Expression of procollagen α1 type I and tenascin proteins induced by HEMA in human pulp fibroblasts

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ABSTRACT

In the dental pulp extracellular matrix, the main macromolecules are collagenous proteins, non-collagenous proteins and proteoglycans. Regulated synthesis of the interstitial collagens, in particular, type I collagen, is important during development and wound healing but also in a number of pathological conditions. Tenascin is also a matrix protein highly expressed during development while it decreases in mature organs. Under pathological conditions such as infections and inflammation, during tumorigenesis and mechanical stress applied to cells in culture or tissue in vivo, the expression of tenascin is increased.

In this study, HEMA, widely used in dentistry, ophthalmology and drug delivery, has been used to study its influence on the expression of procollagen α1 type I and tenascin proteins in the primary cultures of human pulp fibroblasts. Different concentrations of the resin monomer and different times of exposition were tested. The influence of HEMA on the cell viability was evaluated by means of an MTT assay while immunofluorescence and western blotting analysis were performed to detect possible interference with the presence and the synthesis of these proteins.

We observed a strong reduction in cell viability in specimens treated for 96 h and 168 h, especially at concentrations of 1 and 3 mmol/L HEMA. Both immunofluorescence and western blotting analysis demonstrated a reduction of procollagen α1 type I protein and an overexpression of tenascin protein.

Our results showed that long-term exposure and low concentrations of HEMA influence normal cell activity, such as the synthesis of some of the dental pulp extracellular matrix proteins.

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1. Introduction

Dental pulp is a connective tissue having an unusual organization and location. It is composed of cells (fibroblasts, odontoblasts and undifferentiated mesenchymal cells) in contact with a complex chain of macromolecules secreted in the extracellular matrix (ECM) (Linde, 1985). The main ECM macromolecules are collagenous proteins especially type I and III collagen, non-collagenous proteins such as fibronectin, tenascin, osteonectin and osteocalcin, and glycosaminoglycans including hyaluronic acid, chondroitin sulfate, heparin sulfate, and phospholipids (1–4). Collagen makes up nearly 34% of the total ECM proteins, and type I and III collagens are the most predominant types. Type I collagen, most commonly found in dense connective tissue, is necessary for tissue architecture stabilization (Shuttleworth et al., 1980). Regulated synthesis of interstitial collagens, in particular type I collagen, is important

during development and wound healing but also in a number of pathological conditions. Studies on these diseases, as well as various in vitro models, have shown that collagen synthesis and deposition are influenced by cytokines, growth factors and mechanical tension. Inflammatory mediators are important regulatory factors for collagen synthesis in adults (Gressner and Bachem, 1994; Peltonen et al., 1991).

Tenascin is a matrix protein and the regulation of its expression is poorly understood. It is highly expressed during development while it is quite reduced in developed organs; it reappears under pathological conditions caused by infections and inflammations, during tumorigenesis (Chiquet-Ehrismann and Chiquet, 2003) and mechanical stress applied either to cells in culture or to tissues in vivo (Chiquet-Ehrismann et al., 1994; Fluck et al., 2000).

Dental pulp is one of the first tissues of the oral cavity to be involved in damage induced by dental restorative processes in which resin-based materials are utilized.

During these restorative processes, resin-based materials are applied to the dentin, and the monomers released from the

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polymerized resin matrix can reach the dental pulp through dentin tubules, causing several diseases such as dermatitis, pharyngitis, asthma, rhinoconjunctivitis (Kanerva et al., 1995; Lindstrom et al., 2002) and tissue inflammation (Bouillaguet et al., 1998; Gerzina and Hume, 1996). Dental pulp inflammation is characterized by changes in the blood flow (Olgart et al., 1991), immunocompetent cell function (Bergenholtz et al., 1991), and the enzymatic conversion of arachidonic acid into a group of biologically active mediators due to the induction of cyclooxygenase-2 (COX-2) expression (Huang et al., 2005).

HEMA is one of the major components released from resin-modified glass ionomer cement (GIC) and dental adhesives (Geurtsen et al., 1998, 1999). Cytotoxicity of the resin monomers has been evaluated with permanent cell lines or a primary culture of fibroblasts from pulp, gingival, and periodontal ligaments (Issa et al., 2004; Thonemann et al., 2002) and is usually indicated by a decrease in cell proliferation, in mitochondrial activity, and in protein or nucleic acid synthesis (Geurtsen, 2000).

