Abstract: Studies have failed to identify the molecular mechanisms that regulate the genotoxic and cytotoxic effects of methacrylate resins, which are important in the biocompatibility of dental materials. Interleukin (IL)-6 has a crucial role in the control of acute-phase protein response during inflammation. In humans, the synthesis and release of two major acute-phase proteins, C-reactive protein and serum amyloid A, are regulated by IL-6. This study focused on IL-6 and activation of its receptors gp80 and gp130 in human gingival fibroblasts in order to assess the effects of the commercial acid resins Jet Kit, Unifast, and Duralay on control of inflammation.

Keywords: inflammation; interleukin-6; cytotoxicity; cell culture; cytokines.

Introduction

Development of dental materials for clinical applications is a field of considerable interest. An important problem in the use of dental polymers is diffusion of resins that affect the viability and physiological activity of cells (1). Previous studies have investigated the effects of several dental materials on new dentin formation and prevention of secondary caries (2). Moreover, diffusion of monomers residual through dentin, affect the viability of odontoblasts and the physiological activity of dental pulp. Resins reach pulp tissue and penetrate the cytosol of different cell phenotypes, thereby resulting in cytotoxicity, genetic damage, and oxidative stress (3). Moreover, resins may up-regulate interleukin (IL)-6 production (4).

Methacrylates, including acrylic resins, are the most polymeric materials used in dental prostheses. Their chemical composition varies considerably, due to the number of acrylic resins available in various products of the chemical industry. The most commonly used substances include the monomer methyl methacrylate (MMA) and the polymer matrix, poly(methyl methacrylate) (PMMA). The high rate of biocompatibility and low toxicity of PMMA has led to its use in other fields of medicine, as a bone cement, for contact and intraocular lenses, in fastening screws for bone, as a filler for bone cavities and skull defects, and for stabilization of vertebral in osteoporotic patients (5). In dentistry, PMMA is used in individual impression trays, orthodontic devices, implants, and artificial crowns (6).

Studies have failed to identify the molecular mechanisms that regulate the genotoxic and cytotoxic effects of resins, although such effects are important in the biocompatibility of dental materials. The chemical constituents and products released after polymeric conversion of methacrylate are important in the regulation of several adverse responses, including localized and systemic
inflammation, toxicological reactions, and carcinogenic/mutagenic effects. Moreover, residuals released by methacrylate (MMA, PMMA, etc.) induce apoptosis, necrosis, and enhance death in human U937 monoblastoid cells (7). However, gingival inflammation and periodontal bone resorption can occur due to aging and degradation of acrylic resins (porosity, marginal integrity) (7,8).

The polyacrylic resins activate synthesis and release of cytokines, chemokines, and pro-inflammatory proteins in several cell phenotypes (9). In addition, human gingival fibroblasts (HGFs) can synthesize IL-6, C-reactive protein (CRP), and tumor necrosis factor-alpha during gingival disease (10). IL-6 is the most important regulator of hepatic acute-phase protein response during inflammation. In humans, IL-6 causes a dramatic increase (>1,000 fold) in the synthesis of the two major acute-phase proteins, CRP and serum amyloid A (11). Although the liver is the only organ able to induce inflammation processes and the acute-phase protein response, recent studies have found that other tissues, such as adipose tissue and vascular walls, are involved in the transcriptional control of acute-phase markers (12). Indeed, data from the Third National Health and Nutrition Examination Survey show that CRP level is strongly positively associated with cardiovascular disease risk. The effects of IL-6 on target cells occur via a complex receptor system composed of a ligand-binding subunit (IL-6R or gp80) and a signal-transducing glycoprotein (gp130), which are both expressed on the cell surface (13-18). After IL-6 binds IL-6R, the IL-6/IL-6R complex triggers dimerization of the signal-transducing receptor component gp130. This receptor-ligand interaction activates Janus-activated kinases that phosphorylate tyrosine residues of the cytoplasmic portion of gp130, thereby activating signal transducers and activators of the transcription family (19,20). A recent study reported that inflammation had a role in controlling IL-6, gp80, and gp130 gene expression in peripheral blood mononuclear cells harvested from uremic patients undergoing dialysis treatment. Moreover, gp130 gene expression was positively correlated with gp130 protein levels on the cell surface membrane (21). We recently demonstrated that resin monomers have a role in the positive control of IL-6, gp80, and gp130 in human pulp cells (22).

