Title

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Journal

Journal of Endodontics, 39(9)

Authors

Williams, Drake
Wu, Hongkun
Oh, Ju-Eun
et al.

Publication Date

2013-09-01

DOI

10.1016/j.joen.2013.06.004

Peer reviewed

Published in final edited form as:
J Endod. 2013 September ; 39(9): 1156–1160. doi:10.1016/j.joen.2013.06.004.

HEMA inhibits migration of dental pulp stem cells

Drake W. Williams, BS[1], **Hongkun Wu, DDS, PhD**[2], **Ju-Eun Oh, PhD**[1], **Camron Fakhar, BS**[1], **Mo K. Kang, DDS, PhD**[1,3,4], **Ki-Hyuk Shin, PhD**[1,3,4], **No-Hee Park, DMD, PhD**[1,3,4,5], and **Reuben H. Kim, DDS, PhD**[1,3,4,6,*]

[1]UCLA School of Dentistry, Los Angeles, CA, 90095

[2]State Key Laboratory of Oral Diseases West China College of Stomatology, Sichuan University, Chengdu, Sichuan, 610041, P. R. China

[3]UCLA Dental Research Institute, Los Angeles, CA, 90095

[4]UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA, 90095

[5]UCLA David Geffen School of Medicine, Los Angeles, CA, 90095

[6]UCLA Division of Restorative Dentistry, Los Angeles, CA. 90095

Abstract

Objectives—Cell migration is an important step in pulpal wound healing. Although components in the resin-based dental materials are known to have adverse effects on pulp wound healing including proliferation and mineralization, their effects on cell migration have been scarcely examined. Here, we investigated effects of 2-Hydroxyethyl methacrylate (HEMA) on migration of dental pulp stem cells (DPSC) *in vitro*.

Methods—Cell viability was assessed using MTT assay, and cell migration was evaluated using wound scratch assay and transwell migration assay at non-cytotoxic doses. Western blotting was used to examine pathways associated with migration such as focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), and glycogen synthase kinase 3 (GSK3).

Results—There were no drastic changes in the cell viability below 3mM HEMA. When DPSC were treated with HEMA at 0.5, 1.0, and 2.5mM, cell migration was diminished. HEMA-treated DPSC exhibited the loss of phosphorylated focal adhesion kinase (FAK) in a dose-dependent manner. The HEMA-mediated inhibition of cell migration was associated with phosphorylation of p38 but not GSK3, ERK or JNK pathways. When we inhibited the p38 signaling pathway using a p38 inhibitor, migration of DPSC was suppressed.

Conclusion—HEMA inhibits migration of dental pulp cells *in vitro*, suggesting that poor pulpal wound healing under resin-based dental materials may be due, in part, to inhibition of cell migration by HEMA.

*Correspondence to: Reuben H. Kim, DDS, PhD, UCLA School of Dentistry, Division of Restorative Dentistry, Center for the Health Sciences, Room 43-091, 10833 Le Conte Ave, Los Angeles, CA90095. Phone: (310) 825-7312 Fax: (310) 825-2536, rkim@dentistry.ucla.edu.

The authors deny any conflicts of interest

Keywords

HEMA; dental pulp stem cells; migration; pulpal wound healing; p38

INTRODUCTION

2-Hydroxyethyl methacrylate (HEMA) is an important component in dental composite resins and dentin adhesive materials used in dental restorations. HEMA has both hydrophilic and hydrophobic functional groups which, in combination, make it an excellent material to form an interface that allows for mediating the bonding between hydrophilic collagenous dentin and hydrophobic resin materials (1). As such, it plays an important role in increasing bonding strength of dental composite restoration materials.

Although the use of HEMA in dental materials is biomechanically advantageous due to its ability to increase the bonding strength, the biocompatibility of HEMA to pulp tissues is a concerning issue. Evidence shows that pulp tissues have a poor repairing capacity in the presence of dentin adhesives *in vivo* (2,3,4) which suggests that direct contact of the dentin adhesives and their components to pulp tissues leads to adverse effects on pulpal wound healing. Because HEMA accounts for 30–50% of dentin adhesives, cytotoxicity of HEMA has been well documented *in vitro* (5,6). HEMA has been shown to induce apoptosis and cause DNA fragmentation in both human peripheral blood mononuclear cells and mouse macrophages in a dose dependent manner (7). Furthermore, HEMA has been shown to induce an intense pulpal inflammatory response (8), and chronic exposure to the monomer is known to suppress immunological functions *in vivo* (9). Recently, HEMA has been shown to inhibit odontogenic differentiation of stem cells derived from deciduous teeth (10).

