

available at www.sciencedirect.comjournal homepage: www.intl.elsevierhealth.com/journals/dema

Cytotoxicity of resin monomers on human gingival fibroblasts and HaCaT keratinocytes

Keyvan Moharamzadeh^{a,*}, Richard Van Noort^a, Ian M. Brook^a, Andy M. Scutt^b

^a School of Clinical Dentistry, University of Sheffield, Claremont Crescent, Sheffield S10 2TA, UK

^b Department of Engineering Materials, University of Sheffield, Mappin Street, Sheffield S1 3JD, UK

ARTICLE INFO

Article history:

Received 14 July 2005

Accepted 30 November 2005

Keywords:

Dental materials

Resin monomers

Cytotoxicity

Fibroblasts

Keratinocytes

Alamar Blue assay

ABSTRACT

Objectives. The aim of this study was to evaluate and compare the biological effects of three resin monomers on three human gingival fibroblast (HGF) cell lines and immortalised human keratinocytes.

Methods. Primary HGFs and HaCaT keratinocytes were cultured for 24 h and grown to sub-confluent monolayers. Resin monomers were dissolved in dimethyl sulphoxide (DMSO) and diluted with culture medium. Cultures were exposed to different concentrations of monomers (10^{-2} to 10 mM) for 24 h. Cell viability measured by Alamar Blue assay, and cell culture supernatant was examined for the presence of human interleukin-1 β (IL-1 β) using sandwich enzyme-linked immunosorbant assay (ELISA). TC50 values were calculated from fitted dose–response curves.

Results. All monomers showed toxic effects on the HGFs and HaCaT cells and inhibited chemical reduction of Alamar Blue in high concentrations. Statistical analysis of TC50 values by one-way ANOVA followed by Tukey's analysis showed that there is a significant difference in TC50 values between the cell lines ($p < 0.05$), although the rank order of monomer toxicity remained the same for different cell lines. None of these monomers-induced IL-1 β release from HGFs and HaCaT cells.

Significance. Dental resin monomers are toxic to human gingival fibroblasts and HaCaT keratinocytes. However, they cannot induce IL-1 β release from these cells by themselves. Alamar Blue assay is a sensitive method for the evaluation of cytotoxicity and it can detect different sensitivities of different cell lines to the resin monomers.

© 2005 Academy of Dental Materials. Published by Elsevier Ltd. All rights reserved.

1. Introduction

It has been shown that components of dental composite resins can be released into the oral cavity [1] and cause several adverse effects such as mucosal irritation, epithelial proliferation, oral lichenoid reaction, hypersensitivity, and anaphylactoid reactions [2]. Major monomers used in composite resins are triethylene glycol dimethacrylate (TEGDMA), bisphenol A glycerolate dimethacrylate (Bis-GMA), and urethane dimethacrylate (UDMA). Many stud-

ies have examined the cytotoxicity of these monomers on mammalian cell cultures using a wide range of assay techniques and variable levels of cytotoxicity have been reported [3–7]. However, studies that compare the sensitivities of epithelial cell lines harvested from different patients are rare and also no study has been published indicating the use of Alamar Blue assay for biological evaluation of resin monomers. Furthermore, the role of these monomers in the initiation of inflammation is still unclear.

* Corresponding author. Tel.: +44 114 2717924; fax: +44 114 2665326.

E-mail address: k.moharamzadeh@sheffield.ac.uk (K. Moharamzadeh).

0109-5641/\$ – see front matter © 2005 Academy of Dental Materials. Published by Elsevier Ltd. All rights reserved.

doi:10.1016/j.dental.2005.11.039

The Alamar Blue assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. The system incorporates an oxidation–reduction indicator that both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth [8]. Alamar Blue has two advantages over commonly used MTT assay: first, its change in colour can be detected both spectrophotometrically and fluorometrically, which gives greater sensitivity of detection; second, since it is not toxic to the cells, it is possible to assess cell viability on more than one occasion [9].

Interleukin-1 (IL-1) plays a crucial role in inflammatory processes and has been linked to gingivitis and periodontal disease [10]. In recent years, some studies have concentrated on the influence of dental materials and their components on inflammatory markers [11–13]. It has been shown that sublethal exposure of resin monomers for 2 weeks alter TNF- α secretion of THP-1 monocytes [11]. Schmalz et al. demonstrated that inflammatory mediators such as PGE₂, IL-6, and IL-8 were released from human oral tissue culture models after exposure to compounds of dental materials [12].