Several previous studies have demonstrated that, after short-term exposure to resin-based material, the cytotoxic effects depended on concentrations ranging from 100 µmol/L to 10 mmol/L, which are the cytotoxic concentrations responsible for 50% cell death (TC50) (Chang et al., 2005; Mantellini et al., 2006; Moharamzadeh et al., 2007a; Nocca et al., 2007). On the contrary, very few studies have demonstrated the HEMA influence in cells exposed to lower concentrations than TC50 and for long-term exposure (Bouillaguet et al., 2000; Costa et al., 1999) and the effects of these monomers on the expression of specific extracellular matrix proteins (About et al., 2002; Falconi et al., 2007).

The purpose of this study was to analyze the influence of HEMA on procollagen α1 type I and tenascin proteins in primary cultures of human pulp fibroblasts (HPFs) incubated with the monomer at concentrations ranging from 0.5 mmol/L to 3 mmol/L and for long-term exposure. The low dose concentrations tested here for long periods of time simulated the release of resin monomers from polymerized resin matrix in the oral cavity. The leaching of monomers is due to an incomplete polymerization or degradative processes and it occurred even after long periods of time such as 1 or 2 weeks or 4 weeks (Mazzaoui et al., 2002; Moharamzadeh et al., 2007b).

The influence of HEMA on HPF viability was evaluated by means of an MTT assay, while immunofluorescence and western blotting analysis were carried out to detect procollagen α1 type I and tenascin proteins after HEMA exposition. The null hypotheses tested were that (a) different concentrations of HEMA did not effect the synthesis of procollagen α1 type I and tenascin proteins; (b) long-term exposition did not have any effect on the synthesis of procollagen α1 type I and tenascin proteins.

2. Materials and methods

2.1. Establishment of a primary human fibroblast culture

HPFs were isolated from the third molars of healthy normal volunteers during routine oral surgery. Informed consent was obtained from the donors according to the guidelines of the National Bioethics Committee. The central part of the dental papilla was cut into small pieces, washed with PBS and incubated in Dulbecco's Modified Essential Medium (DMEM)/F12, containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin and 1% fungizone. Monolayer cultures were maintained at 37 °C in a humidified atmosphere of 5% (v/v) CO$_2$. Cultured HPFs in passage numbers between four and eight were used for this study.

2.2. HEMA treatment

For each experiment, HEMA was dissolved in ethanol (6 M stock solution) and then diluted in Dulbecco's Modified Essential Medium (DMEM) to form a serum free medium containing HEMA in concentrations of 0.5 mmol/L, 1 mmol/L and 3 mmol/L. In all the exposure media, the final ethanol concentration was 0.3%

2.3. MTT assay

HPFs (1×10^4) were seeded into each well of a 96-well culture plate in DMEM containing 10% FBS, 1% penicillin and streptomycin and 1% fungizone to measure cell viability. After 24 h, the medium was changed to a fresh one containing 0.5 mmol/L, 1 mmol/L and 3 mmol/L of HEMA. The cells were incubated for 72 h, 96 h and 168 h, refreshing the medium every day during the 7 days of culture. They were then washed with phosphate buffered saline (PBS) and incubated with fresh medium containing 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h at 37 °C. The formazan produced was dissolved by solvent solution (0.1 N HCl in isopropanol) and the optical density was read at 570 nm by Microplate Reader (Model 680, Biorad Lab Inc., CA, USA).

The MTT data were presented as the mean (±S.D.) of triplicate experiments. Statistical differences was assessed by one-way ANOVA ($p < 0.05$) and Dunnett's Multiple Comparison Test ($p < 0.05$). The statistical analysis was performed with GraphPad Prism 5.0 software (San Diego, CA, USA).

2.4. SDS–PAGE and Western blotting

HPFs were exposed to 0.5–1–3 mmol/L HEMA for the same periods of time as previously described. Cytosolic extracts were then prepared with the use of a RIPA modified lysis buffer (50 mmol/L Tris–HCl pH 7.4; 1% NP-40; 150 mmol/L NaCl; 2 mmol/L EDTA; 0.1% SDS; 1 mmol/L EGTA; 1 mmol/L PMSF; 0.15% βME) supplemented with a 25 µmol/L protease inhibitor cocktail (Sigma Aldrich, St. Luis, Missouri, USA). Total proteins were resolved on 8% SDS polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred into a nitrocellulose membrane using a wet blotting apparatus (Mini Tank Electroblotting System, Owl, Portsmouth, UK). The membranes were blocked with 2.5% dry milk (Bebilac, Sicura, France) in TBS–Tween buffer (Tween 20 0.1%, NaCl 0.15 mol/L, Tris-Base 0.01 mol/L) pH 7.5 for 1 h at room temperature (RT) and were then incubated with the primary antibodies for 2 h at 37 °C .The primary antibodies were: anti-procollagen α1 type I antibody (Santa Cruz Biotechnology, INC., Santa Cruz, CA, USA) diluted 1:5000, anti-β tubulin antibody (Sigma Aldrich, Saint Luis, Missouri, USA) diluted 1:10000 and anti-human tenascin antibody (Sigma Aldrich, Saint Luis, Missouri, USA) diluted 1:250. After several washes in TBS–Tween buffer, the membranes were incubated with the specific HRP (horseradish peroxidase) conjugated antibodies. For procollagen α1 type I protein, the secondary HRP conjugated antibody was diluted 1:80000 (Santa Cruz Biotechnology, INC., Santa Cruz, CA, USA) for 90 min at 37 °C while, for the tenascin protein, the secondary antibody was diluted 1:50000 (Sigma Aldrich, Saint Luis, Missouri, USA) for 90 min at RT. Signals were detected by the enhancement chemiluminescence system (ECL plus, Amersham Biosciences, Little Chalfont Buckinghamshire, UK). Images were obtained by Image Station 2000R (Kodak, NY, USA).

