°C with 5% CO_2. Growth factors (100 ng/mL) were added at the time of plating on 24-well plates for experiments. Experiments were performed on cultures 7 to 9 days *in vitro*.

Preparation of Dental Materials

Flow Line and Durafill VS were prepared according to the manufacturer's instructions. Briefly, they were dispensed on a sterile glass slab and light cured with a visible light curing gun from 3M Unitek for 60 seconds and cut into uniformly sized pieces.

Exposure of Dental Materials to Cell Cultures

Freshly prepared dental materials were placed into 96-well plates in 250 μL of media similar to plating media except lacking serum. After 24 hours, the media was removed from the 96-well plates and placed on the cells growing on 24-well plates for 6 hours (glutathione assays) or for 48 hours (toxicity assays). For experiments in which glutathione monoethylester was tested, it was present during the 48-hour exposure of the cells to the dental material treated media. The weights of the materials used were the following: Flow Line, 9.5 $\pm$ .4 mg, and Durafill VS, 10.0 $\pm$ .4 mg.

Cell Viability Assessment

Cell injury was quantified by the measurement of the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) to produce a dark blue formazan product (20). MTT was added to each well 48 hours after the beginning of insult to the cells. After a 30-minute incubation, the media was removed, and cells were dissolved in dimethyl sulfoxide. The formation of formazan was measured as the amount of reaction product by absorbance change at a wavelength of 590 nm by using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). Levels of formazan formation from cultures exposed to 10 μmol/L of the calcium ionophore A23187 (100% cell death) were subtracted from insult formazan levels, and results were normalized to control (12, 21). The possibility exists that cultures prepared from different teeth have different properties. To mitigate this potential complication, all experiments include control conditions on the same 24-well plate. Therefore, cell death for each experimental condition is compared with a control from the same pulp source.

^{14}C-Cystine Uptake

The uptake of cystine was measured by exposure of cultures to ^{14}C-cystine (0.1 μCi/mL) for 20 minutes. After the exposure to ^{14}C-cystine, the cultures were washed three times and dissolved in 1% sodium dodecyl sulfate (250 μL). An aliquot (200 μL) was removed and added to scintillation fluid for counting. Values were normalized to control ^{14}C-cystine uptake (20-minute exposure to ^{14}C-cystine without growth factor or dental material treatment).

Monochlorobimane Assay of Cellular Glutathione

Cellular glutathione levels were measured by monochlorobimane (MCB) fluorescence. MCB forms a fluorescent compound when it reacts with glutathione through a reaction catalyzed by glutathione-S-transferase (22). MCB was added to the media after a 6-hour treatment with dental materials, a time point before gross cell death occurs. After 30 minutes, the cultures were excited at a wavelength of 355 nm and emission measured at a wavelength of 460 nm using a Thermo Labsystems Fluoroskan microplate reader (Waltham, MA). Background (no MCB added) was subtracted and values normalized to control (MCB but no dental material present).

Statistical Analysis

Statistical calculations of the continuous variables assessed in the studies were performed using one-way analysis of variance followed by the Bonferroni correction post hoc test. Statistics were calculated using Sigma-Stat software (Systat Software, San Jose, CA). P values $<.05$ were considered to indicate significant differences.

Results

Durafill VS and Flow Line both caused significant toxicity after 48 hours of exposure as measured by the MTT metabolism assay (Fig. 1*A*). The toxicity of the dental materials was no longer observed in cultures that were treated with IGF-1 or TGF-β (Fig. 1*B* and *C*). Because the growth factors can alter the levels of MTT metabolism caused by changes in cell growth and differentiation, the effects of the dental materials are compared with the starting level of MTT metabolism with each growth factor treatment.

The toxicity of Durafill VS and Flow Line was attenuated by the addition of the cell permeable form of glutathione, glutathione monoethylester (Fig. 2*A*). This finding suggests a specific role for glutathione depletion in Durafill VS and Flow Line toxicity and raises the possibility that the protective effects of IGF-1 and TGF-β may be mediated by enhancing cellular glutathione levels. In support of this idea, we found that IGF-1 and TGF-β were also protective against toxicity induced by buthionine sulfoximine, an inhibitor of glutathione synthesis (Fig. 2*B*). A potential mechanism for this protection is provided by the result that IGF-1 and TGF-β both increased ^{14}C-cystine uptake (Fig. 3). The increased cystine uptake appears to be mediated by system xc- because the selective inhibitor of that system, sulfasalazine, blocked the stimulated uptake (Fig. 3). Because the levels of cellular cystine are the rate-limiting step for the synthesis of glutathione, it seemed possible that the protective effects of IGF-1 and TGF-β could be caused by an increased cystine uptake leading to the maintenance of cellular glutathione levels during an insult. Six-hour exposure to Durafill VS and Flow Line caused a significant decrease in cellular glutathione as measured by the fluorescent dye MCB (Fig. 4*A*). At this time point, there was no overt cell death as determined by a lack of release of the cytosolic enzyme lactate dehydrogenase (data not shown), a well-established method of assessing gross cell death (21). In cultures treated with IGF-1 or TGF-β, Durafill VS and Flow Line still caused a significant