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Original Article

Effects of root-end filling materials on vascular endothelial cell proliferation and tube formation

Etsuko Matsuzaki [a,b*], Haruna Hirose [a], Kazuma Matsumoto [a], Noriyoshi Matsumoto [a], Seishiro Fujimasa [a], Junko Hatakeyama [c], Hisashi Anan [a]

[a] *Section of Operative Dentistry and Endodontology, Department of Odontology, Fukuoka Dental College, Fukuoka, Japan*
[b] *Oral Medicine Research Center, Fukuoka Dental College, Fukuoka, Japan*
[c] *Section of General Dentistry, Department of General Dentistry, Fukuoka Dental College, Fukuoka, Japan*

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CS-BG-Multi;
Mineral trioxide aggregate;
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Super EBA cement;
Tube formation of vascular endothelial cell;
4-META/MMA-TBB resin

Abstract *Background/purpose:* Regarding root-end filling materials in apical surgery, sealing ability and biocompatibility are useful for treatment. Angiogenesis, which occurs in the process of periapical wound healing, is closely related to bone formation. In this study, we investigated the effects of root-end filling materials on vascular endothelial cell proliferation and angiogenesis.
Materials and methods: Mineral trioxide aggregate (MTA), 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butyl borane (4-META/MMA-TBB) resin, Super EBA, and CS-BG-multi, bioactive glass-related materials, were used. After curing, each material was soaked in a medium for 1 or 7 days, and then cultured for 1–7 days to investigate the effects on human umbilical vein endothelial cell (HUVEC) proliferation, angiogenesis, and vascular endothelial growth factor receptors (VEGFRs) mRNA expression.
Results: In the 1-day soaked sample, there was significantly less proliferation in MTA and Super EBA on day 7 of culture. In the 7-day soaked sample, there was significantly less proliferation in Super EBA and CS-BG-multi on day 7 of culture. Tube formation was significantly high in MTA in both the 1-day and 7-day soaked samples, significantly high in SB in the 1-day soaked sample, and significantly low in Super EBA in both the 1-day and 7-day soaked samples. CS-BG-multi was comparable to the control. VEGFR-1 and VEGFR-2 mRNA expressions showed an upward trend in MTA, and a trend similar to the control in SB.

* Corresponding author. Section of Operative Dentistry and Endodontology, Department of Odontology, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka, 814-0193, Japan.
E-mail address: matsuzaki@college.fdcnet.ac.jp (E. Matsuzaki).

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Conclusion: MTA and 4-META/MMA-TBB resin had a higher pro-angiogenic effect while Super EBA had a less pro-angiogenic effect. CS-BG-multi had low toxicity on tube formation of HUVEC.

Introduction

During apical surgery, root-end filling is performed with the aim of blocking irritation from the root canal system. The prognosis of apical surgery is based on the fact that bone formation by osteoblasts will occur in the bone cavity once the source of infection is removed. The healing period may take up to 1 year for large bone defects. In addition, a follow-up period of 4 years is necessary to determine the postoperative prognosis.[1] Therefore, it will be beneficial that the root-end filling material not only has sealing properties but is also biocompatible, and in particular, induces the activation of cells involved in the healing of the periapical tissue, including bone formation.

Amalgam was conventionally used as a material for root-end filling. However, many materials with excellent sealing properties and stability have been developed and are currently being used. Super EBA and IRM, which are reinforced zinc oxide eugenol cements, are frequently used instead of amalgam.[2–5] It is generally believed that Super EBA is a biocompatible material for root-end filling because of its high clinical success rate.[3] On the other hand, the cytotoxicity of Super EBA has been pointed out because it contains free eugenol.[6] The liquid component of Super EBA is based on 32% eugenol and 68% EBA, whereas the content of eugenol is about one-third of IRM. Resin composite and methyl methacrylate (MMA)-based resin are also used as root-end filling materials with good sealing ability. These can be bonded to tooth structures by treating the tooth surface. 4-Methacryloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butyl borane (4-META/MMA-TBB) resin cement such as Super-Bond are more biocompatible than resin composite because of its high polymerization rate and less elution of components including monomers and polymerization initiators.[7] Mineral trioxide aggregate (MTA) is a hydraulic cement developed in the 1990s. It produces calcium hydroxide by mixing with sterilized water, and exerts hard tissue induction ability and an antibacterial effect.[8] Therefore, it has been widely applied clinically not only as a root-end filling material but also as a direct pulp capping material and a perforation sealing material.[9–11] On the other hand, the root canal sealer using bioactive glass (BG), Nishika Canal Sealer BG (CS-BG), has been developed.[12] BG binds directly to hard tissue and exhibits biocompatibility.[13] Recently, a BG-based material, Nishika Canal Sealer BG multi (CS-BG-multi), which can be used for various purposes by mixing CS-BG with BG-based powder to adjust viscosity and curing speed, has been marketed, and its usefulness as a root-end filling material has been reported.[14]