2.5. Immunofluorescence

HPFs were grown in monolayers on cover glasses and treated with 0.5–1 mmol/L and 3 mmol/L HEMA for 72 h, 96 h and 168 h.

Two cover glasses were prepared for each treatment and the entire experiment was performed three times. Samples were washed three times in PBS and fixed with 4% formalin/0.1% Triton X-100 in PBS for 20 min at 4 °C. After three washes in PBS for 10 min each, the samples were blocked in 1% dry milk (for the procollagen α1 type I protein immunodetection) (Bebilac, Sicura, France) in the same buffer (blocking reagent) for 30 min at room temperature (RT) while the specimens were blocked in 2.5% dry milk (for the tenascin protein detection) (Bebilac, Sicura, France) in PBS for 90 min at RT. The cover glasses were then incubated with anti-human procollagen α1 type I antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:400 in blocking reagent for 1 h at 37 °C, or with an anti-human tenascin antibody (Sigma Aldrich, Saint Luis, Missouri, USA) diluted 1:100 in blocking reagent for 1 h at 37 °C. After three rinsings in PBS for 10 min each, all the samples were incubated respective with CY$_3$-conjugated anti-goat IgG antibody (Sigma Aldrich, Saint Louis, Missouri, USA) and CY$_3$-conjugated anti-mouse IgG antibody (Sigma Aldrich, Saint Louis, Missouri, USA) both diluted to 1:2000 in blocking reagent. Finally, the slides were washed three times in PBS and then mounted in VECTASHIELD® mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). The slides were observed by a fluorescence microscope (Nikon Eclipse E800, Tokyo, Japan).

2.6. Controls

Each of the above described experiments was performed on HPFs exposed to only HEMA solvent (the final dilution of ethanol was 0.3%) to assay the influence of the solvent on cell expression and the synthesis of procollagen α1 type I and tenascin proteins.

For immunofluorescence labeling, the control specimens consisted in HPFs fixed with 4% formalin/0.1% Triton X-100 and incubated only with the specific secondary antibody CY$_3$-conjugated to check the presence of a non-specific interaction between the antibody and the free aldehyde groups of the fixative.

3. Results

3.1. MTT assay

The MTT assay of the primary cultures of HPFs exposed to 0.5–1–3 mmol/L HEMA for 0 h, 72 h, 96 h and 168 h is shown in Fig. 1.
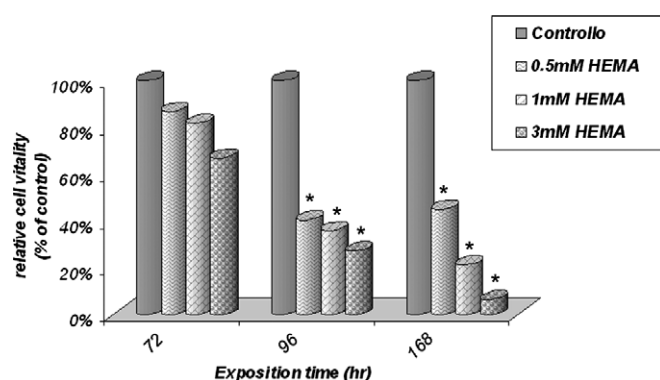

Fig. 1. Cell viability of HPFs exposed to 0.5–1–3 mmol/L HEMA for 0 h, 72 h, 96 h and 168 h. Values of cell viability higher than 50% were observed after 72 h of treatment for all the concentrations tested while a mild reduction was detectable after 96 h of treatment. In specimens exposed for 168 h, a stronger reduction in cell viability was particularly observed in samples incubated with 1 and 3 mmol/L. The data represent the mean (±S.D.) of triplicate experiments per condition and are expressed as a percentage of the control value. The MTT data were statistically analyzed by one-way ANOVA followed by the Dunnet test. *Statistically significant differences between groups ($p < 0.05$).