Pulpal wound healing is a complex process that is orchestrated by discrete but overlapping multiple steps of migration, proliferation, and mineralization of cells in dental pulp (11). Although migration is one of the required steps during the pulp wound healing process before the dental pulp mesenchymal cells become repopulated and mineralized in the wounded area, roles of dental materials, particularly HEMA, on migration of dental pulp cells are poorly characterized. The aim of this study is to examine the effects of HEMA on the migration of dental pulp stem cells (DPSC).

MATERIALS AND METHODS

Reagents and antibodies

p-FAK(Y397) (#44-624G) was purchased from Invitrogen (Carlsbad, CA). FAK (#558),GSK3α/β (#7291), pGSK3α/β(Y297/Y216) (#81496), GAPDH (#25778) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). p-p38(T180/Y182) (#4511), p38 (#9212), p-Erk1/2 (T202/Y204) (#4370), Erk1/2 (#9102), p-JNK(T183/Y185) (#4668), and JNK (#9528) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). 2-Hydroxyethyl methacrylate (HEMA; CAS: 868-77-9) was purchased from Sigma-Aldrich (St. Louis, MO). PD169316 (4-(4-Fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole), p38-Inhibitor, was purchased from CalBioChem/EMD Biosciences (San Diego, CA).

Cell cultures

Primary DPSC were kindly provided by Dr. Songtao Shi (Herman Ostrow School of Dentistry, USC). Cells were cultured in α-MEM (Invitrogen) supplemented with 10%FBS (Invitrogen) and 5μg/mL Gentamycin Sulfate (Gemini Bio-Products, West Sacramento, CA).

MTT assay

Actively proliferating DPSC cells were plated at 4×10^3 cells per well in 96-well plates. One day after plating, cells were treated with different amounts of HEMA. After two days, the cell viability was measured using MTT Cell Proliferation Assay Kit (ATCC, Manassas, VA) according to manufacturer's protocol. Briefly, MTT reagents were added and incubated for 4 hours. After confirmation of purple precipitates under the microscope, detergents were added and cells were further incubated in the dark overnight. The colorimetric analysis was performed using the microplate reader at 570nm. For each HEMA concentration, we performed the assay in triplicate.

Scratch Assay

DPSC were grown to 90% confluency in 100mm culture dishes. Cells were then serum starved in 0.1%FBS (Invitrogen) for 24 hours. Medium was changed with the indicated HEMA amounts, and a scratch was made with a 200μL pipet tip. The initial scratched areas were uniform across the different samples and permanently marked; the marked areas were photographed at 0, 6, and 15 hours after the scratch. The photographs were taken using an Olympus DP72 microscope. The migration of the cells was determined by measuring the distance between the wounded edges. Three different areas were measured for each time point.

Boyden Chamber Assay

DPSC (2×10^4cells per well) were plated on a porous membrane (pore size: 8μm; Corning, Cat. #3422) with or without HEMA (0, 0.5, 1.0, 2.5mM) for 48 hours. Cells were fixed with 10% formaldehyde and stained with 2.5%Crystal violet overnight. Cells on the top of the transwell were removed, and the cells that migrated through the transwell membrane were photographed. Cells were counted and the cell numbers were normalized to the control. This assay was performed in triplicate. The same procedures were followed when checking migration patterns of DPSC in the presence of a p38-inhibitor at the indicated amounts.

Western blot

Cells were lysed using lysis buffer (1%Triton X-100, 20mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM sodium orthovandate and PMSF). Lysates (40–50μg) from cells were fractionated by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon protein membrane (Millipore, Billerica, MA). Immobilized membrane was incubated with primary antibodies and probed with the respective secondary antibodies. The membrane was exposed to the HyGLO Chemiluminescent HRP antibody detection reagent (Denville Scientific, South Plainfield, NJ) and scanned using ChemiDoc System (Bio-Rad, Hurcules, CA, USA).

Statistical analysis

The results are expressed as means ± standard deviation. For the comparison, the outcome measurements were compared to the control group using the Student *t*-test. p-values that are less than 0.05 are considered significant.

