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Proliferation and differentiation potential of pluripotent mesenchymal precursor C2C12 cells on resin-based restorative materials

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This study investigated the proliferation and differentiation potential of pluripotent mesenchymal cells on three resin-based restoratives using a typical pluripotent mesenchymal precursor cell line, C2C12. C2C12 cells were cultured for 3-21 days on cured specimens of a Bis-GMA/TEGDMA-based composite resin (APX; Clearfil AP-X), a 4-META/MMA-based resin cement (SB; Superbond C&B) or a HEMA-containing resin modified glass-ionomer (LC; Fuji Ionomer Type II LC). To examine the influences on differentiation potential, alkaline phosphatase (ALP) activity of the cells cultured on each material was determined. On APX and SB, cells adhered and proliferated well, and no significant influences on ALP activity were observed. In contrast, poor cell proliferation and significant suppression of ALP activity were observed for cells cultured on LC, similar to those cultured on a zinc oxide EBA cement used as a control material. Bis-GMA/TEGDMA-based composite resin and 4-META/MMA-based resin exhibited better biocompatibility for C2C12 cells than HEMA-containing resin modified glass-ionomer, suggesting a potential advantage of the former two resins to show smaller influences on regeneration of periapical or periodontal tissue.

Keywords: Restorative materials, Resins, Mesenchymal cells

INTRODUCTION

Resin-based restorative materials have the potential to be utilized for various dental-treatment modalities, in addition to the restoration of missing tooth structures, because they can produce hermetic seals to prevent re-infections. These include root-end filling after apicoectomy[1-5], sealing of perforations[6] or adhesion of fractured roots[7-10]. However, histological studies have reported conflicting results of tissue responses to resin-based restoratives[1,2,4], and it remains to be determined if they are biocompatible with the periapical or periodontal tissue.

Previously, we have demonstrated that composite resins based on bisphenol-A glycidyl methacrylate (Bis-GMA)/triethyleneglycol dimethacrylate (TEGDMA), or 4-methacryloxyethyl trimellitate anhydride (4-META)/methyl methacrylate (MMA)-based resins had less effects on the proliferation and differentiation of osteoblastic MC3T3-E1 cells than a resin-modified glass-ionomer containing hydroxyethyl methacrylate (HEMA)[11]. We also confirmed that the release characteristics of unpolymerized monomers from cured resins had a significant impact on osteoblastic activities and that elution of HEMA primarily contributed to the adverse effects of resin-modified glass-ionomer on osteoblasts[12]. These findings suggest that Bis-GMA/TEGDMA-based composite resins or 4-META/MMA-resins may be more biocompatible in terms of bone regeneration when they are used in direct contact with the surrounding periapical or periodontal tissue. However, healing of tooth-supporting tissue contains regeneration of cementum and periodontal ligament in addition to alveolar bone, and diverse cells at various stages of differentiation are involved in this complicated regeneration process. Responses to resin components differ among cell types[13-17], and thus, it is of importance to thoroughly investigate the influence of resinous materials on tissue regeneration using a variety of cell types. Here, we examined the proliferation and differentiation potential of pluripotent mesenchymal cells cultured on three resinous restorative materials using a typical pluripotent mesenchymal precursor cell line, C2C12.

MATERIALS AND METHODS

Materials tested

Three resin-based restoratives, including a Bis-GMA/TEGDMA-based composite resin (APX; Clearfil AP-X, Kuraray Medical Inc., Tokyo, Japan), a 4-META/MMA-based resin cement (SB; Superbond C&B, Sun Medical Co., Moriyama, Japan) and a resin modified glass-ionomer containing HEMA (LC; Fuji Ionomer Type II LC, GC, Tokyo, Japan) were used in this study (Table 1). An aluminium-reinforced zinc oxide eugenol cement altered by addition of ethoxybenzoic acid (EBA; Super EBA, Bosworth Co., Chicago, IL, USA), which has been reported to demonstrate adverse effects on osteoblasts[11], was included as a control material.

The monomer, powder and catalyst of SB were mixed according to the manufacturer's instruction. For LC and EBA, the powder and liquid were mixed for 30-60 sec with P:L ratio=3.2:1.0 and 2.6:0.3, respectively.