We investigated IL-6 and activation of its receptors gp80 and gp130 in HGFs, to determine the effects of the commercial acid resins Jet Kit, Unifast, and Duralay on inflammation.

Materials and Methods
Culture of HGFs
Gingival tissue was obtained from healthy adults aged 20 to 30 years, after informed consent was received and the study was approved by the Institutional Review Board of University of Napoli Federico II No. 226/14. A surgical blade was used to dissect the connective layer from gingival samples. Tissue fragments were washed twice in phosphate-buffered saline (PBS), transferred to tissue culture dishes in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL of penicillin, and 100 mg/mL of streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in air. After 10 days, the fragments were removed, and the released fibroblasts began proliferating. At confluence, the cells were washed with PBS, detached from the culture dishes using a brief treatment with trypsin/EDTA for 5 min, and re-cultured until a confluent monolayer was again obtained. In all experiments, cells were used between passages 4 and 6 (22). Reagents were purchased from Lonza (Trevischio, Italy).

Preparation of resins
Jet Kit (Lang Dental Mfg. Co., Wheeling, IL, USA), Unifast (GC Corp., Tokyo, Japan), and Duralay (Reliance Dental Mfg. Co., Alsip, IL, USA) were prepared by mixing the materials within the well plates. A cytotoxic assay (MTT) was used to identify the optimal resin concentration for the experiments. The cytotoxic experiment used resins at a concentration from 0.001 to 1 mM. The assay was performed using two time ranges: 3 and 30 min after mixing the materials. Then, the well plates were covered with HGF-specific medium for 24 h, to demonstrate release of resin residuals. After that, HGFs were stimulated with the conditioned medium for 24 h. Finally, the cytotoxic assay (MTT) was used to assess cell viability.

MTT assay
MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, Milan, Italy) measures mitochondrial dehydrogenase activity. HGF cells were seeded into 96-well flat-bottomed tissue culture plates at 10⁴ cells/well. After 24 h of incubation, the culture medium was replaced with 200 µL/well of dilutions. After an additional 24 h, the medium was substituted with 100 µL/well of MTT solution (1 mg/mL) in PBS, and the cells were incubated for an additional 1 h at 37°C in a 5% CO₂ atmosphere. After the solution was removed, 100 µL/well of dimethyl sulfoxide (DMSO) was added, and the plates were swirled gently for 10
The optical density of each well was immediately measured with a spectrophotometer (Sunrise, Tecan, Mannedorf/Zurich, Switzerland) at 590 nm. The optical density of cells cultured in medium plus saline solution without the miniscrew extracts was used as the control for 100% cell viability and as the reference for determination of cytotoxicity (%) in the assay. At least four independent experiments were performed in quadruplicate, and data from the four independent experiments were statistically analyzed (Mann-Whitney U test, *P* < 0.05) (22).

**RNA extraction and cDNA synthesis**

Total RNA was isolated from HGF culture cells, using an RNeasy Micro-Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer’s instructions. Samples were treated with RNase-free DNase (Qiagen GmbH) to prevent amplification of genomic DNA. One microgram of RNA was subjected to cDNA synthesis for 1 h at 37°C using the Ready-to-go You-Prime First-Strand Beads kit (GE Healthcare UK Ltd., Buckinghamshire, UK; cod. 279264D-100) in a reaction mixture containing 0.5 mg random hexamers (GeneAmp RNA PCR Random Hexamers Set N808-0127 Applied Biosystems, Foster City, CA, USA).

**Quantitative real-time reverse-transcriptase-polymerase chain reaction analysis**

Quantitative real-time reverse-transcriptase-polymerase chain reaction (qRT-PCR) analysis was performed using Taq-Man technology (qRT-PCR StepOne, Applied Biosystems). This assay uses a specific oligonucleotide probe and annealing between the two primer sites, which are labelled with a reporter fluorophore and a quencher. Cleavage of the probe by exonuclease activity of Taq polymerase during strand elongation releases the reporter from the probe, which increases reporter emission intensity owing to its separation from the quencher. This increment in net fluorescence is monitored in real-time during each PCR amplification. The cDNA are used for real-time PCR performed in 48-well optical reaction plates with cDNA equivalent to 100 ng RNA in a reaction volume of 25 mL containing Taqman Universal Master Mix (Applied Biosystems, cod. 4304437). The qRT-PCR assay, was performed using specific concentrations of FAM-labelled probe and specific forward and reverse primers for the IL-6, gp80, and gp130 genes (Applied Biosystems) from Assay on Demand. The results were analyzed using a comparative method, and the values were normalized to β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, as endogenous controls (22,23).

