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

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IGF-1 and TGF-B Stimulate Cystine/Glutamate Exchange Activity in Dental Pulp Cells

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Abstract

Introduction: The growth factors IGF-1 and TGF-β are protective to dental pulp cells in culture against the toxicity of the composite materials Durafill VS and Flow Line. Since 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 enhancement of an endogenous antioxidant mechanism.

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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 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. Since 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.

Keywords: dental pulp, toxicity, cystine, system xc-, glutathione

Introduction

Death of dental pulp cells following exposure to the environment, and how it may be prevented, is 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 following 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, while $Ca(OH)_2$ 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 (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 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 upregulating system xc-.

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Material and Methods

Materials

Serum was obtained from Atlanta Biologicals (Atlanta, GA, USA). Flow Line and Durafill VS were obtained from Henry Schein Inc. (Melville, NY, USA). Growth factors were obtained from ProSpec-Tany Technogene (Rehorot, Israel). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

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 6 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's medium supplemented with 20% fetal calf serum/100 μM L-ascorbic acid 2-phosphate/2 mM L-glutamine/100 units/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 7–9 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 where 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: Flow Line: 9.5 ± .4 mg; Durafill VS: 10.0 ± .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. 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 to a control from the same pulp source.

^{14}C-Cystine Uptake

Uptake of cystine was measured by exposure of cultures to ^{14}C-cystine (0.1 μCi/ml) for 20 minutes. Following the exposure to ^{14}C-cystine, the cultures were washed 3 times and dissolved in 1% SDS (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 (MCB) assay of cellular glutathione

Cellular glutathione levels were measured by 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 following a six 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. 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 ANOVA followed by the Bonferroni correction post-hoc test. Statistics were calculated using Sigma-Stat software. P-values <0.05 were considered to indicate significant differences.

Results

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

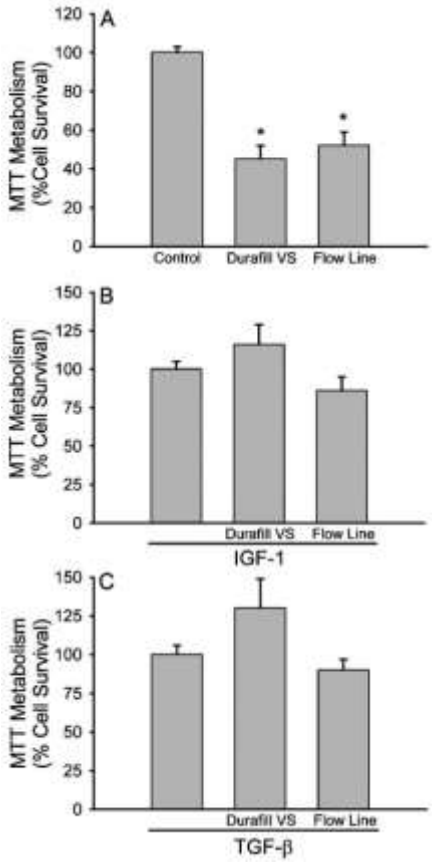

Figure 1 Durafill VS and Flow Line cause significant toxicity to dental pulp cells (A) which is eliminated by treatment with IGF-1 (B) or TGF-β (C). Dental materials were incubated in media for 24 hours at which time the conditioned media was placed on the cultures for 48 hours. Bars show % cell survival (mean + SEM, n=12–16) quantified by inhibition of MTT reduction. Control represents MTT levels in untreated cultures (no dental materials) and is defined as 100% cell survival. * indicates significant difference from control ($P < 0.05$).

The toxicity of Durafill VS and Flow Line was attenuated by addition of the cell permeable form of glutathione, glutathione monoethylester (Fig 2A). 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 (BSO), an inhibitor of glutathione synthesis (Fig 2B). 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-, as the selective inhibitor of that system, sulfasalazine, blocked the stimulated uptake (Fig 3). Since 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 due to 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 4A). At this time point there was no overt cell death as determined by 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 decrease in cellular glutathione, but the decrease was attenuated by the growth factor treatment (Fig 4B & C).