The viability of the HPFs is expressed as the percentage relative to pulp cells not exposed to HEMA (0 mmol/L). Values of cell viability higher than 50% were observed after 72 h of treatment for all the concentrations tested while a mild reduction was detectable after 96 h of treatment. In specimens exposed for 168 h, a stronger reduction in cell viability was particularly observed in samples incubated with 1 and 3 mmol/L.

3.2. Western blot analysis for procollagen α1 type I and tenascin proteins

Western blot analysis, carried out to demonstrate the effect of HEMA on procollagen α1 type I protein, showed a low decrease of the protein signal in specimens exposed to 1 mmol/L HEMA for 72 h and a significant decrease in the sample treated with 3 mmol/L HEMA (Fig. 2A). In specimens exposed to 1 and 3 mmol/L HEMA for 96 h, the signal was almost absent (Fig. 2B). No signal was detectable in the specimens treated for the longest periods of time (168 h) for all three concentrations tested (Fig. 2C).

The effect of HEMA on the production of tenascin proteins is shown in Fig. 3. Control samples always demonstrated a low signal corresponding to the tenascin protein. After 72 h of exposition, a slight increase of the protein was observed in all the samples examined (Fig. 3A) while, after 96 h of exposition, the protein
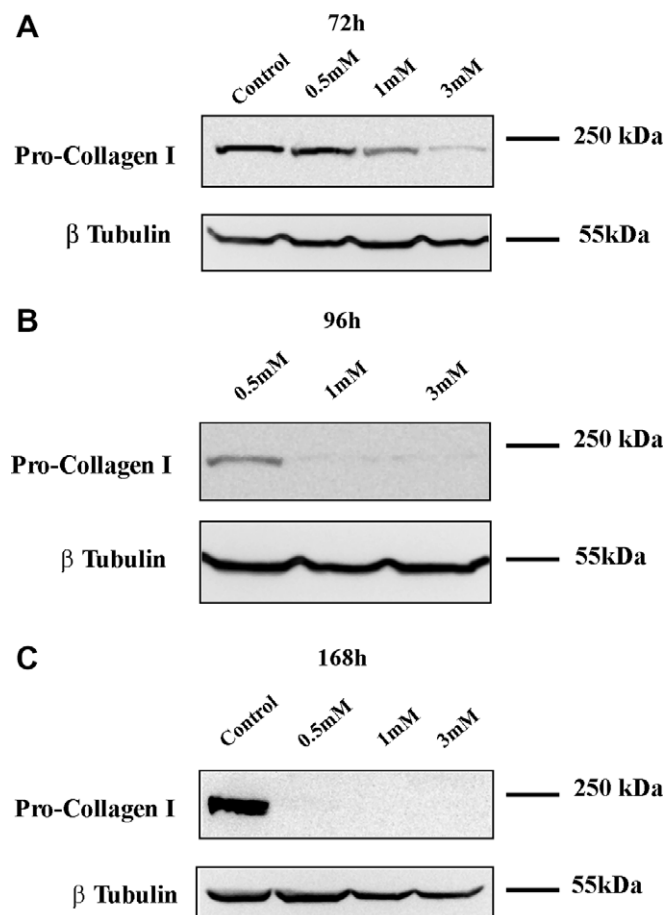