RESULTS

HEMA inhibits migration of DPSC

To examine effects of HEMA on migration of DPSC, we first determined viability of DPSC in response to HEMA treatment. After normalization to the control, we found that the viability of DPSC decreased dramatically after about 3mM HEMA (Fig. 1A). Using non-

cytotoxic HEMA concentrations (below 3mM), migratory patterns of DPSC were observed. During the course of 6 and 15 hours after scratches were made, inhibition of cell migration was evident in a dose-dependent manner (Fig. 1B and 1C). To further confirm anti-migratory effects of HEMA, we utilized the transwell migration assay that is more stringent and devoid of proliferation effects. When treated with HEMA, the migration of DPSC decreased markedly (Fig. 1D). Quantification of the migrated cells revealed that the migration was inhibited in a dose-dependent manner (Fig. 1E), indicating that HEMA inhibits migration of DPSC at non-cytotoxic doses.

Suppression of FAK and p38 phosphorylation is associated with HEMA-induced inhibition of cell migration

Activation of FAK by autophosphorylation at Tyr397 is an important initial signaling pathway in promoting cell migration. Therefore, we examined the phosphorylation status of FAK in response to HEMA treatment. In untreated DPSC, FAK is highly phosphorylated at Tyr397 (Fig. 2A) which was consistent with active migration (Fig. 1). When cells were treated with HEMA, the phosphorylation of FAK was suppressed (Fig. 2A). HEMA had no effects on phosphorylation of GSK3α and GSK3β (Fig. 2B), suggesting anti-migratory effects of HEMA is GSK3-independent. When the phosphorylation status of proteins involved in the MAP kinase pathways were examined, there was a dose-dependent reduction of phosphorylated p38 in response to HEMA treatment while Erk1/2 and JNK showed no correlation (Fig. 2C). These data suggest that HEMA-induced inhibition of cell migration is associated with FAK and p38 pathways.

Inhibition of p38 MAPK suppresses migration of DPSC

To determine the role of p38 in the migration of DPSC, we treated DPSC with PD169316, a known p38-specific inhibitor, and performed the transwell migration assay. PD169316-treated DPSC showed dose-dependent decrease in migration (Fig. 3A and 3B). To rule out the cytotoxic effects of the doses that we used to perform the migration assay, we examined the cell viability with different doses of PD169316 and found that, at 5 and 10μM, no changes in cell viability were observed (Fig. 3C). These data suggest that anti-migratory effect of HEMA on DPSC may be mediated, in part, through the p38 signaling pathway.

DISCUSSION

Although the cytotoxic effects of HEMA on dental pulp cells have been well documented, its effect on cell migration has not been examined. Here, we provide evidence that HEMA exerts anti-migratory effects on dental pulp cells, and that such effects are not the result of cellular cytotoxicity. We also showed that its anti-migratory effects are associated with inhibition of FAK and p38 MAPK phosphorylation.

One of the main goals of pulp therapy is to regenerate hard tissue barriers on iatrogenic or pathologic pulp exposures to protect pulp tissues from further exogenous insults. The utilization of dentin adhesives was initially considered to be a viable treatment option for pulp exposure provided that bacterial contamination is well-isolated (12,13,14). In contrast, more recent studies suggest that the utilization of dentin adhesives for direct pulp capping causes adversary effects to pulp healing *in vivo* (2,3,4). Indeed, several *in vivo* studies found that no dentinal bridges were found under the dentin adhesives even in the absence of bacterial contamination (15,16,17,18), indicating that dentin adhesives are generally not well-tolerated for direct pulp capping procedures.

Formation of hard tissue barriers, or reparative dentin, is mediated by the pulp wound healing process. This is a complex process comprised of multiple discrete but overlapping

steps of migration, proliferation, and mineralization of pulp cells (11). Therefore, ordered orchestration of these steps yields reparative dentin formation and successful outcome of pulp therapy. Our data suggests that HEMA inhibited the migration of cells (Fig. 1). Cell migration is an important step in pulpal wound healing and is indispensable particularly when dental pulp mesenchymal cells are expected to migrate into the wounded areas before they repopulate and differentiate to form reparative dentin. Clinical studies have demonstrated that exposed pulp areas capped with dentin adhesives containing HEMA lacked reactionary dentin deposition without the presence of odontoblast-like cells or newly-formed odontoblast layers, suggesting that pulp cells were not only failed to undergo mineralization but also inhibited to migrate to the exposed areas *in vivo* (16). This finding, along with our study, suggests that HEMA may be associated with inhibition of cell migration in areas of exposed pulp.

Our data showed that HEMA-mediated inhibition of cell migration occurred at sub-cytotoxic doses (Fig. 1). The primary cellular responses to HEMA cytotoxicity *in vitro* are suggested to be apoptosis as demonstrated in multiple cell types including peripheral blood mononuclear cells, gingival fibroblasts, gingival epithelial cells and pulp cells (7,19,20). However, there is a lack of direct evidence that apoptotic responses actually occur *in vivo* (e.g., TUNEL positive cells). This implies that dentin adhesives may have sub-apoptotic effects *in vivo* and that inhibition of cell migration may play a more important role in poor pulpal wound healing in response to dentin adhesives *in vivo*.