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Table 1 Materials used in this study

Materials	Manufacturer	Code	Compositions
Clearfil AP-X	Kuraray Medical Inc., Tokyo, Japan	APX	Bis-GMA[a], TEGDMA[b], silica filler, camphorquinone
Super Bond C&B	Sun Medical Co., Moriyama, Japan	SB	MMA[c], PMMA[d], 4-META[e]
Fuji Ionomer Type II LC	GC, Tokyo, Japan	LC	fluoro-alumino silicate glass, HEMA[f], polyacrylic acid, water, camphorquinone
Super EBA	Bothworth Co., Chicago, IL, USA	EBA	zinc oxide, alumina, eugenol, ortho-ethoxy benzoic acid

[a] 2,2-bis[p-2'hydroxy-3'methacryloxy-propoxy]phenyl]propane
[b] triethyleneglycol dimethacrylate
[c] methyl methacrylate
[d] polymethyl methacrylate
[e] 4-methacryloxyethyl trimellitate anhydride
[f] 2-hydroxyethyl methacrylate

Disc-shaped specimens (10-mm diameter and 2-mm thick) of each material were fabricated by placing the paste in a mould and covering with a glass slide. APX and LC specimens were polymerized by light-irradiation for 40 sec each from the top and bottom surfaces using a light activation unit (Optilux 501, Kerr Corporation, Orange, CA, USA). SB and EBA specimens were allowed to set for 30 min at 25°C. All discs were sterilized by ethylene oxide gas for 7 h at 55°C followed by degassing for 12 h.

Scanning electron microscopy (SEM) observation

C2C12 cells were obtained from a commercial source (RIKEN Cell Bank, Tsukuba Science City, Japan) and maintained in Dulbecco's Modified Eagle Medium (Nacalai Tesque, Tokyo, Japan) supplemented with 10% fetal calf serum (HyClone, South Logan, UT, USA), 100 units/mL penicillin (Invitrogen Corporation, Carlsbad, CA, USA) and 100 μg/mL streptomycin (Invitrogen Corporation). Onto a sterilized disc of each material placed in a 48-well tissue culture plate (Corning Inc., New York, NY, USA), the cells were seeded at an initial density of 5×10^4 cells/well and incubated for 3, 7, 14, or 21 days at 37°C under humidified 5% CO_2. The culture media was changed every 3 days to reproduce monomer elution characteristics as in the previous study which examined the responses of osteoblasts[11].

After each incubation period, the samples were rinsed with phosphate buffered saline (PBS, pH 7.2) to eliminate unattached cells, fixed with half-strength Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde, pH 7.4) for 30 min at 4°C and dehydrated in an ascending ethanol series (50, 70, 80, 90, 96, and 100%). After being freeze-dried and sputter-coated with platinum, the specimens were evaluated by scanning electron microscopy (SEM; JEM-840A, JEOL, Tokyo, Japan). Cells cultured without any disc served as controls. Using additional sterilized specimens, it was confirmed by SEM that surface morphology before culture did not differ among the materials.

MTT assay

Cells were cultured following the same incubation parameters as for SEM observation. After each incubation period, 50 μL of MTT solution at 5 mg/mL (Sigma Chemical Co., St. Louis, MO, USA) was added to each well, and the plates were incubated for 4 h at 37°C and 5% CO_2. The plates were further incubated for 8 h after the addition of 500 μL of 2% SDS-0.01 N HCl (Nacalai Tesque), and the absorbance at 570 nm was measured. Five specimens from each material group were tested at each time point.

Alkaline phosphatase (ALP) activity measurement

After 3, 7, 14, and 21 days of culture, the ALP activity of the cells cultured on each material was evaluated as previously described[18]. The cells were rinsed twice with PBS and detached from discs using a rubber scraper after the addition of 0.02% (w/v) Triton X (Wako Pure Chemicals Industries, Osaka, Japan). A commercial kit (Alkaline Phosphate Substrate Kit, Bio-Rad Laboratories, Hercules, CA, USA) was used to determine ALP activity. Eighty μL of substrate was added to 20 μL of each sample and the mixture was incubated for 30 min at 37°C. The reaction was terminated by the addition of 100 μL of 0.4 N NaOH, and the optical density at 405 nm was measured and compared with a series of *p*-nitrophenol standard values. Total cell protein was measured using a commercial kit (BCA Protein Assay Kit, Pierce, Rockford, IL, USA), and ALP activity was calculated in nanomoles of *p*-nitrophenol produced per min per μg of protein. Five specimens from each material group were tested at each time point.