**Results**

**MTT assay**

The results of the MTT assay of HGFs treated with Jet Kit, Unifast, or Duralay for 3 or 30 min are shown in Figs. 1 and 2. The viability of HGFs was assessed in relation to resin concentration. High concentrations of Jet Kit, Unifast, and Duralay were associated with decreased cell viability. Therefore, on the basis MTT assay results, a concentration of 0.01 mM was selected for the experiments because the viability of HGFs was greater than 80%. Moreover, this concentration was consistent with conditions in dental practice.

**qRT-PCR**

qRT-PCR was used to analyze gene expressions of IL-6 and its receptors gp80 and gp130 in HGFs treated with specific medium conditioned by Jet Kit, Duralay, or Unifast resin, as described above (indirect method: 3 and 30 min after mixing). The first experiment was conducted with resins after 3 min of mixing (Figs. 3-5).
qRT-PCR showed no change in gene expression for IL-6, gp80, or gp130 in HGFs treated with medium exposed to Jet Kit, Unifast, or Duralay. After 30 min of mixing (Figs. 3-5), IL-6, gp80, and gp130 gene expressions were consistently higher in HGFs treated with Jet Kit and Unifast but not with Duralay. However, gene expressions of IL-6, gp80, and gp130 were associated with the resin used: IL-6, gp80, and gp130 gene expressions were higher in HGFs treated with Jet Kit (by 4-fold) than in the control (Figs. 3-5). In contrast, no differences were observed between HGFs treated with Duralay and HGF control in gene expressions of IL-6, gp80, and gp130. Expressions of IL-6 and its receptors (22) were always higher (by 3-fold) in HGFs processed with Unifast than in the control.

**Discussion**

Temporary fixed prostheses are an important adjunct because they protect prepared teeth and prevent exposure of dental tissues. The most commonly used temporary restorative materials in prosthodontics are PMMA, MMA, and the more modern bis-acryl composites. The main techniques for temporary restorations use custom-made and preformed materials. Both are adapted to dental tissues by means of direct clinical, indirect laboratory, and/or combined direct/indirect techniques.

In direct clinical technique, acrylic resins are in close contact with soft tissue during the polymerization phase (3-5 min), and complete polymerization requires 24-72 h. In this report, we studied the effects of three commercial polyacrylic resins (Jet Kit, Unifast, and Duralay) on transcriptional control of gene expressions of human IL-6 and its receptors gp80 and gp130 in HGFs. As compared with HGFs processed with resins polymerized for 3 min, gene expression was increased in HGFs treated when these materials were polymerized for 30 min. Moreover, Jet Kit and Unifast had the greatest ability to upregulate IL-6, gp80, and gp130 gene expression. In contrast, Duralay had no effect on transcriptional control of IL-6 or its receptors.

Polyacrylic resins induce an inflammatory mechanism in HGFs, and this process is mediated by IL-6 through the gp80 and gp10 receptors. We demonstrated that upregulation of the IL-6, gp80, and gp130 genes in HGFs differs, perhaps because of variation in the characteristics of the acid resins, namely, the amount of the materials released, the composition of the materials, and polymerization time. All these factors have crucial roles in the control of DNA transcription.

In conclusion, the present findings suggest we cannot exclude the involvement of these signals in the activation of inflammatory processes in the human gingiva. The present data should be regarded as preliminary and will require further study and experiments, which are currently underway. Specifically, our target is to identify the role of polyacrylic acid resins in the control of acute-phase
response. Our group is particularly interested in determining whether upregulation of IL-6 and its receptors in HGFs (as described in this report) leads to activation of acute-phase response mainly by means of upregulating CRP and the serum amyloid A genes, which are positive markers of the acute phase. Moreover, we plan to analyze downregulation of the albumin, pre-albumin and transferrin genes, which are negative markers of the acute phase.

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Conflict of interest
The authors have no conflict of interest to declare.