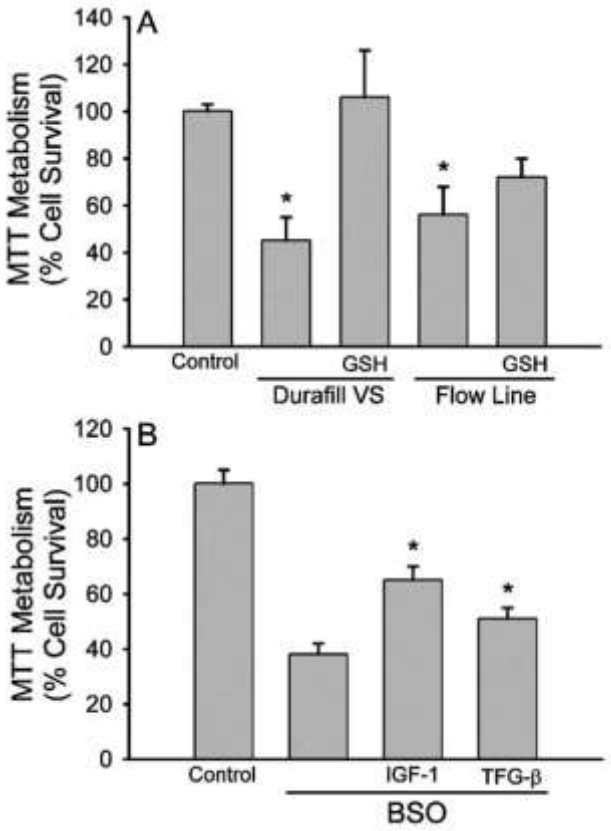

Figure 2 The toxicity of Durafill VS and Flow Line is attenuated by the cell permeable glutathione monoethylester (GSH, 100 μM) (A), while IGF-1 and TGF-β protect against the toxicity induced by inhibition of glutathione synthesis induced by 1

mM buthionine sulfoximine (BSO)(B). Control represents MTT levels in untreated cultures (no dental materials) and is defined as 100% cell survival. Bars show % cell survival (mean + SEM, n=8–16) quantified by inhibition of MTT reduction. * indicates significant difference from control (P < 0.05).