During wound healing, angiogenesis by endothelial cells is observed. In angiogenesis, endothelial cell proliferation, migration, and tube formation are promoted after the basement membrane of the vessel is degraded. The activity of endothelial cells is regulated by the balance of angiogenesis-promoting factors and angiogenesis-inhibiting factors, which affect angiogenesis. In recent years, it has become clear that vascular endothelial cells are deeply involved in bone maturation and regeneration because they are linked to bone growth.[15] Regarding in vitro studies on root-end filling materials, there have been reports on their effects on osteoblast proliferation from the viewpoint of cytotoxicity,[6,16,17] but there have been no studies on their effects on vascular endothelial cells. In this study, we investigated the effects of MTA, 4-META/MMA-TBB resin, Super EBA, and CS-BG-multi on the proliferation of vascular endothelial cells and angiogenesis.

Materials and methods

Materials

MTA (ProRoot MTA: Dentsply Maillefer, Ballaigues, Switzerland), 4-META/MMA-TBB resin (Super-Bond (SB): Sun Medical Co., Ltd, Shiga, Japan), EBA cement (Super EBA Cement: Harry J. Bosworth Co., Skokie, IL, USA), and CS-BG-multi (Nippon Shika Yakuhin Co., Ltd., Yamaguchi, Japan) were used in this study.

Preparation of cells and sample medium

Human umbilical vein endothelial cells (HUVEC: PromoCell GmbH, Heidelberg, Germany) were used for the experiments. Specifically, 0.1 g of each material was used, molded into 96-well plates, and stored at 37 °C in an incubator with 5% CO_2 for 24 h for curing. Subsequently, cell culture medium (Endothelial Cell Basal Medium: PromoCell GmbH) was added to each well, and allowed to soak and stand. The supernatant was collected and diluted 20-fold with the cell culture medium, as reported by Costa et al.,[18] and used as a sample medium for the experiments.

Cell viability assay

The effect of various materials on the proliferation of HUVECs was investigated: 5×10^4 cells were seeded in 96-well plates and cultured in sample medium soaked with various materials at 37 °C in an incubator containing 5% CO_2. Cell counts were measured on days 1, 2, 3, and 7 of the culture using a Countess chamber slide glass (C10228; Thermo Fisher Scientific Inc., Waltham, MA, USA) with a Countess II FL (AMQAF1000; Thermo Fisher Scientific Inc.).

Angiogenesis assay

Angiogenesis was assessed using the Endothelial Tube Formation Assay (Cell Biolabs, Inc., San Diego, CA, USA). Briefly, 20 μl of ECM gel was added to 96-well plates and incubated at 37 °C in an incubator with 5% CO_2 for 30 min. Next, the plates were incubated with 3×10^4 HUVECs and each sample medium for 18 h in the same incubator. Tube formation was observed using a fluorescence microscope (BZ-X710: Keyence Co., Osaka, Japan) after calcein staining. Tube formation was evaluated when the elongated cells were uninterruptedly connected among the branch points spreading from the aggregated cells. After scanning the image, the number of branch points was measured manually by one co-author in a blind test.

Real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed according to our previous report.[19] A total of 500 ng of first-strand cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan) after extraction of total RNA using RNAiso Plus (Takara Bio Inc.). GAPDH was used as an internal control. The primers used were synthesized based on sequences obtained from the GenBank database. The sequences are shown in Table 1.

Statistics

Data were expressed as means ± standard error (SE). Significance tests were performed using Student's t-test (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA, USA) and $p < 0.05$ was considered statistically significant.

Results

Effects of various materials on the proliferation of HUVECs

The effect of various materials on the proliferation of HUVECs was investigated using sample mediums of MTA, SB, Super EBA, and CS-BG-multi soaked for 1 or 7 days. For all materials, after 1 day of soaking, cell proliferation was similar to that of the control on days 1−3 of culture, but on day 7 of culture, cell proliferation in MTA and Super EBA was significantly lesser than that of the control (Fig. 1A). In addition, after 7 days of soaking, cell proliferation in all materials was similar to that of the control after 1−3 days of incubation, but after 7 days of incubation, cell proliferation in Super EBA and CS-BG-multi was significantly lesser than that of the control (Fig. 1B). We also examined the effect of CS-BG, a basis of CS-BG-multi, on cell proliferation, resulting in the similar tendency to CS-BG-multi (Supplementary Fig. 1A, B).