Fig. 2. Western blot analysis for procollagen α1 type I in HPF exposed to 0.5–1–3 mmol/L HEMA for 0 h, 72 h, 96 h and 168 h. There was a slight decrease of the protein signal in specimens exposed to 1 mmol/L HEMA for 72 h and a significant decrease in the sample treated with 3 mmol/L HEMA (A). In the specimens exposed to 1 and 3 mmol/L HEMA for 96 h, the signal was almost absent (B). No signal was detectable in the specimens treated for the longest periods of time (168 h) for all concentrations tested (C). β Tubulin represents the loading control.
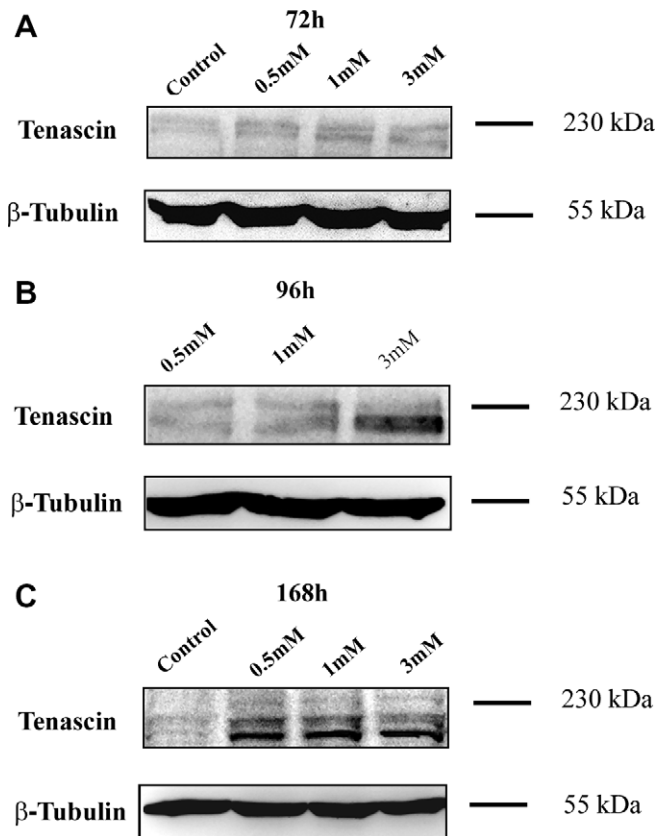
Fig. 3. Western blot analysis for tenascin in HPFs exposed to 0.5–1–3 mmol/L H-EMA for 0 h, 72 h, 96 h and 168 h. Control samples always demonstrated a low signal corresponding to the tenascin protein. After 72 h of exposition, a slight increase of the protein was observed in all the samples examined (A) while, after 96 h of exposition, the protein signal was marked in the sample treated with 3 mmol/L HEMA (B). A strong increase in the tenascin protein was easily detectable in all the specimens exposed for 168 h (C). β Tubulin represents the loading control.

signal was marked in the sample treated with 3 mmol/L HEMA. A strong increase of the tenascin proteins was easily detectable in all the specimens exposed for 168 h.

3.3. Immunofluorescence for procollagen α1 type I and tenascin proteins

To test the interference of HEMA with the production of fibroblastic proteins, immunostaining was carried out for procollagen α1 type I and tenascin proteins in HPFs with and without HEMA exposure. Fig. 4A, E and I show samples without any treatment with the labeling corresponding to procollagen α1 type I organized in small clusters around the cell nucleus. The specific labeling pattern almost vanished in HPFs after 168 h of exposure for all three concentrations of HEMA tested (L, M, N) while it was strongly reduced in samples exposed for 72 h and 96 h, especially with 1 and 3 mmol/L (C, D, G, H).

Fig. 5 presents the labeling pattern of the tenascin proteins after HEMA incubation. The fluorescence signal increased with an increase in HEMA concentration and in exposition time. Indeed, in samples exposed to 3 mmol/L for 72 h and 96 h (D, H) and to 0.5–1–3 mmol/L for 168 h (L, M, N), a strong increase in the fluorescence signal was detectable.

4. Discussion

HEMA is a commonly used constituent of dental restorative materials in amount ranging from 30% to 55% (Geurtsen et al.,

1998). The use of high percentages of HEMA in the bonding agent allows high diffusivity of the monomer which blends into the very hydrophilic substrate, i.e. the dentin collagen meshwork.

Other than in dentistry, HEMA is widely utilized in ophthalmology for the production of contact lenses (Goda and Ishihara, 2006; Lord et al., 2006), in drug delivery and in tissue engineering (Mei et al., 2005).

Despite the extensive use of HEMA on biomedical materials, its biocompatibility and biological safety are still a matter of study. Several studies demonstrated that HEMA and other monomers are rapidly released from the polymerized matrix (Cetinguc et al., 2007), reach dental pulp and induce adverse effects (Chang et al., 2005; Nocca et al., 2007). According to Ferracane and Condon (1990), most unbound substances are liberated in high amounts from polymerized resins within 24 h, but a low leaching of dental monomers is detectable even after 30 days (Moharamzadeh et al., 2007b; Polydorou et al., 2007).

In the current study, low dose concentrations of HEMA for long-term expositions were analyzed to detect the cytotoxicity of the monomer in a primary culture of human pulp fibroblasts. We have already described (Falconi et al., 2007) the inhibitory effect of 3 mmol/L HEMA on the synthesis of the procollagen α type I protein in human gingival fibroblasts. In this study, we analyzed lower concentrations of HEMA (0.5 mmol/L, 1 mmol/L, 3 mmol/L) for longer periods of time (until 168 h) to simulate (in vitro) a low dose release of the monomer which was observed even 1 week after the polymerization process.

Previous studies demonstrated the effects of these monomers at low concentrations but only for short-term exposition ranging from minutes to 24 h (Nocca et al., 2007). Nevertheless, few data are available on the influence of HEMA at concentrations near the dose estimated to be released from a polymerized resin matrix and for long-term exposure (Chang et al., 2005; Mantellini et al., 2006).