Statistical analysis

For MTT evaluations and ALP activity measurements, statistical differences were analyzed by ANOVA followed by a post hoc Scheffe's *F*-test. Statistical significance was determined as $p<0.05$.

RESULTS

SEM observation

After 3 days of culture, the C2C12 cells attached and spread well on APX and SB discs, exhibiting spindle- to polygonal-shapes similar to the control cells. On these materials, the density of the cells increased in a time-dependent manner, and the surfaces were fully covered with cells after 14 days of incubation and thereafter similarly to the control culture without any material (Fig. 1). On LC and EBA discs, attachment of cells with rounded or collapsed appearances was observed after 3 days of culture. Cells grew poorly on these materials and sparse attachment of rounded cells was observed even after 7 to 21 days (Fig. 1).

MTT assay

Figure 2 demonstrates the results of MTT assay. For

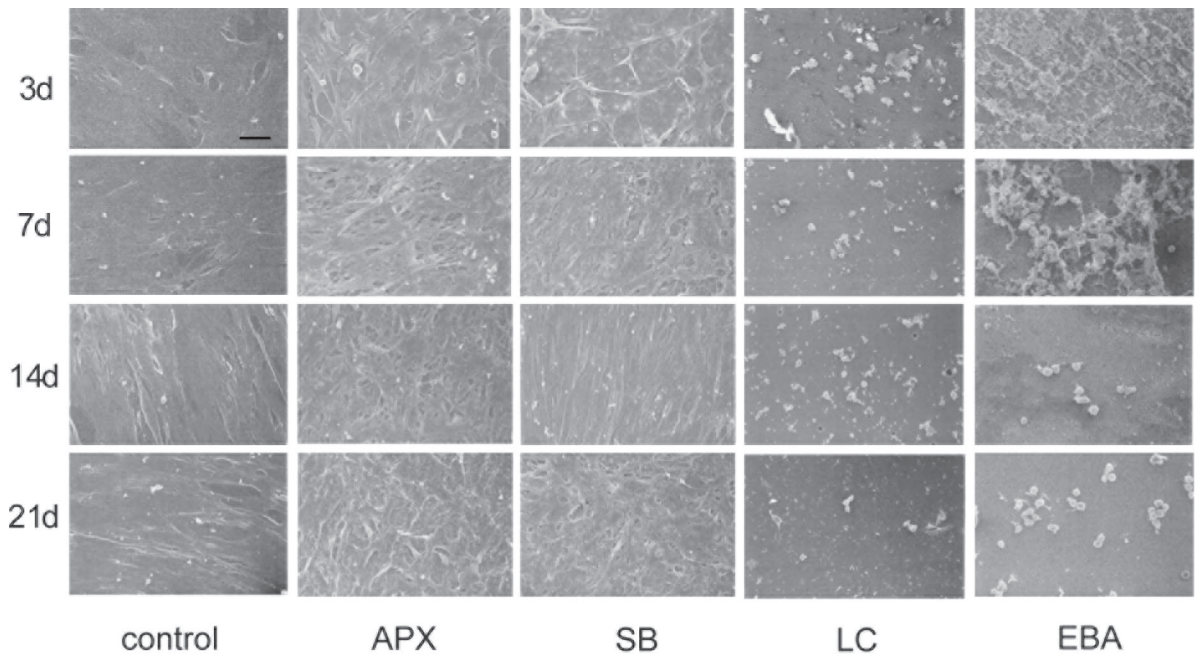

Fig. 1 SEM images of C2C12 cells cultured for 3-21 days on APX, SB, LC, and EBA discs and a control culture plate (×350). The bar indicates 50 μm.