Effect of various materials on HUVEC angiogenesis

Fig. 2A shows fluorescence microscopy images of tube formation and the number of tubes formation in the 1-day-soaked sample; MTA and SB had significantly more tubes formed than the control, and Super EBA had significantly fewer tubes. The number of tube formation by CS-BG-multi was comparable to the control. In the 7-day-soaked sample as shown in Fig. 2B, the number of tube formation was significantly higher in MTA than in the control, and the branching trunk was thicker in MTA. SB had a tendency to increase, but no statistically significant difference was observed compared to the control. On the other hand, Super EBA had significantly fewer tubes, and the continuity of the tubes was interrupted, resulting in an incomplete shape. The number of tube formation by CS-BG-multi and CS-BG were comparable to the control (Fig. 2B, Supplementary Fig. 1C).

Next, we examined the effects of various materials on the mRNA expression of vascular endothelial growth factor receptor (VEGFR)-1 and VEGFR-2, which are expressed on vascular endothelial cells and are reported to be upregulated during the process of angiogenesis.[20] In the samples soaked for 1 day, MTA showed an increasing trend of VEGFR-1 and VEGFR-2 mRNA expression compared with the control (Fig. 3A and B), while the effect of SB was similar to that of the control. Super EBA showed a decreasing tendency of VEGFR-1 mRNA expression. In CS-BG-multi, VEGFR-1 mRNA expression tended to increase, but the difference was not statistically significant; CS-BG was similar to the control (Supplementary Fig. 1D). VEGFR-2 mRNA expression in Super EBA, CS-BG-multi and CS-BG were comparable to the control (Fig. 3B, Supplementary Fig. 1D). In the 7-day-soaked samples, VEGFR-1 and VEGFR-2 mRNA expression was not stable, with some samples being below the detection limit for MTA, SB, and EBA (Data not shown).

Discussion

In the case of apical surgery, approximately 44% of the failures were likely caused by unimplemented root-end filling.[21] Therefore, the implementation of root-end filling has become the gold standard. The properties required for root-end filling materials are sealing ability, biocompatibility, and ease of use. The clinical success rate of the materials is 95% for Super EBA and 91% for IRM compared to

Table 1 List of primers used for RT-qPCR. VEGFR-1; vascular endothelial growth factor receptor-1, VEGFR-2; vascular endothelial growth factor receptor-2, GAPDH; glyceraldehyde-3-phosphate dehydrogenase.

GenBank ID	Target gene	Primer sequence forward/reverse
2321	Human VEGFR-1	5′-TGGCAGCGAGAAACATTCTTTTAT-3′/5′-CAGCAATACTCCGTAAGACCACAC-3′
2791	Human VEGFR-2	5′-CTCTTGGCCGTGCCTTTG-3′/5′-GTGTGTTGCTCCTTCTTTCAAC-3′
2597	Human GAPDH	5′-ATCAAGAAGGTGGTGAAGCAGG-3′/5′-GTCATACCAGGAAATGAGC-3′

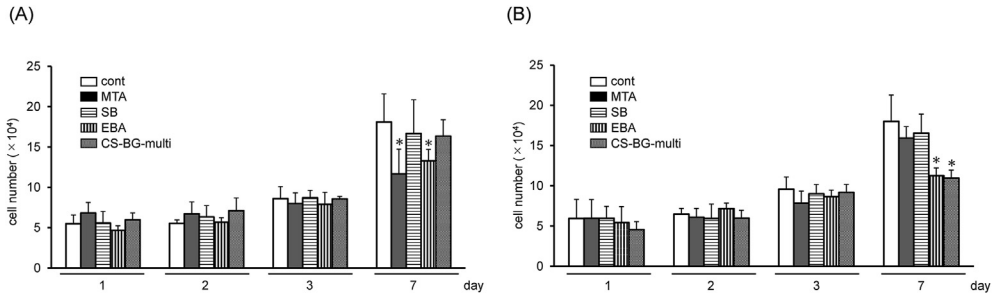

Figure 1 Effect of root-end filling material on the proliferation of HUVECs (5×10^4 cells) were cultured for 1–7 days. Sample medium that was soaked for one day (A) and 7 days (B) in each material were used, and the cell numbers were counted. Values are expressed as means ± SE ($n = 9$). *$p < 0.05$ vs control by Student's t-test.