The aim of this study was to evaluate and compare the biological effects of three resin monomers on three different HGF cell lines and immortalised HaCaT keratinocytes using Alamar Blue assay and enzyme-linked immunosorbant assay (ELISA) for human IL-1 β .

2. Materials and methods

2.1. Fibroblast culture

Primary human gingival fibroblasts (HGFs) were obtained from stocks stored in liquid nitrogen. These cells had already been harvested from the patient biopsies under ethics committee approval. The cells were cultured in Dulbecco's modified Eagle medium (Sigma, UK) supplemented with 10% fetal calf serum (Biowest Ltd., UK), 2% L-glutamine (Sigma), 100 U/ml penicillin, (Sigma), and 100 μ g/ml streptomycin (Sigma). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. Fibroblasts were split with a mixture of 0.02% trypsin (Sigma) and 0.02% EDTA (Sigma), and incubated for 2–5 min at 37 °C. Three different HGF cell lines were serially passaged at confluence to P-6 and then they were used in cytotoxicity assay.

2.2. HaCaT keratinocyte culture

Immortalised human skin HaCaT keratinocytes were gifts from Professor Fusenig (German Cancer Research Centre, Heidelberg, Germany). Cell culture method for HaCaT cells was the same as the procedure described for oral fibroblasts except for incubating the cultures with trypsin/EDTA for 15 min to split them. HaCaT cells in passage 69 were used for cytotoxicity assay.

2.3. Monomer solution preparation

Three dental composite resin monomers; triethylene glycol dimethacrylate, bisphenol A glycerolate dimethacrylate, and

urethane dimethacrylate were purchased from Sigma–Aldrich Co., Dorset, UK. Monomers were dissolved in DMSO (Sigma) and diluted with culture medium, and seven different concentrations of monomers from 10⁻² to 10 mM were obtained by serial dilutions. The maximum concentration of DMSO used was 0.5%.

2.4. Cytotoxicity assay

In 48-well tissue culture plates, 5 \times 10⁴ cells in 1 ml DMEM per well were cultured and grown to sub-confluent monolayers for 24 h. Then cultures were exposed to different concentrations of monomers by medium change. Control groups were cells with 0.5% DMSO and cells without DMSO (N=4). After 24 h incubation, the medium was removed and stored in the freezer for inflammatory marker measurement, and 1 ml solution of 10% Alamar Blue (Biosource, Camarilo, CA, USA) in DMEM was added to each well. After 5 h incubation, 200 μ l of each medium was placed into 96-well plates in duplicates, and fluorescence intensity was measured using a fluorescent plate reader (FLUOstar Galaxy, BMG Lab Technologies) for excitation at 530 nm and emission at 580 nm. Alamar Blue reduction percent of untreated control for each dilution was calculated and plotted versus the logarithm of the concentration of the monomer. TC50 values (concentrations which reduce the cell viability by 50%) for each monomer were calculated from fitted dose–response curves using Minitab regression analysis. One-way ANOVA followed by Tukey's analysis was used to compare the sensitivity of HaCaT cells and three different HGF cell lines to resin monomers.

2.5. Inflammatory marker measurement

Cell culture supernatant was examined for the presence of IL-1 β using enzyme-linked immunosorbant assay (R&D systems, Minneapolis, MN, USA) after 24 and 72 h incubation with resin monomers in separate experiments. Supernatants were filter sterilised using a 0.22 μ m filter and used to quantify IL-1 β in duplicate samples, according to the manufacturer's instructions. A total volume of 100 μ L was used to perform these quantitative analyses. Plates were read at 450 nm and analysed using FLUOstar Galaxy plate reader (BMG Lab Technologies). Using this ELISA kit, the minimum detectable concentration for IL-1 β is <1 pg/ml, as reported by the manufacturer.

3. Results

3.1. Alamar Blue assay

During incubation, the colour of Alamar Blue changed from blue to red in control groups and low concentrations of the monomers. However, in high concentrations, all monomers showed toxic effects on the HGFs and HaCaT cells and inhibited chemical reduction of Alamar Blue.

