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Periodontal tissue regeneration using the cytokine cocktail mimicking secretomes in the conditioned media from human mesenchymal stem cells

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ABSTRACT

Secretomes in the conditioned media from human mesenchymal stem cells (MSC-CM) were previously demonstrated to promote periodontal tissue regeneration. By mixing insulin-like growth factor-1, vascular endothelial growth factor-A, and transforming growth factor-β1 which were included in MSC-CM, we made the cytokine cocktail (CC) mimicking MSC-CM, and then evaluated its efficacy on periodontal tissue regeneration. *In vitro*, CC promoted the migration of dog bone marrow-derived stem cells and periodontal ligament cells, and the tube formation of human umbilical vein endothelial cells. *In vivo*, class II furcation defects were surgically created at premolars in dogs. After 4 weeks of vinylpolysiloxane-induced inflammation, defects were filled with or without CC mixed in hydroxypropyl cellulose, or enamel matrix derivative (EMD). After 8 weeks, periodontal tissues were evaluated histologically and immunohistochemically. CC showed promotional effects on angiogenesis and formation of new bone and cementum. Osteogenesis by CC was greater than that by EMD and cementogenesis by CC was as well as that by EMD. CC may be promising for periodontal tissue regeneration.

1. Introduction

Periodontitis is an inflammatory disease that causes the destruction of periodontal tissues and ultimately teeth loss. There are many reports on regenerative therapies for destroyed periodontal tissues, such as guided tissue regeneration, tissue engineering by periodontal ligament-derived cell sheets [1], periosteum sheets [2], cytokines [3,4] and enamel matrix derivative (EMD). EMD is a protein fraction derived from pigs that includes an amelogenin family protein secreted from the Hertwig's epithelial root sheath in dental root formation. EMD is commonly used in today's regenerative therapy because its efficacy is supported by abundant evidence [5–9].

We have focused on secretomes in the conditioned media from human mesenchymal stem cells (MSC-CM), which were known to include many kinds of cytokines and other factors. We have reported that MSC-CM promote angiogenesis, the migration of endogenous stem cells and the regeneration of bone and periodontal tissues [10–14]. When MSC-CM were applied to one-wall critical-size, intrabony periodontal defects in dogs, MSC-CM group displayed significantly greater bone and cementum regeneration than control groups [11]. Indeed, MSC-CM can overcome several problems of cell transplantation such as safety and quality management, cost, and difficulties of handling cells. However, cell-derived products including MSC-CM still have difficulties of its standardization as well as cell transplantation. In fact, MSC-CM need pre-culture of human mesenchymal stem cells (MSCs) before application. There is also an ethical problem when a MSC-CM product secreted by a foreign donor is applied to a patient. In addition, the effective components in MSC-CM remain unclear. These things have impeded the development of MSC-CM as a commercially available product. We have also reported that MSC-

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CM include insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF)-A, and transforming growth factor (TGF)-β1 [11,12]. In this study, these cytokines were mixed at the concentrations detected in MSC-CM, and the cytokine cocktail (CC) was made mimicking MSC-CM. The regenerative effect of CC on periodontal tissues was evaluated and compared with that of EMD using a dog class II furcation defect model.

2. Materials and methods

2.1. Cytokine cocktail

Recombinant human IGF-1 (Astellas Pharma Inc., Tokyo, Japan), VEGF-A (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and TGF-β1 (R&D Systems, Inc., Minneapolis, MN, USA) were mixed at the concentrations of 1400, 500, and 350 pg/ml, respectively, and used as CC in this study. These concentrations were decided based on the concentrations of each cytokine included in MSC-CM [12]. Then, by mixing CC with hydroxypropyl cellulose (HPC) (NISSO HPC M; Nippon Soda Co., Ltd., Tokyo, Japan) and Dulbecco's phosphate-buffered saline (PBS) (Life Technologies, Inc., Paisley, U.K.), a viscous CC solution in 8% HPC (CC/HPC) was produced.

2.2. Cell isolation

Four healthy male beagle dogs, 12–18 months of age and weighting approximately 10 kg, were used in this experiment. All experiments were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine (Approval ID No.27464). Dog bone marrow-derived mesenchymal stem cells (dBMMSCs) and dog periodontal ligament cells (dPDLCs) were isolated from dogs and expanded according to published techniques [15,16]. Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). All cells were cultured at 37 °C with 5% CO_2 and 95% air in a humidified incubator and cells in the third passages were used in this experiment.