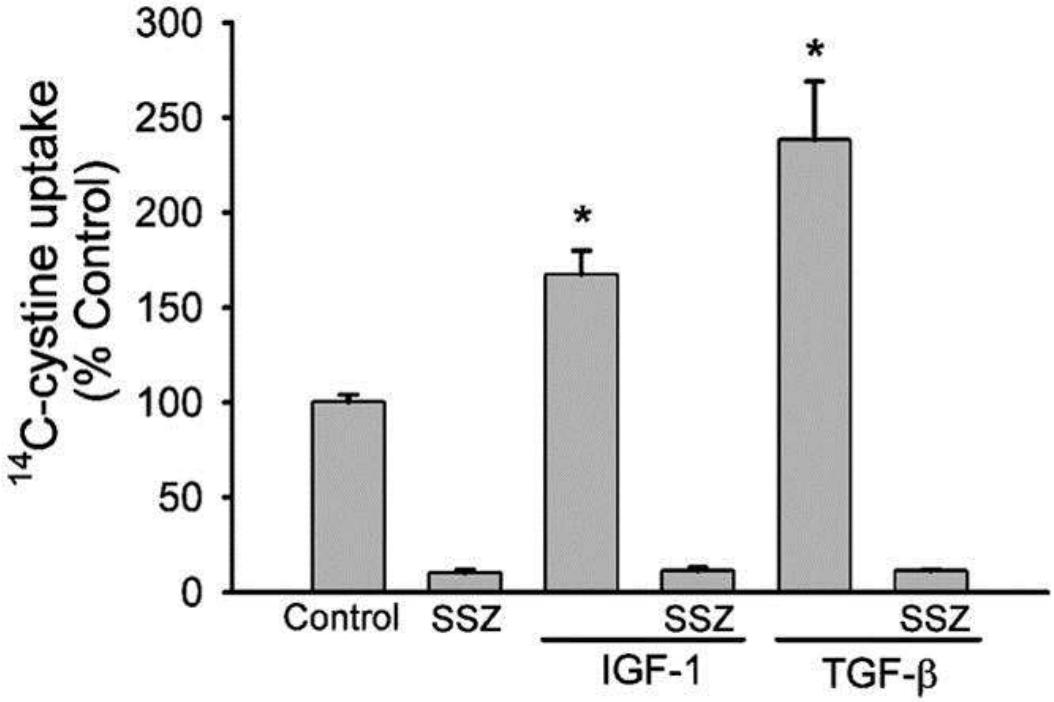

Figure 3 IGF-1 and TGF-β-glutamate exchange (system xc-) mediated 14C-cystine uptake. Sulfasalazine (SSZ, 200 μM) selectively inhibits cystine-glutamate exchange mediated cystine uptake. Bars show % 14C-cystine uptake normalized to control uptake (mean + SEM, n=12–24). *indicates significant difference from control 14C-cystine uptake (P < .05).

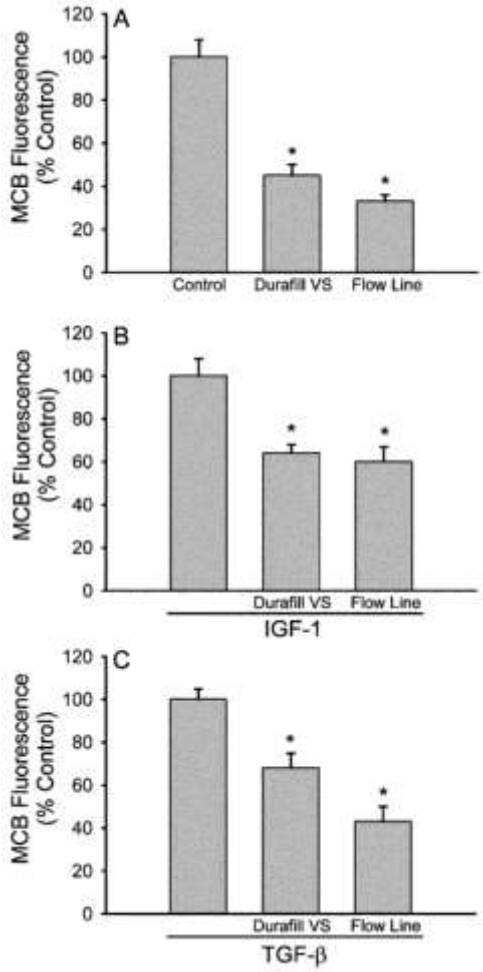

Figure 4 The decrease in cellular glutathione levels induced by Durafill VS and Flow Line (A) is attenuated by treatment with IGF-1 (B) or TGF-β (C). Total reduced glutathione is determined by MCB fluorescence. Bars show % MCB fluorescence normalized to control fluorescence (no dental material) (mean + SEM, n=8–16). * indicates significant difference from control ($P < .05$).

Discussion

The current studies demonstrate a novel mechanism of growth factor actions. That is, growth factors stimulate system xc- mediated cystine uptake in dental pulp cells making them resistant to cell death mediated by oxidative stress. We had shown previously that exposure to Durafill VS or Flow Line caused significant toxicity to dental pulp cells as measured by the MTT metabolism assay and that the toxicity of the dental materials was prevented by treatment with the growth factors IGF-1 and TGF-β (12). In the current studies we repeated

those experiments, with the change that to avoid possible physical damage to the cells from the dental materials, they are no longer placed directly on the cells but are instead incubated in media for 24 hours and that media is then placed on the cell cultures for 48 hours (12). The main finding of the present study is that the mechanism of the protective effect of the growth factors is the stimulation of system xc- function. This result has broad implications due to the critical effects of system xc- on cell function and survival.