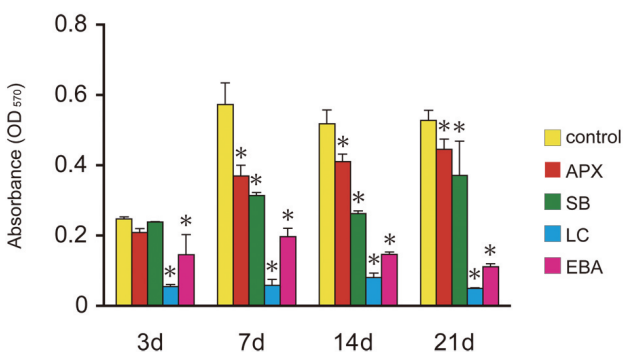

Fig. 2 Mitochondrial dehydrogenase activities of C2C12 cultured on APX, SB, LC, and EBA discs. Cells cultured without a resin disc served as a control. The values are shown as mean+standard deviation (n=5). * indicates a significant difference ($p<0.05$, Scheffe's *F*-test) between the control at each time point.

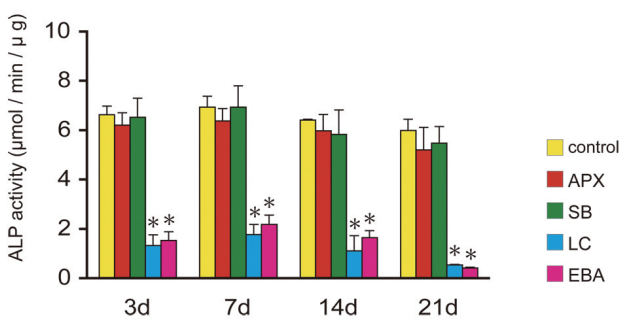

Fig. 3 ALP activity of C2C12 cultured on APX, SB, LC, and EBA discs. Cells cultured without a resin disc served as a control. The values are shown as mean+standard deviation (n=5). * indicates a significant difference ($p<0.05$, Scheffe's *F*-test) between the control at each time point.

APX and SB, no difference in the absorbance between the control was observed after 3 days of culture. The absorbance increased after 7 days on APX and SB similarly to the control cells, showing promotion of proliferation, although the values were significantly lower than the control on day 7 and thereafter ($p<0.05$). Cells cultured on LC and EBA exhibited significantly lower values than the control cells on day 3. Increase in the absorbance was not observed for these two materials even after culture for 7 days or over, and the proliferation values were significantly lower than APX or SB ($p<0.05$).

ALP activity measurement

No significant differences in ALP activity were observed between the control cells and the cells cultured on APX or SB discs ($p>0.05$) from 3 to 21 days. Contrarily, ALP activity was significantly suppressed by LC and EBA throughout the experimental period (Fig. 3).

DISCUSSION

SEM observation revealed that APX and SB supported the attachment and proliferation of C2C12 cells, while significantly fewer cells with impaired morphologies attached to LC surface during 21 days of culture. The results of the MTT assays, which measure mitochondrial dehydrogenase activity and reflect cell viability, corresponded with those of the SEM evaluations, showing a significant inhibition of cell growth by LC throughout the experimental period. In the present study, EBA was used as a control material since this cement has been demonstrated to hamper the attachment and proliferation of osteoblast-like cells[11] and to cause rapid death of periodontal and gingival fibroblasts upon culturing in pulverized form[19] due to release of eugenol. The negative influences on C2C12 observed for LC by morphological observation and MTT assay were similar to those for EBA. Accordingly, among the three resinous materials tested, Bis-GMA/TEGDMA-based composite resin and 4-META/MMA-resin were more cytocompatible and suitable for proliferation of C2C12 cells than the HEMA-containing resin modified glass-ionomer. We previously demonstrated that APX and SB were less toxic to the proliferation of osteoblast-like MC3T3-E1 cells as compared with LC[11]. The present findings for C2C12 demonstrate similar behaviors as MC3T3-E1 cells when cultured on the three resin materials. By determining the release of unreacted components from cured specimens and culturing MC3T3-E1 in the presence of monomers at the release concentrations, we confirmed that the different effects on the cells as stimulated by these three resinous materials were attributable to the elution characteristics of the unreacted monomers[12]. The proliferation of MC3T3-E1 was inhibited by HEMA at 200 μg/mL and above, while the cells grew normally in the presence of 100 μg/mL of TEGDMA or 10 μg/mL of MMA, indicating that impairment of the proliferation by LC was caused by the elution of unpolymerized HEMA[12]. Although little information regarding the toxic concentrations of unpolymerized Bis-GMA, TEGDMA, MMA or HEMA on mesenchymal cells is available and the sensitivity to resin monomers differs between cell species[13-17,20], it is reasonable to consider that the elution of HEMA from the LC specimen suppressed proliferation of C2C12 cells in this study. Additionally, as described previously[12], it is possible that acidification of the medium due to the release of polyacrylic acid from LC contributed to cell damage. However, the reason why the cells showed rounded shape on LC even after 21 days, at which period elution of HEMA is greatly reduced[11], is unknown. Detailed mechanism of cell damage by LC, including the influences on cell cycle and apoptosis, remains to be determined.