2.3. Migration assay

Transwell dishes with 8.0-μm pore membranes (BD BioCoat™ Control Inserts; Becton Dickinson and Co., Franklin Lakes, NJ, USA) were used. dBMMSCs (5×10^5 cells/cm^2) were seeded into the upper chamber. CC/HPC or EMD (Emdogain®; Straumann, Basel, Switzerland), diluted twice with Dulbecco's modified Eagle's medium (DMEM) (Gibco®), was added to the lower chamber. DMEM with 30% FBS or without serum were used as controls. For dPDLCs, the same procedure was followed using α-minimum essential media (α-MEM) (Gibco®) instead of DMEM.

After 48 h incubation, cells on the upper side of membranes were removed by washing with PBS and wiping with a cotton swab. The migrated cells on the lower side were stained with hematoxylin, and counted in three randomly selected fields using a light microscope (CL-40; Olympus, Tokyo, Japan) at 200× magnification (n = 9 per group).

2.4. Tube formation assay

ECM gel solution (50 μL) (CBA-200; Cell Biolabs, San Diego, CA) was applied to a 96-well culture plate and incubated at 37 °C for 1 h to solidify. HUVECs (3×10^4 cells/well) were seeded onto the ECM gel and cultured in endothelial cell medium (EM) (ScienCell Research Laboratories) with or without the CC or VEGF (500 ng/mL) for 10 h. Tube formation was evaluated using a light microscope (CL40; Olympus) at 40× magnification (n = 5 per group).

2.5. Animal experimental model

All surgical procedures were performed by one surgeon (K.S.) who was experienced in the technique, monitored by another author (W.K.), and under general anesthesia using isoflurane (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and local anesthesia using 2% lidocaine hydrochloride and 1/80,000 epinephrine (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan).

After acclimatization of 30 days, furcation class II defects were surgically created at the buccal aspect of premolars except for the first premolars. Following elevation of mucoperiosteal flaps, alveolar buccal bone was removed with steel burs, and exposed cementum was removed using Gracey curettes. The furcation defects were measured at 5 mm in the apical-occlusal direction and 3 mm in the buccolingual direction with a probe. Vinylpolysiloxane impression materials (GC Co., Tokyo, Japan) were placed in these defects to induce inflammation. The flaps were repositioned and sutured. After 4 weeks, following the elevation of the flaps, the impression materials and inflammatory granulation tissues were removed and root planing was carried out. Small 1/2 rounded steel burs were then used to make horizontal landmark grooves showing the base of the defects on each root surface. Exposed root surfaces were demineralized with 24% ethylenediaminetetraacetic acid (PrefGel®; Straumann) for 3 min and washed with saline. Thirty two defects were randomly assigned to one of the following four groups: Defect, HPC, CC/HPC, and EMD groups (n = 8 per group). In the experimental groups, the defects were filled with 100 μl of each gel solution. The flaps were repositioned and sutured.

After the operations, azithromycin (Zithromac® Tablets; Pfizer Japan Inc., Tokyo, Japan) and meloxicam (Mobic® Tablets; Nippon Boehringer Ingelheim, Co., Ltd., Tokyo, Japan) were given for 3 days and soft diets were served for 7 days. Teeth cleaning with local application of 0.5% chlorhexidine gluconate (Yakuhan Pharmaceutical Co., Ltd., Hokkaido, Japan) was carried out twice per week, based on the published method [17].

2.6. Histological processing

Eight weeks after the second operations, the animals were anaesthetized and their jaws were fixed by perfusion of 10% formalin applied through the carotid arteries. The jaw blocks were cut out, decalcified at 4 °C for 98 h with the decalcifying solution [18], dehydrated, and embedded in paraffin. Serial sections (4-μm thick) were cut in the mesiodistal plane throughout the buccolingual extension of the teeth.