System xc- mediates two important actions: the uptake of cystine and the release of glutamate. The levels of intracellular cystine are the rate limiting step in the production of the main intracellular free radical scavenger glutathione. Depletion of cellular glutathione has been implicated in multiple processes including neurodegenerative diseases and schizophrenia (23,24). An important aspect of the current studies is that growth factors are able to protect dental pulp cells against insults involving oxidative stress. Oxidative stress is a final common pathway of toxicity of many compounds, including dental materials (25). Oxidative stress has also been shown to be involved in the death of pulp cells induced by infection (26). Interestingly, we have found that IGF-1 and TGF-β are also protective against amalgam toxicity (11). While it is unclear whether amalgam toxicity is mediated by oxidative stress, mercury toxicity does involve oxidative stress (27). Also, we have found that amalgam toxicity can be mediated by release of zinc (28), and zinc toxicity involves oxidative stress (29). Many dental materials contain zinc, usually in the form of zinc oxide, which may account for some of their toxicity. Thus the ability to attenuate oxidative stress induced cell death is of general importance. The mechanism of protection by IGF-1 and TGF-β appears to be through increased uptake of cystine leading to better maintenance of cellular glutathione during oxidative insults. While there was still a decrease in glutathione levels caused by Flow line and Durafill VS after growth factor treatment, the decrease was attenuated. In three of the conditions (Flow Line after IGF-1 or TGF-β treatment and Durafill VS after IGF-1 treatment) the decrease in glutathione was significantly attenuated ($P < .05$), while in one of the conditions (Flow Line after TGF-β treatment) there was only a trend toward less glutathione decrease with growth factor treatment.

The other action of system xc- is the release of glutamate into the extracellular space in exchange for cystine. This is likely of great importance in the central nervous system where glutamate acts as the main excitatory neurotransmitter, but where excess extracellular glutamate can cause neuronal death through excitotoxicity. Release of glutamate from system xc- can play a role in this toxicity. For example, activated astrocytes release glutamate via system xc- that can kill cortical neurons (30) and microglia can release glutamate via xc- that can kill cerebellar granule cells (31). However, such toxicity is unlikely to occur in dental pulp cells since excitotoxicity is not known to occur in these cells. Dental pulp cells do have glutamate receptors so the altered extracellular glutamate associated with upregulation of system xc- may alter dental pulp cell function or differentiation. An example of such an action is in the immune system where glutamate release from dendritic cells via system xc- inhibits T cell activation through actions on mGluR5 receptors (32). Interestingly, mGluR5 receptors are also present in dental pulp, both on nerve endings and on odontoblasts (33).

Growth factors have the potential for multiple uses involving dental pulp cells. They have the ability to differentiate pulp cells into odontoblast-like cells and may potentially play a role in total tooth regeneration (34). They may also be useful for the specific application of stimulating the formation of reparative dentin making them potentially useful in pulp capping procedures. It is possible that the ideal pulp capping treatment will be a combination of growth factors and a bioinert compound. The idea being that the growth factors induce differentiation of the pulp cells to provide the formation of reparative dentin and protection against toxicity of restoration materials, while the bioinert compound provides physical protection of the cells. Both IGF-1 and TGF-β have been shown to stimulate reparative dentin formation in animal models (19, 35). Also, TGF-β expression is increased at the odontoblastic-subodontoblastic layer in teeth with irreversible pulpitis (Piattelli et a., 2004), suggesting that endogenous TGF-β is involved in dentine repair during pulp inflammation. The finding that IGF-1 and TGF-β increase cystine uptake, while also attenuating oxidative stress induced cell death and glutathione depletion, suggests that they may have broad protective effects. Since oxidative stress is a common final mechanism of cell death under multiple conditions, the results suggest that IGF-1 and

TGF-β should be considered during situations in which growth factors are being used to differentiate dental pulp cells because those cells would be more resistant to cell death in a variety of conditions.

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Footnotes: The authors deny any conflicts of interest.

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