To analyze the influence of low dose concentrations of HEMA for long-term expositions, we first carried out an MTT assay to test cell viability, followed by western blot analysis and immunofluorescence to check the effect of the monomer on the synthesis of procollagen α1 type I and tenascin proteins, two of the main proteins of the extracellular matrix.

The toxicity of resin monomers released from the polymerized matrix depends on the type of monomer, the concentration, the time of exposition and the kind of cell line in contact with the material (Geurtsen et al., 1998). Indeed, many data about the cytotoxicity of resin monomers are different and contradictory due to the different experimental conditions and the different cell lines utilized for the experiments (Cetinguc et al., 2007; Lee et al., 2006; Spagnuolo et al., 2006).

In view of this, we carried out an MTT assay on human pulp fibroblasts to detect the sub-lethal concentrations of HEMA for the different times of exposition. The MTT results showed that HPF viability is time and dose dependent. In fact, samples treated even with 0.5 mmol/L demonstrated a cell viability of higher than 50% after 96 h of exposition but it decreased to under 50% after 168 h of exposure. However, the concentrations of 0.5 mmol/L, 1 mmol/L and 3 mmol/L were not lethal after 72 h and 96 h of treatment while these concentrations become toxic after 168 h of exposition.

From these results, we utilized 0.5 mmol/L, 1 mmol/L and 3 mmol/L to test the interference of low dose concentrations of HEMA on the synthesis of procollagen α1 type I and tenascin proteins using western blotting analysis followed by immunofluorescence analysis. The results obtained with both techniques are in agreement. Indeed, western blot analysis for procollagen α1 type I protein demonstrated a decrease of the protein with an increase in time-exposure and HEMA concentration. In particular, after
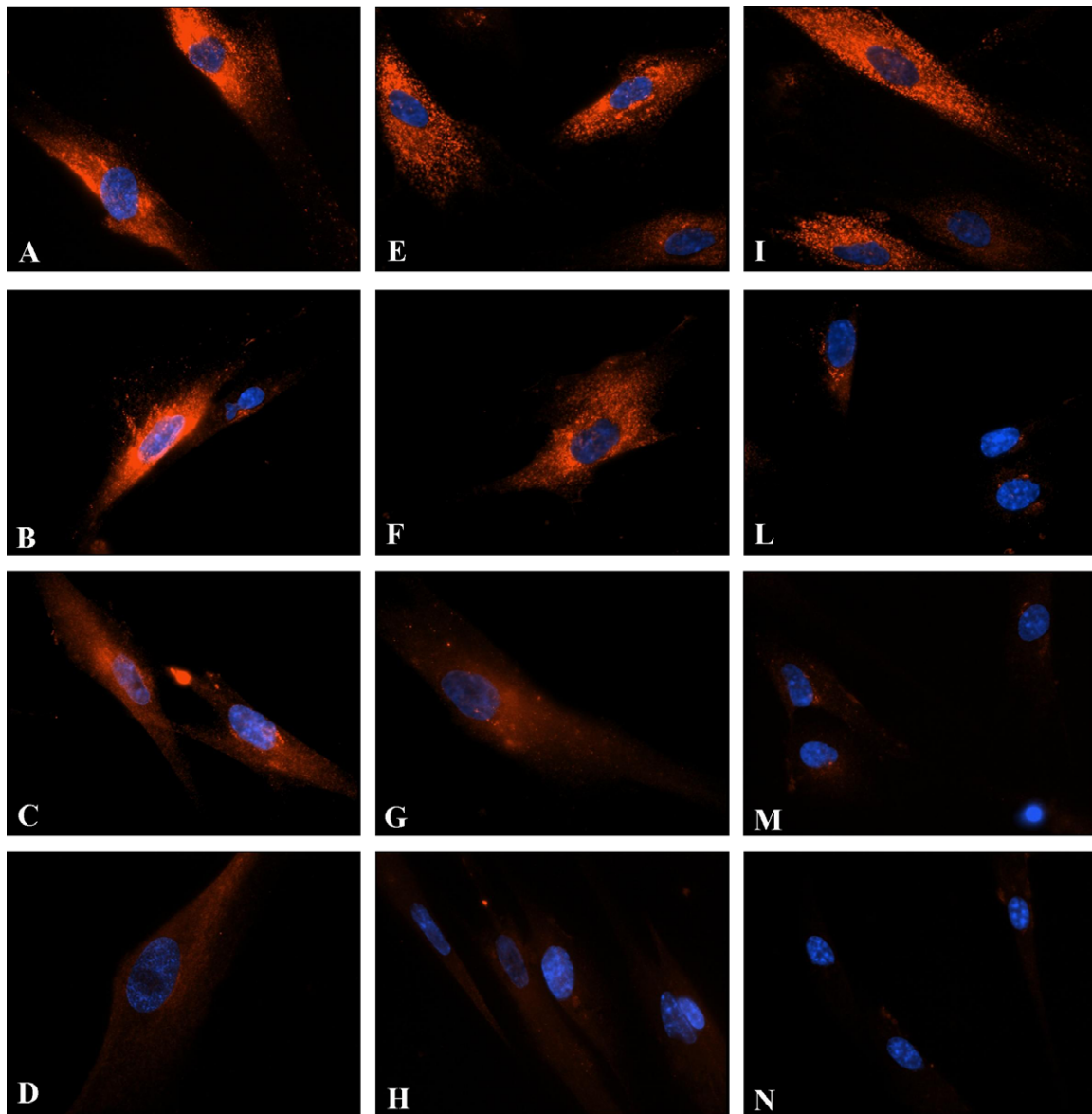
Fig. 4. Immunocytochemical localization of procollagen α1 type I in HPF treated with three different concentrations of HEMA and at different periods of time. CY₃-conjugated anti-mouse IgG antibody was used to detect the localization of the protein. All samples were counterstained with DAPI. (A, E, I) showed HPFs without any treatment. A CY3 signal was localized in one area around the cell nucleus (600×). It was strongly reduced in samples exposed for 72 h and 96 h, especially with 1 and 3 mmol/L (C, D, G, H). The fluorescence signal was reduced with the increase in time and the clusters around the nucleus disappeared (600×). (L, M, N) HPFs after 168 h of HEMA treatment. The specific labeling pattern had almost vanished in this time-exposure for all three concentrations of HEMA tested.