Statistical analysis by one-way ANOVA showed that there was no significant difference in Alamar Blue values between two control groups. Therefore, either of them could be used to calculate Alamar Blue reduction percent for each dilution of

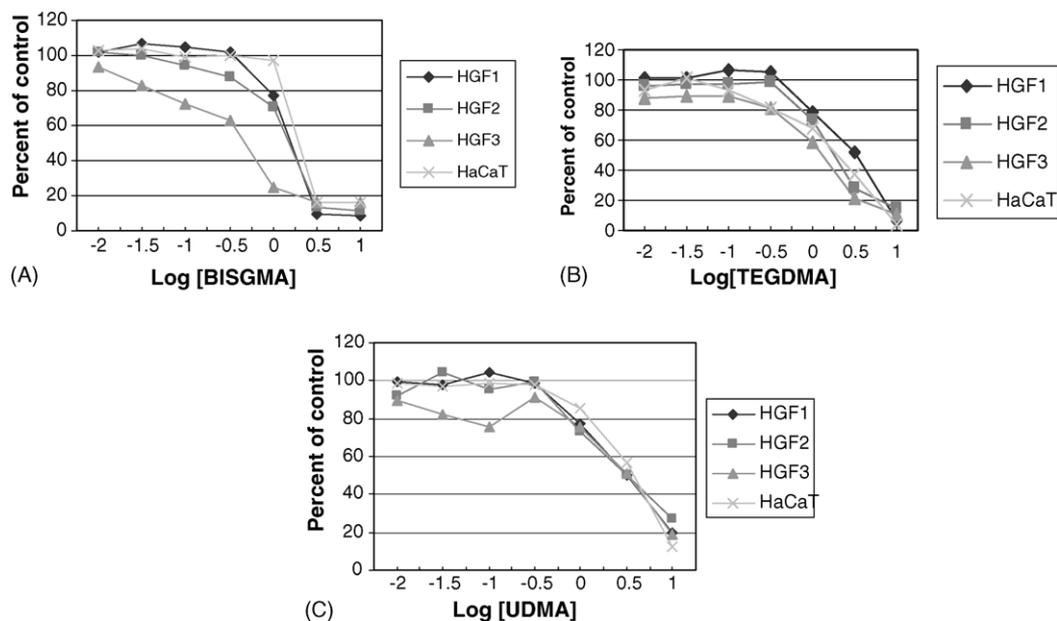


Fig. 1 – Dose–response curves for different cell lines treated with: (A) BisGMA, (B) TEGDMA, and (C) UDMA.

monomers. Dose–response curves for different cell lines and different monomers are illustrated in Fig. 1.

Regression analysis was used to produce equations for the fitted dose–response curves and then using these equations, TC50 values were calculated.

The mean TC50 values and standard deviations for each monomer on different cell lines are graphically represented in Fig. 2. Statistical analysis of TC50 values by one-way ANOVA followed by Tukey's test showed that there was a significant difference in TC50 values between the cell lines ($p < 0.05$). However, the rank of monomer toxicity was the same for each cell line. BisGMA was the most toxic and UDMA was the least toxic of the monomers tested.

3.2. Inflammatory marker measurement

Using the data obtained from the spectrophotometer and plotting the standard curve, human IL-1 β was not detectable in cell culture supernatants after 24 and 72 h incubation with resin monomers.

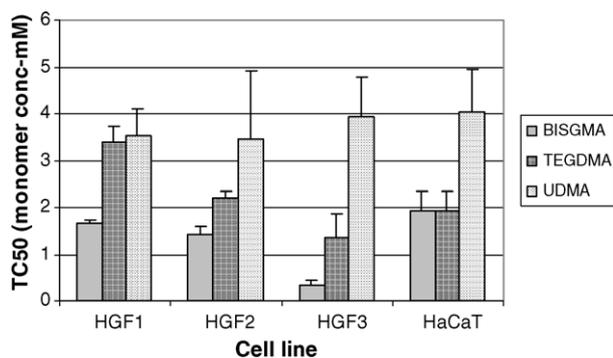


Fig. 2 – Mean TC50 for resin monomers on different cell lines.

4. Discussion

Human gingival fibroblasts were used for cytotoxicity testing because they are in close proximity with restorative dental materials in the oral cavity and more clinically relevant. Also HGFs are sensitive cells that can be easily isolated and cultured in normal culture medium [14]. HaCaT cells are immortalised human skin keratinocytes and suitable substitutes for oral keratinocytes because they can be easily grown and passaged indefinitely. Human fibroblasts and keratinocytes are frequently used in the biological assessment of dental materials [15,16].

Among numerous options available for biological endpoints, two assays were employed in the present study: Alamar Blue assay and human IL-1 β ELISA.

Alamar Blue assay was chosen because it provides an accurate index of cell viability and proliferation and as already mentioned, since its change in colour can be measured fluorometrically, it is more sensitive than the commonly used MTT assay. As the results of the present study show, Alamar Blue assay could differentiate between sensitivities of three HGF cultures isolated from different patients. This has not previously been reported using MTT assay.

Human IL-1 β as the main inflammatory marker was measured in order to investigate the possible link between resin monomers and clinical parameters such as inflammation.