2.7. Histological and histomorphometric analysis

The sections that was located 1000 μm and 1500 μm from the most buccal section of the furcation defect in the lingual direction (buccal sight and center, respectively) were stained with hematoxylin and eosin, and photographed using a light microscope. The histomorphometric measurements on the defect area (mm^2), the defect length (mm), the new bone area (mm^2), and the new cementum length (mm) were performed for each section using the ImageJ 1.50a software package (National Institutes of Health, Bethesda, MD, USA). The new bone formation rate (NBR) is the ratio of the new bone area to the defect area, whereas the new cementum formation rate (NCR) is the ratio of the new cementum length to the defect length. They were calculated, as shown in Fig. 1. All histomorphometric analyses were performed independently by three different investigators (K.S., W.K., and M.O.) without any information about the specimen, and data were collected and averaged. Before starting analyses, investigators shared the rules for histological evaluation.

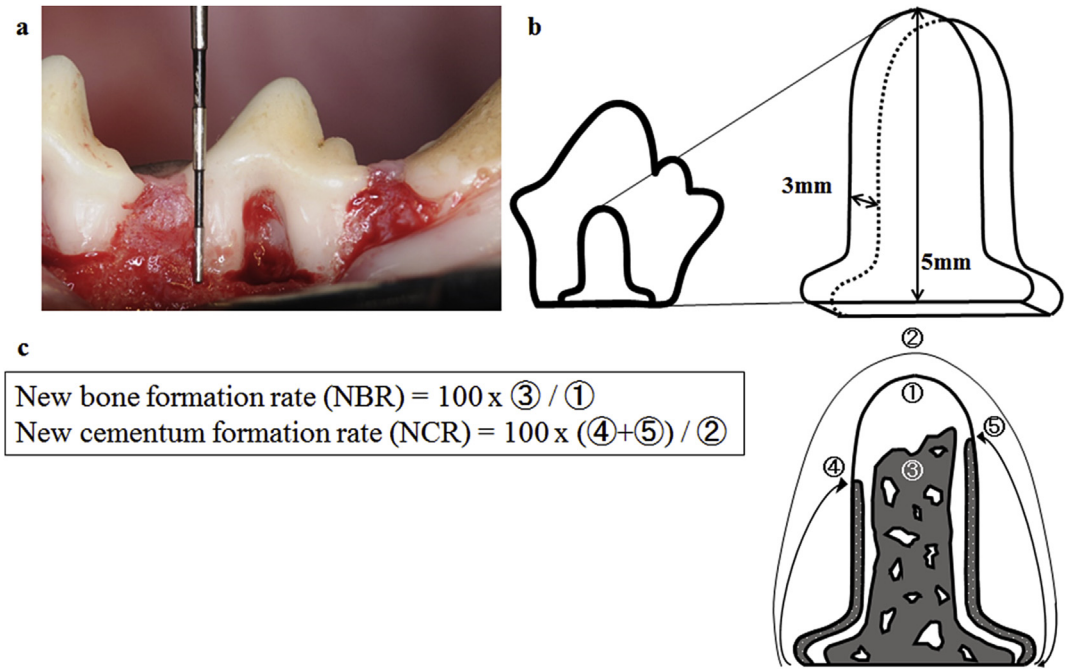

Fig. 1. (a) Clinical photograph of the class II furcation defect. (b) Schema of mesiodistal view of the defect for the histomorphometric analyses. (c) The formulas used to calculate new bone formation rate (NBR) and new cementum formation rate (NCR). ① Defect area (mm^2); ② Defect length (mm); ③ New bone area (mm^2); ④,⑤ New cementum length (mm).

2.8. Immunohistochemical evaluation

Sections located at the buccal sight were immunohistochemically stained using anti-human polyclonal rabbit antibody for von Willebrand factor (vWF) (Dako Japan Co., Ltd., Tokyo, Japan) as a marker for endothelial cells. EnVision + System-HRP-labelled polymer anti-rabbit (Dako Japan Co., Ltd.) was used as a secondary antibody. Stained sections were observed using a light microscope.

2.9. Statistical analysis

Statistical analysis by means of Tukey's honesty significant difference test was performed using the SPSS software (ver.23.0, SPSS, Chicago, IL, USA). All values are expressed as mean ± standard deviation. If $p < 0.05$, the result obtained was considered statistically significant.

3. Results

3.1. Migration assay

The numbers of migrated dBMMSCs were 22.44 ± 3.71, 148.11 ± 4.54, 27.78 ± 2.54, and 255.11 ± 29.83 cells in DMEM, CC/HPC, EMD, and DMEM with 30% FBS groups, respectively. The numbers of migrated dPDLCs were 10.56 ± 1.81, 75.67 ± 14.90, 7.33 ± 1.00, and 162.44 ± 11.22 cells in α-MEM, CC/HPC, EMD, and α-MEM with 30% FBS groups, respectively (Fig. 2a). Migrated cell numbers of dBMMSCs and dPDLCs in CC/HPC group was larger than those in EMD group.