Previous reports examining migration of pulp cells showed that cell migration is mediated through different signaling pathways (21,22,23). In particular, recent studies suggest that GSK3 is essential for cell polarization and migration (24). However, we did not find any link between GSK3 and HEMA-mediated migration inhibition (Fig. 2B). Rather, HEMA inhibited phosphorylation of FAK (Fig. 2A). FAK is known to play a crucial role in cell motility (25). FAK-deficient mice exhibited a general defect of mesoderm development and reduced cell motility of embryonic cells *in vitro* (26). Conversely, 'SuperFAK,' a constitutively active form of FAK, has been shown to increase the migration of cells (27), indicating that the loss of phosphorylated FAK in HEMA-treated cells is an important signal that leads to the inhibition of cell migration.

Previous studies showed that MAP kinases including p38, Erk and JNK are known to play important roles in cell migration (21, 23,28), and our results suggest that p38 MAP kinase is involved in HEMA-mediated inhibition of DPSC migration (Fig. 3). Conflicting results have been shown in other studies where HEMA treated cells exhibited increased phosphorylation of MAP kinases including p38 (29). Increased phosphorylation of Erk1/2 was also noted when cells were treated with HEMA (30). The differences in MAPK phosphorylation may be attributed to treatment durations (hours vs. days), cell culture conditions (DMEM vs. α-MEM) and cell types (glandular cells or fibroblasts vs. dental pulp stem cells). On the other hand, it has been demonstrated that increased phosphorylation of p38 is directly associated with increased FAK phosphorylation and cell migration (31). Indeed, inhibition of the p38 signaling pathway has been shown to suppress enhanced migration of pulp cells by hepatocyte-growth factor (23). It would be worthwhile to examine the phosphorylation status of MAPKs in pulp cells *in vivo*.

In this study, we demonstrated that HEMA inhibits the migration of DPSC at non-cytotoxic doses and such inhibition was associated with the FAK and p38 signaling pathways. Therefore, mitigating these signaling pathways may provide therapeutic options to improve pulp regeneration in the presence of the resin-based dental materials containing HEMA.

Acknowledgments

This study was supported in part by the grants (K08DE17121, K03DE30304 to R.H.K.) from NIDCR/NIH and the Faculty Research Grant (to R.H.K.) from the UCLA Council on Research of the Academic Senate.

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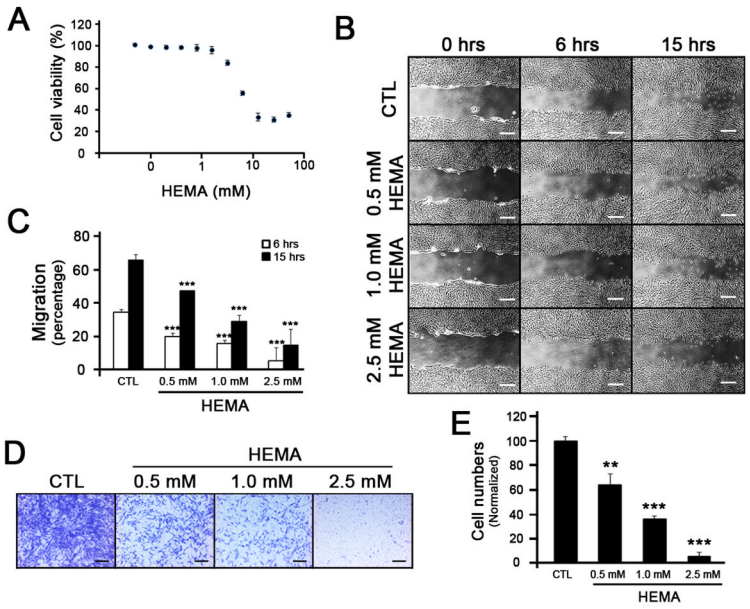

Figure 1. Effects of HEMA on migration of DPSC using wound scratch assay

(A) DPSC were treated with different doses of HEMA for 2 days, and MTT assay was performed to examine cell viability. Each dose was triplicated and the error bars indicate standard deviation. (B) Cells grown up to 90% confluency were serum-starved for 24 hours, and scratches were made with a 200 μL pipet tip. The medium containing different doses of HEMA was changed, and photographs were taken at the indicated hours. The white bar represents 4 μm. (C) The migrated distance was measured and presented as bar graphs. Three different fields were taken and the standard deviations were obtained. *** $p < 0.001$. (D) DPSC were plated on the top of the transwell membrane with different amounts of HEMA. Cells were allowed to migrate through the transwell membrane for 2 days. Cells were stained with Crystal Violet, and the cells on the top of the transwell membrane were removed. The migrated cells on the bottom of the transwell membrane were photographed using a microscope. The black bar represents 2 μm. (E) The numbers of migrated cells were counted and plotted as a bar graph. ** $p < 0.01$, *** $p < 0.001$.