Monomers tested in this study were basic monomers used in composite resins and almost all types of commercially available composite resins employ one or a combination of these monomers in their products.

Exposure time is another important parameter in cytotoxicity testing. In this experiment different cell lines were exposed to different concentrations of resin monomers for 24 h, because it has been shown that monomer release from

composite resins is complete in 24 h [17]. Therefore, most toxic effects from composite resins occur during the first 24 h.

TC50 values have been frequently used in cytotoxicity studies and a wide range of TC50 values have been reported for resin monomers [7,18–20]. The results of statistical analysis in this study showed that there was a significant difference in TC50 values between three HGF cultures and between HaCaT cells and HGF cell lines. Difference in monomer-sensitivities of HGF cultures isolated from different patients were an important finding of this study that can explain the variations in reported TC50 values for resin monomers from other studies. The difference between similar cell types isolated from different human donors is often neglected in cytotoxicity testing of dental materials and this can significantly compromise the reproducibility of cell culture tests. In line with this study it has been demonstrated that different HGF cultures isolated from different patients responded differently in a glutathione determination assay [21]. However, the results of this study showed that the rank of monomer toxicity was the same for all cell lines and similar to those reported by other studies [20,22,23]. Therefore, this study confirms that the rank of relative toxicity of tested materials is a more reproducible parameter than TC50 values in cytotoxicity tests.

In this study BisGMA was the most toxic monomer to the cell cultures. A suggested mechanism of monomer cytotoxicity is the alteration of the lipid layers of cell membranes, which affects the permeability of the membrane [24]. Based on this concept, Issa et al. investigated the correlation between monomer toxicity and log P (water/octanol partition) value and they linked the higher toxicity of BisGMA to its liposolubility [20]. However, TEGDMA is less liposoluble than UDMA but it was more cytotoxic in the present study. This finding supports other reported mechanisms of TEGDMA-induced toxicity such as apoptosis induction [25] or interference with glutathione activity and production of radical metabolites [26,27].

Although BisGMA was the most toxic monomer, studies [1,28] have shown that BisGMA and UDMA are released from composite resins in much lower levels than toxic concentrations found in this study. However, it has been demonstrated that TEGDMA leaches at comparable levels to toxic concentrations observed in this study [1]. Our findings are thus consistent with the other studies and further explain the adverse reactions induced by TEGDMA-based restorative materials.

Monomers did not induce IL-1 β release from different HGF cell lines and HaCaT keratinocytes. This finding has not previously been observed and yet would suggest that these monomers are not able to initiate inflammation by cytokine release from target cells. However, there are contradictory results in the literature with regard to inflammatory mediator release from different cell types. Consistent with the present study, it has been reported that TEGDMA and HEMA cannot induce TNF- α release from THP-1 monocytes by themselves, but they suppress LPS-induced TNF- α secretion [11]. However, it has been shown that other inflammatory mediators such as IL-6 and IL-8 are released from 3-D cultures of TR146 cells exposed to TEGDMA [12]. There might be two explanations for this finding: first, interleukin release might be due to abnormal characteristics of TR146 carcinomatous cells compared to normal epithelial cells. Second, three-dimensional cell culture models might be better than monolayer cell culture models in

the evaluation of inflammatory markers release. To find out the correct answer, further research is necessary to investigate parameters affecting cytokine release from single layer and three-dimensional cultures of normal and carcinomatous epithelial cells.

5. Conclusions

Dental resin monomers are toxic to human gingival fibroblasts and HaCaT keratinocytes. However, they cannot induce IL-1 β release from these cells by themselves.

Alamar Blue assay is a sensitive method for evaluating the cytotoxicity of resin monomers.

Different human gingival fibroblast cell lines and HaCaT keratinocytes show different sensitivities to dental resin components.