72 h of treatment and at 0.5 mmol/L and 1 mmol/L, a weak band corresponding to the protein is still detectable. After 168 h and for all the concentrations tested, the protein signal is totally absent. Immunofluorescence for procollagen α1 type I protein confirmed these data. The fluorescence signal corresponding to the protein is almost absent after 168 h of exposition for all the concentrations tested.

In our previous study (Falconi et al., 2007), an inhibitory effect of 3 mmol/L HEMA on the synthesis of procollagen α1 type I protein was demonstrated after 96 h of exposition. In this study, we demonstrated that the inhibitory effect is still present even at lower concentrations of HEMA (0.5 mmol/L and 1 mmol/L) and for longer-term exposure, suggesting a dependence of HEMA cytotoxicity on the concentration and time of treatment (Spagnuolo et al., 2006).

Pulp cells are important for the homeostatic function of pulp connective tissue and are responsible for healing processes when

pulp tissues are insulted by mechanical or chemical injury, or microbial irritants. After injury, pulp tissue exerts its inherent potential of repairing and regenerating. Our results suggest that HEMA could impair pulp cell function due to an interaction with the procollagen α1 type I protein. According to David and Bernfield (1981), type I collagen protein may be involved in the assemblage of the basal lamina proteoglycans to stabilize the lamina components. The mechanism by which collagen reduces the rate of glycosaminoglycan (GAG) degradation and promotes the accumulation of lamina materials is unclear. In accordance with our results, we hypothesized that a reduction of the production of procollagen α1 type I in the pulp cells could enhance degradation of basal lamina proteoglycans and, thus, the loss of lamina integrity.