References
1. Tammaro L, Vittoria V, Calarco A, Petillo O, Riccitiello F, Peluso G (2014) Effect of layered double hydroxide intercalated with fluoride ions on the physical, biological and release properties of a dental composite resin. J Dent 42, 60-67.
2. Calarco A, Di Salle A, Tammaro L, De Luca I, Mucerino S, Petillo O et al. (2015) Long-term fluoride release from dental resins affect STRO-1+ cell behavior. J Dent Res 94, 1099-1105.
3. Schweikl H, Spagnuolo G, Schmalz G (2006) Genetic and cellular toxicology of dental resin monomers. J Dent Res 85, 870-877.
4. Diomede F, Caputi S, Merciaro I, Frisone S, D’Arcangelo C, Piattelli A et al. (2014) Pro-inflammatory cytokine release and cell growth inhibition in primary human oral cells after exposure to endodontic sealer. Int Endod J 47, 864-872.
5. Frazer RQ, Byron RT, Osborne PB, West KP (2005) PMMA: an essential material in medicine and dentistry. J Long Term Eff Med Implants 15, 629-639.
6. Bhola R, Bhola SM, Liang H, Mishra B (2010) Biocompatible denture polymers--a review. Trends Biomater Artif Organs 23, 129-136.
7. Cimpan MR, Matre R, Cresssey LI, Tysnes B, Lie SA, Gjertsen BT et al. (2000) The effect of heat- and auto-polymerized denture base polymers on clonogenicity, apoptosis, and necrosis in fibroblasts: denture base polymers induce apoptosis and necrosis. Acta Odontol Scand 58, 217-228.
8. Oppenheimer BS, Oppenheimer ET, Danishefsky I, Stout AP, Eirich FR (1955) Further studies of polymers as carcinogenic agents in animals. Cancer Res 15, 333-340.
9. Kalbgarzi V, Sravya L, Warad S, Vijayalaxmi K, Sejal P, Hazeil DJ (2014) Role of systemic markers in periodontal diseases: a possible inflammatory burden and risk factor for cardiovascular diseases? Ann Med Health Sci Res 4, 388-392.
10. Khalaf H, Lönn J, Bengtsson T (2014) Cytokines and chemokines are differentially expressed in patients with periodontitis: possible role for TGF-β1 as a marker for disease progression. Cytokine 67, 29-35.
11. Thorn CF, Lu ZY, Whitehead AS (2004) Regulation of the human acute phase serum amyloid A genes by tumour necrosis factor-alpha, interleukin-6 and glucocorticoids in hepatic and epithelial cell lines. Scand J Immunol 59, 152-158.
12. Calabrò P, Willerson JT, Yeh ET (2003) Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells. Circulation 108, 1930-1932.
13. Tata T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T et al. (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell 58, 573-581.
14. Ford ES (1999) Body mass index, diabetes, and C-reactive protein among U.S. adults. Diabetes Care 22, 1971-1977.
15. Mendall MA, Strachan DP, Butland BK, Ballam L, Morris J, Sweetnam PM et al. (2000) C-reactive protein: relation to total mortality, cardiovascular mortality and cardiovascular risk factors in men. Eur Heart J 21, 1584-1590.
16. Ridker PM, Hennekens CH, Buring JE, Rifai N (2000) C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med 342, 836-843.
17. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR (2002) Comparison of C-reactive protein and low-density lipoprotein-cholesterol levels in the prediction of first cardiovascular events. N Engl J Med 347, 1557-1565.
18. Tice JA, Brown R, Tracy RP, Cummings SR (2003) The relation of C-reactive protein levels to total and cardiovascular mortality in older U.S. women. Am J Med 114, 199-205.
19. Schuringa JJ, Jonk LJ, Doktor WH, Vellenga E, Kruijer W (2000) Interleukin-6-induced STAT3 transactivation and Ser727 phosphorylation involves Vav-1 and the kinase SEK-1/MKK-4 as signal transduction components. Biochem J 347, 89-96.
20. Neels JG, Olefsky JM (2006) Inflamed fat: what starts the fire? J Clin Invest 116, 33-35.
21. Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 343, 297-314.
22. Procino A, Simeone M, Spagnuolo G (2015) Role of IL-6 and its receptors (gp80, gp130) in the pro-inflammatory cytokine production. Int J Mol Sci 6, 742-753.