C2C12 cells used in this study are a subclone of C2 myoblasts, which were originally isolated from the thigh muscle of a C3H mouse. This pluripotent mesenchymal precursor cell line has the capacity to differentiate into osteoblasts, chondroblasts, myoblasts or adipocytes[21], and has been used to investigate the differentiation of mesenchymal cells in the periodontal ligament[22]. Many studies reported that differentiation pathway of C2C12 cells was directed into the osteoblast or chondroblast lineage by stimulation with BMP-2 or other molecules, showing elevated ALP activity[22-26]. ALP, which hydrolyzes the ester bond of organic phosphate compounds under alkaline conditions, plays an important role in the calcification. ALP activity correlates with matrix formation in osteoblasts prior to the initiation of mineralization[27] and is also a typical marker of the differentiation of osteoblasts and hypertrophic chondroblasts[28,29]. Although the present culture condition used was not specific to induce osteblastic differentiation since no stimulating factors were added, C2C12 cells cultured on three restoratives exhibited ALP activities and comparison of the differentiation potential was possible. On the APX and SB discs, ALP activity was not hampered but maintained throughout 21 days of culture. Contrarily, significant suppression of ALP activity was observed for cells cultured on LC similarly to EBA. Our previous study indicated that HEMA, at the concentrations released from LC, inhibited differentiation and mineralization of MC3T3-E1 cells, and ALP activity was significantly suppressed, while the effects of TEGDMA or MMA were much smaller or negligible[12]. In the present study, C2C12 cells responded in a similar manner as osteoblastic cells, indicating that APX and SB potentially provide preferable surfaces for mesenchymal cells to differentiate and express phenotypic characteristics.

Sawase *et al.*[30] found that Ti surfaces modified to attain photocatalytic hydrophilicity led to a significant increase in the attachment, spreading and proliferation of C2C12 cells, and such effects seemed to accelerate the early bone apposition to the implant surface. The present results obtained by culturing C2C12 directly on the cured materials indicate the possible advantage of

Bis-GMA/TEGDMA-based composite resins or 4-META/MMA-based resins to induce better biological responses in tissue regeneration. Morohoshi *et al.*[31] transplanted pulp tissue applied with SB beneath the renal capsule in rabbits and found proliferation of mesenchymal cells and ALP activity expression throughout the entire transplanted pulp area after 3 days. MMA is known to have even milder influences on cells, including osteoblasts, than Bis-GMA or TEGDMA[14,17], and therefore, SB may be further advantageous as a biomaterial for tissue regeneration.

While the LC specimen containing HEMA provided an unfavorable surface for the attachment and proliferation of mesenchymal cells, poly-HEMA hydrogel has been used as a scaffold for tissue engineering[32]. Bryant *et al.*[33] reported elongation, spreading and fibrillar formation of C2C12 cells cultured on a poly-HEMA hydrogel with immobilized type I collagen, demonstrating its benefit as a degradable scaffold. It is considered that, by controlling the polymerization rate, resinous materials can be applied diversely for tissue regeneration. However, polymerization of monomer components of dental resinous materials, which are cured in situ, is primarily influenced by environmental conditions. Difficulty in moisture control or limited direction of light irradiation may result in a compromise of curing. Animal usage tests, focusing on the use of dental resins in specific conditions, are needed to evaluate their clinical usefulness. Clearly, the development of ideal resinous materials with the capacity of regenerating periapical or periodontal tissue is an area of important future study.

CONCLUSION

A Bis-GMA/TEGDMA-based composite resin and a 4-META/MMA-based resin supported attachment, proliferation and ALP activity of C2C12 cells as compared to that exhibited on a resin-modified glass-ionomer containing HEMA, suggesting a biocompatibility for pluripotent mesenchymal cells of the former two materials.

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