On the contrary, western blot analysis for tenascin proteins showed an increase in the protein depending on HEMA concentration and time of exposure. In particular, the protein signal clearly
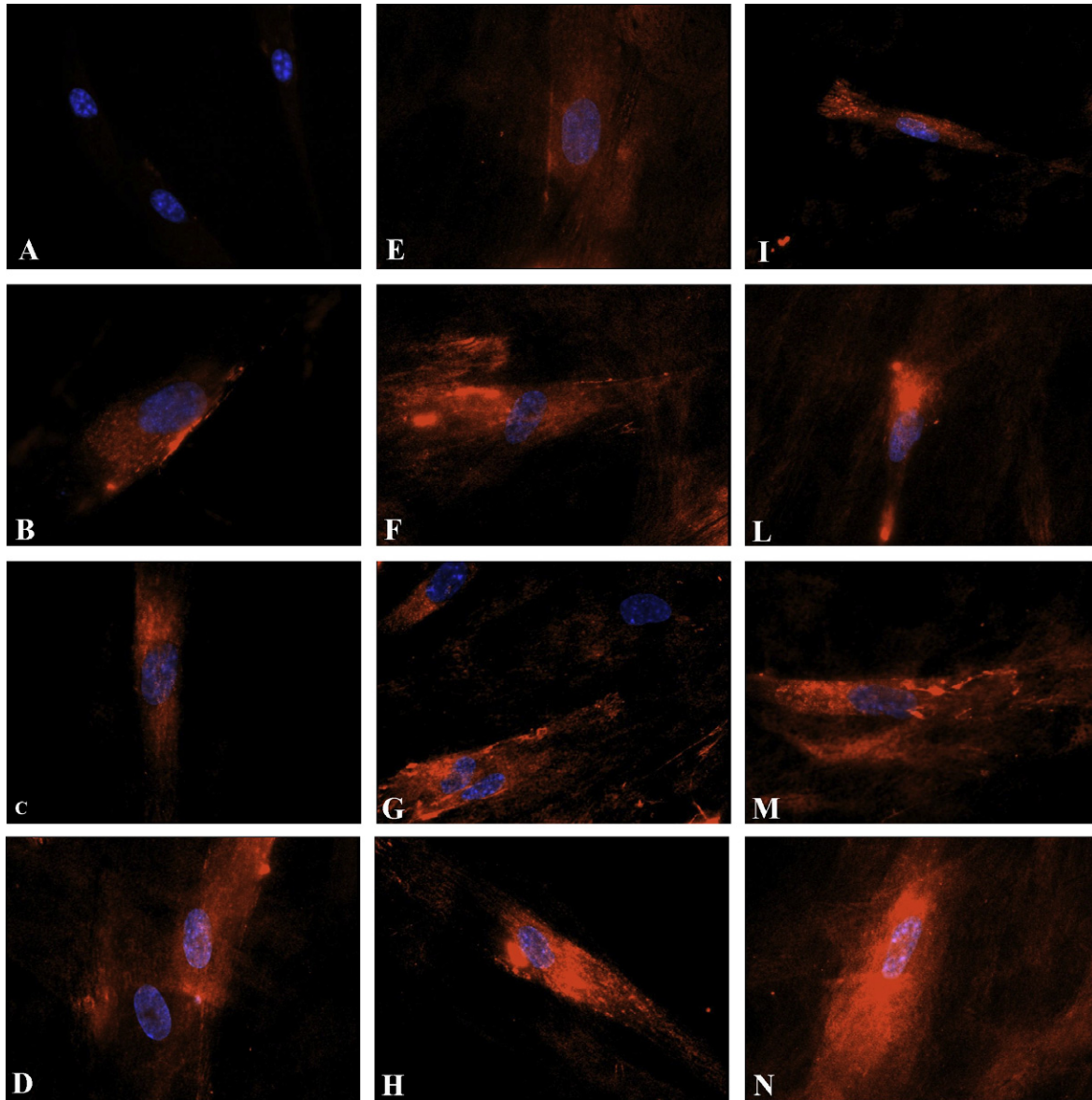
Fig. 5. Immunocytochemical localization of tenascin protein in HPFs treated with three different concentrations of HEMA and at different times of exposure. CY_3-conjugated anti-mouse IgG antibody was used to detect the localization of the protein. All samples were counterstained with DAPI. (A, E, I) showed HPFs without any treatment. The fluorescence signal increased with the increase in HEMA concentration and with the exposition time. In samples exposed to 3 mmol/L for 72 h and 96 h (D, H) and to 0.5–1–3 mmol/L for 168 h (L, M, N), a strong increase in the fluorescence signal was detectable.

increased in samples treated with 1 mmol/L for 96 h and in all the concentrations tested for 168 h. Immunofluorescence analysis confirmed these data, demonstrating an increase of the fluorescence signal particularly in samples exposed to all three concentrations tested for 168 h.

Several studies demonstrated that tenascin is the one of the main ECM proteins involved in wound healing (Chiquet-Ehrismann, 1990). Enhanced expression of tenascin is correlated to the conditions of mechanical and pathological stress (Chiquet-Ehrismann and Chiquet, 2003) which are connected with irreversible inflammation and tumors in connective tissue. In accordance with these studies, our data suggest that the concentration of 3 mmol/L HEMA for long-term exposure determines a stressful condition for HPFs and could therefore be responsible for the future insurgence of pulp inflammation and/or oral tumors.

In conclusion, the null hypotheses tested in this study were rejected since HEMA reduced the synthesis of procollagen α1 type I protein and increased tenascin proteins in human pulp fibroblasts exposed to low concentrations of the monomer for long-term periods of time.

The HEMA concentrations tested in this study and the long-term exposures had the aim of simulating a low but constant release of dental monomers from a polymerized resin matrix. Our results showed that, even though after short-term treatment (72 h) there were no strong inhibitory effects of the monomer on protein synthesis, its influence definitely increased with an increase in the concentration and with long-term exposition (168 h). This dependence of resin monomers should not be underestimated because it could be responsible for weak but constant cell suffering.

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