3.2. Tube formation assay

The numbers of branch points were 25.00 ± 10.51, 54.80 ± 9.83, and 91.00 ± 18.53 points in EM only, EM with CC, and EM with VEGF, respectively. Tube lengths were 14330.03 ± 3183.40, 21833.07 ± 3224.26, and 35970.03 ± 3335.54 μm in EM only, EM with CC, and EM with VEGF, respectively (Fig. 2b). Tube formation of HUVECs in CC/EM group was greater than that in EM group.

3.3. Clinical observation

No suppuration, abscess formation, or increased teeth mobility was observed at all sites throughout the duration of the experiment.

3.4. Histological and immunohistochemical evaluations

Histologically, at the buccal sight in Defect and HPC groups, a small amount of new bone and cementum were locally formed around the bottom of the defects (Fig. 3a and b). Connective tissues were mainly observed in the middle portion of the defects (Fig. 3e and f). In CC/HPC group, new bone and cementum was observed from the bottom towards the middle portion of the defects (Fig. 3c and g). In EMD group, a small amount of new bone was observed around the bottom of the defects, and new cementum was observed from the bottom towards the middle portion of the defects (Fig. 3d and h).

At the center, new bone and cementum were tended to be observed more than those at the buccal sight in all groups. Especially, in CC/HPC and EMD groups, new cementum was observed from the bottom to the top of the defects (Fig. 3o and p). New bone was observed all over the defects in CC/HPC group (Fig. 3k), whereas it was locally observed below the middle portion of defects in EMD group (Fig. 3l). No foreign body reaction, inflammation or direct contact of bone and dentin was observed in any of the defects.

Tube formations of vWF-positive cells were seen on the periphery of the periodontal ligaments and the surfaces of bones and roots in CC/HPC and EMD groups (Fig. 3s and t). In Defect and HPC groups, those features were not observed to any great extent