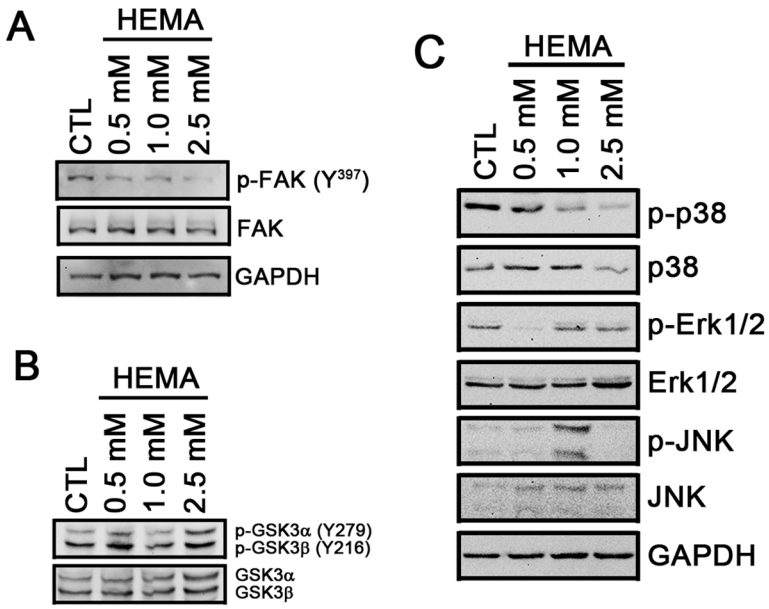

Figure 2. HEMA-induced inhibition of cell migration is mediated through suppression of FAK and p38 phosphorylation

DPSC were treated with different doses of HEMA, and western blotting was performed to examine phosphorylation of FAK (A), GSK (B) or MAPK (C). GAPDH was used as a control.

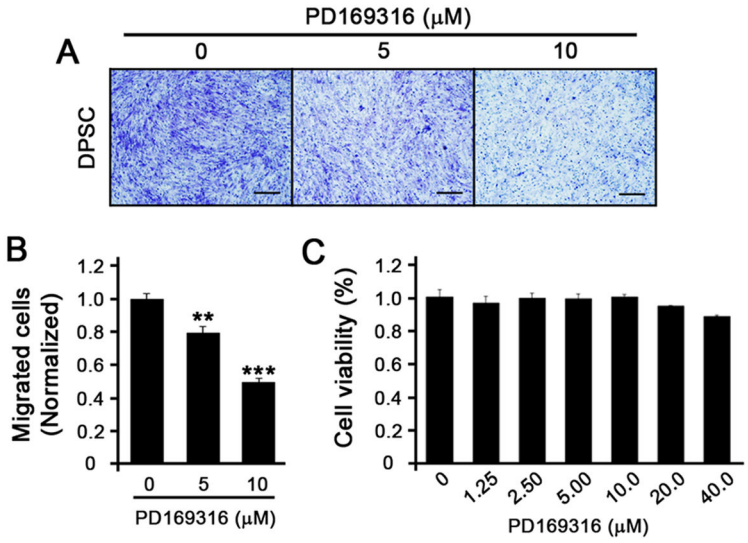

Figure 3. Inhibition of p38 suppresses the migration of DPSC

(A) DPSC were plated on the top of the transwell membrane with different amounts of p38 inhibitor, PD169316. Cells were allowed to migrate through the transwell membrane for 2 days. Cells were stained with Crystal Violet, and the cells on the top of the transwell membranes were removed. The migrated cells on the bottom of the transwell membrane were photographed using a microscope. The black bar represents 2 μm. (B) The numbers of migrated cells were counted and plotted as a bar graph. ** $p < 0.01$, *** $p < 0.001$. (C) DPSC were treated with different doses of PD169316 for 2 days, and MTT assay was performed to examine cell viability. Each group of cells was performed in triplicate and the error bars indicate standard deviation.