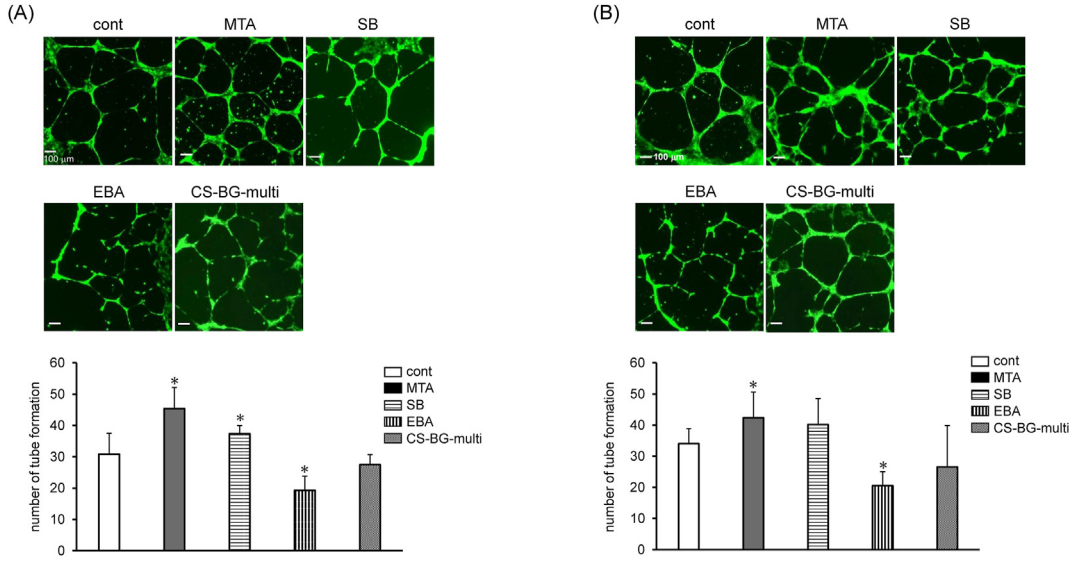

Figure 2 Effect of root-end filling material on HUVEC angiogenesis. Endothelial tube formation assay was performed on HUVECs (3×10^4 cells) using a sample medium soaked for 1 day (A) and 7 days (B). They were subjected to 18 h of incubation, and then the number of tube formation was counted manually under a fluorescence microscopy by calcein staining. Values are expressed as means ± SE ($n = 6$). *$p < 0.05$ vs control by Student's t-test.

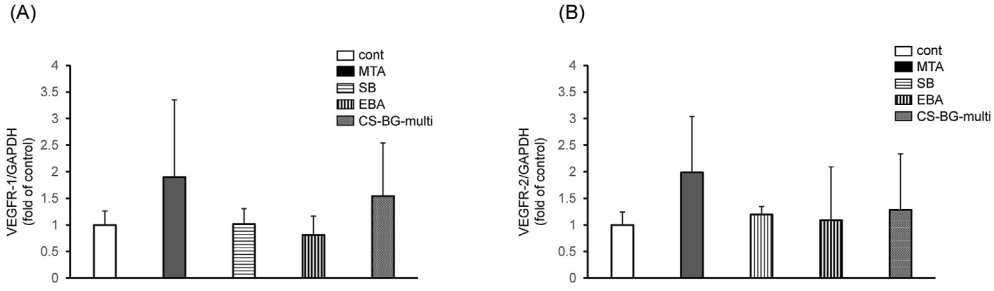

Figure 3 Effect of root-end filling material on VEGFR (A: VEGFR-1, B: VEGFR-2) mRNA expression in HUVECs. HUVECs were cultured in a sample medium that was soaked in each material for 1 day, and RNA was extracted. mRNA levels for each gene were quantified by RT-qPCR and compared with that of the controls. Values are expressed as means ± SE ($n = 3$).

75% for amalgam.[2–5] Super EBA has been reported in papers since 1990, and has a strong clinical record; therefore, it is a material covered by insurance in Japan. The treatment results of MTA are as good as or better than those of Super EBA,[3,22] and its sealing properties are superior to those of other materials.[22–24] However, in Japan, it is approved only for pulp wound surfaces. Resin composite and 4-META/MMA-TBB resin also have excellent sealing properties as a root-end filling material because they can be bonded to tooth structures by treating the tooth surface.[7]