REFERENCES

- [1] Spahl W, Budzikiewicz H, Geurtsen W. Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. *J Dent* 1998;26:137–45.
- [2] Hensten-Pettersen A. Skin and mucosal reactions associated with dental materials. *Eur J Oral Sci* 1998;106:707–12.
- [3] Bouillaguet S, Shaw L, Gonzalez L, Wataha JC, Krejci I. Long-term cytotoxicity of resin-based dental restorative materials. *J Oral Rehabil* 2002;29:7–13.
- [4] Willershausen B, Schafer D, Pistorius A, Schulze R, Mann W. Influence of resin-based restoration materials on cytotoxicity in gingival fibroblasts. *Eur J Med Res* 1999;4:149–55.
- [5] Schmalz G. The biocompatibility of non-amalgam dental filling materials. *Eur J Oral Sci* 1998;106:696–706.
- [6] Thonemann B, Schmalz G, Hiller KA, Schweikl H. Responses of L929 mouse fibroblasts, primary and immortalized bovine dental papilla-derived cell lines to dental resin components. *Dent Mater* 2002;18:318–23.
- [7] Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *J Biomed Mater Res* 1998;41:474–80.
- [8] Nociari MM, Shalev A, Benias P, Russo C. A novel one-step, highly sensitive fluorometric assay to evaluate cell-mediated cytotoxicity. *J Immunol Methods* 1998;213:157–67.
- [9] Fields RD, Lancaster MV. Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. *Am Biotechnol Lab* 1993;11:48–50.
- [10] Jandinski JJ. Osteoclast activating factor is now interleukin-1 beta: historical perspective and biological implications. *J Oral Pathol* 1988;17:145–52.
- [11] Noda M, Wataha JC, Lockwood PE, Volkmann KR, Kaga M, Sano H. Sublethal, 2-week exposures of dental material components alter TNF-alpha secretion of THP-1 monocytes. *Dent Mater* 2003;19:101–5.
- [12] Schmalz G, Schweikl H, Hiller KA. Release of prostaglandin E2, IL-6 and IL-8 from human oral epithelial culture models after exposure to compounds of dental materials. *Eur J Oral Sci* 2000;108:442–8.
- [13] Heil TL, Volkmann KR, Wataha JC, Lockwood PE. Human peripheral blood monocytes versus THP-1 monocytes for in vitro biocompatibility testing of dental material components. *J Oral Rehabil* 2002;29:401–7.

- [14] Hensten-Pettersen A, Helgeland K. Sensitivity of different human cell line in the biologic evaluation of dental resin-based restorative materials. *Scand J Dent Res* 1981;89:102-7.
- [15] Polyzois GL. In vitro evaluation of dental materials. *Clin Mater* 1994;16:21-60.
- [16] Theilig C, Tegtmeier Y, Leyhausen G, Geurtsen W. Effects of BisGMA and TEGDMA on proliferation, migration, and tenascin expression of human fibroblasts and keratinocytes. *J Biomed Mater Res* 2000;53:632-9.
- [17] Ferracane JL, Condon JR. Rate of elution of leachable components from composite. *Dent Mater* 1990;6:282-7.
- [18] Reichl FX, Walther UI, Durner J, Kehe K, Hickel R, Kunzelmann KH, Spahl W, Hume WR, Benschop H, Forth W. Cytotoxicity of dental composite components and mercury compounds in lung cells. *Dent Mater* 2001;17:95-101.
- [19] Ratanasathien S, Wataha JC, Hanks CT, Dennison JB. Cytotoxic interactive effects of dentin bonding components on mouse fibroblasts. *J Dent Res* 1995;74:1602-6.
- [20] Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. *Dent Mater* 2004;20:12-20.
- [21] Engelmann J, Leyhausen G, Leibfritz D, Geurtsen W. Effect of TEGDMA on the intracellular glutathione concentration of human gingival fibroblasts. *J Biomed Mater Res* 2002;63:746-51.
- [22] Yoshii E. Cytotoxic effects of acrylates and methacrylates: relationships of monomer structures and cytotoxicity. *J Biomed Mater Res* 1997;37:517-24.
- [23] Geurtsen W, Spahl W, Leyhausen G. Residual monomer/additive release and variability in cytotoxicity of light-curing glass-ionomer cements and compomers. *J Dent Res* 1998;77:2012-9.
- [24] Lefebvre CA, Schuster GS, Rueggeberg FA, Tamareselvy K, Knoernschild KL. Responses of oral epithelial cells to dental resin components. *J Biomater Sci Polym Ed* 1996;7:965-76.
- [25] Spagnuolo G, Galler K, Schmalz G, Cosentino C, Rengo S, Schweikl H. Inhibition of phosphatidylinositol 3-kinase amplifies TEGDMA-induced apoptosis in primary human pulp cells. *J Dent Res* 2004;83:703-7.
- [26] Stanislawski L, Lefevre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A. TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. *J Biomed Mater Res* 2003;66A:476-82.
- [27] Lefevre M, Bourd K, Loriot MA, Goldberg M, Beaune P, Perianin A, Stanislawski L. TEGDMA modulates glutathione transferase P1 activity in gingival fibroblasts. *J Dent Res* 2004;83:914-9.
- [28] Ortengren U, Wellendorf H, Karlsson S, Ruyter IE. Water sorption and solubility of dental composites and identification of monomers released in an aqueous environment. *J Oral Rehabil* 2001;28:1106-15.