On the other hand, biocompatibility is required not only for root-end filling material but also for materials that are placed in vivo. In the healing process after root-end filling, angiogenesis by vascular endothelial cells, osteogenesis by osteoblasts, and formation of new cementum and regeneration of periodontal ligament by activation of stem cells in the periapical tissue are important. Therefore, it is desirable to have a bioactive material that can integrate with the surrounding tissue during angiogenesis and promote healing. Additionally, it is necessary to evaluate cytotoxic effects of materials for biocompatibility. In previous study, the effects of MTA and bioceramic-related materials on cytotoxicity were examined in the 1-day-soaked sample as the early setting stage using HUVECs,[18] human mesenchymal stem cells,[18] human odontogenic stem cells.[25] Furthermore, neurotoxic evaluation of MTA and Super EBA after 7 days of setting has been reported.[26] Regarding MTA, it has been reported that the soaking period correlates with the release of calcium and hydroxyl ions, affecting in the cytotoxicity.[27] Since the final setting time of MTA is reported to be 7 days,[27] the 7-day-soaked sample was also used in this study. The proportion of tube formation was significantly high for MTA after both 1 and 7 days of soaking. In addition, VEGFR-1 and VEGFR-2 mRNA expression showed an upward trend, although no significant difference was observed compared with the control. Since VEGF is produced by macrophages under inflammatory conditions in the clinical setting, it is possible that increased VEGF signaling through these receptors may act to promote endothelial cell differentiation in vivo. However, there were no significant differences between the controls and each material, nor between all materials. The precise mechanism of angiogenesis in root-end filling materials remains to be elucidated. On the other hand, MTA significantly inhibited cell proliferation on day 7 of culture in samples soaked for 1 day. This result is consistent with the report that the cell proliferation of HUVECs peaked on day 4 of culture and showed a decreasing trend on day 7 of culture in the MTA-soaked 1-day sample.[18] MTA has been shown to become highly alkaline at 3 h after kneading.[28] Although there is no report showing a relationship between high alkalinity and suppression of vascular endothelial cell proliferation, it is possible that the high pH value in the early stage of curing may affect proliferation. However, since the proliferation by MTA was similar to that by the control on day 7 of culture in the sample soaked for 7 days, the long-term biocompatibility of MTA is considered to be good. Furthermore, osteoblasts cultured on MTA showed better cell growth and adhesion than those on amalgam or Super EBA.[16,29] Since angiogenesis is important for bone maturation and regeneration,[15] MTA may be actively involved in the promotion of alveolar bone regeneration after root-end filling through its effects of increasing angiogenesis and osteoblast proliferation. 4-META/MMA-TBB resin showed the same level of proliferation as the control, and angiogenesis was significantly higher in the sample after 1 day of soaking. As in previous reports,[7,30] it showed biocompatibility with vascular endothelial cells from an early stage. Super EBA showed significantly less cell proliferation and tube formation in both the 1-day and 7-day soaked samples. Although there are reports of cytotoxicity and delayed osteogenic repair due to free eugenol,[31,32] the biocompatibility of Super EBA is considered to be relatively excellent.[2] In this study, the number of tube formation in the sample on day 7 of soaking increased compared to the sample on day 1. Therefore, it is necessary to study under long-term soaked conditions in the future. Regarding the biocompatibility of CS-BG-multi, there is a report that CS-BG-multi did not affect the proliferation of cementoblast-like cells.[14] On the contrary, there is no report on vascular endothelial cells. Tube formation was observed within 24 h after cell seeding under in vitro conditions; therefore, the results of long-term cell culture are not reflected. However, tube formation in CS-BG-multi did not differ significantly from that in the control in the sample after 7 days of soaking, suggesting that its toxicity to vascular endothelial cells is low. Although there was no significant increase in VEGFRs mRNA expression, BG itself increased VEGF production in cardiomyocytes, inducing angiogenesis in a paracrine manner,[33] and promoting bone regeneration via HIF-1α and TNF-α signaling in HUVECs.[34] On the other hand, cell proliferation was significantly less on day 7 of culture in CS-BG-multi, and CS-BG soaked samples on day 7. It has been reported that the pH value of CS-BG-multi is 10.5 until 48 h of soaking, and that of CS-BG is 9.1 after 10 min of kneading, which becomes almost neutral on day 7, and continues to be slightly alkaline until 30 days thereafter,[14,35] suggesting that slightly alkaline is a pH suitable for survival of cementoblast-like cells, periodontal ligament cells, and osteoblasts. The reason for the discrepancy is currently unknown. However, in the process of periapical wound healing, it is speculated that CS-BG-multi has a bioactive effect on cells involved in hard tissue regeneration than on vascular endothelial cells.

In conclusion, the root-end filling materials MTA and 4-META/MMA-TBB resin were found to play a pro-angiogenic role while Super EBA had a less pro-angiogenic effect. In addition, CS-BG-multi had low toxicity on tube formation of vascular endothelial cells, and is expected to be further studied as a root-end filling material.

Declaration of competing interest

The authors declare no conflict of interest in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2021.12.006.

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