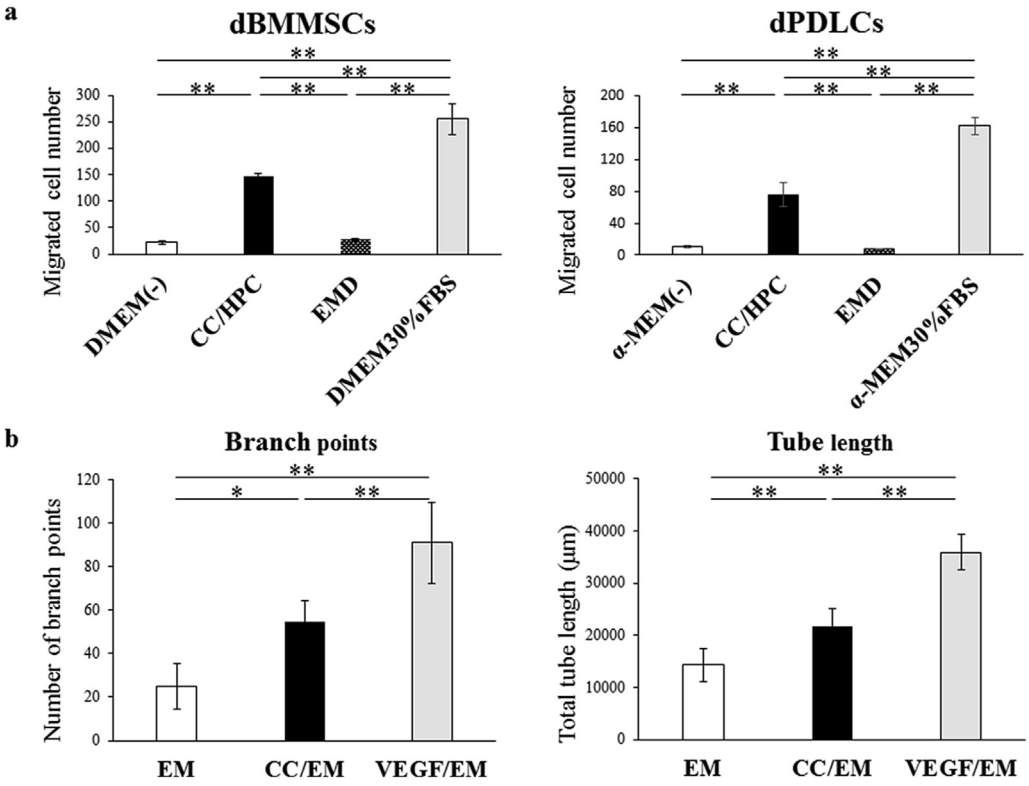

Fig. 2. (a) Transwell migration assay. The migrated cell numbers were counted. $^{**}p < 0.01$. n = 9. (b) Tube formation assay. The number of branch points and tube lengths were evaluated. $^{*}p < 0.05$; $^{**}p < 0.01$. n = 5.

(Fig. 3q and r).

3.5. Histomorphometric analysis

At the buccal sight, the NBRs were 33.10 ± 11.81, 40.86 ± 8.58, 60.80 ± 12.54, and 37.14 ± 18.44% in Defect, HPC, CC/HPC, and EMD groups, respectively (Fig. 4a). The NCRs were 38.34 ± 10.50, 49.88 ± 8.60, 65.68 ± 9.78, and 68.58 ± 17.38% in Defect, HPC, CC/HPC, and EMD groups, respectively (Fig. 4b). At the center, the NBRs were 56.84 ± 11.76, 67.57 ± 11.95, 84.30 ± 5.96, and 69.50 ± 5.24% in Defect, HPC, CC/HPC, and EMD groups, respectively (Fig. 4c). The NCRs were 60.75 ± 15.38, 70.21 ± 11.64, 88.54 ± 6.67, and 89.65 ± 6.91% in Defect, HPC, CC/HPC, and EMD groups, respectively (Fig. 4d). The NBR was higher in CC/HPC group than in the other groups at both sight. The NBR was higher in EMD group than in Defect group at the center. The NCRs were higher in CC/HPC and EMD groups than in Defect and HPC groups, and there was no significant difference in the NCR between CC/HPC and EMD groups at both sight.

4. Discussion

EMD was previously reported on its high cementogenic potential [5–9]. In this study, the NCR in EMD group was high, and there was no significant difference in the NCR between CC/HPC and EMD groups. This suggest that cementogenic potential of CC may be as well as that of EMD. The NBR in CC/HPC group was greater than in EMD group and this suggests osteogenic potential of CC may be greater than that of EMD. Some groups suggested that EMD alone may have little osteogenic potential [19,20], and our results were in agreement with them. *In vitro*, the numbers of migrated dBMMSCs and dPDLCs in CC/HPC group were also greater than in EMD group. Abundant endogenous stem cells recruited by applied reagents may differentiate into osteoblasts, and/or may also release cytokines and then exert paracrine effects which promote osteogenesis, such as the recruitment of other stem cells and the differentiation of them. Thus, the difference in osteogenic potential may be partially explained by the difference in the promotional potential on stem cell migration between CC/HPC and EMD. Moreover, it was previously suggested that EMD may contain TGF-β or TGF-β-like molecules, while cells exposed to EMD may upregulate the expression of TGF-β1 [21]. Another group reported that osteoblasts cultured in EMD released not IGF-1 and bone morphogenetic protein 2 (BMP-2) but TGF-β1 [22], and EMD-induced osteogenesis may be via TGF-β signaling pathway. On the other hand, CC includes other osteogenic cytokines in addition to TGF-β1 and has the potency of promoting other osteogenic signaling pathways and also exerting their cooperative effects. This may be another of the reasons for the superiority of osteogenesis by CC/HPC.

In general, cytokines have dose-dependent effects and most researchers have used them at higher concentrations to achieve adequate regeneration. Among the commercially available cytokine products, BMP-2 (INFUSE® Bone Graft; Medtronic, Memphis, TN) and PDGF-BB (GEM21s®; Osteohealth, Shirley, NY) have been used at concentrations of 1.5 mg/ml and 300 ng/ml, respectively, i.e., approximately thousands-fold greater than the concentration of the cytokines in CC. A cytokine applied at high concentrations may induce inflammation-related disorders such as oral edema, mouth pain, and oral erythema, as reported for BMP-2 [23,24]. Cytokine combinational approaches have also been reported in bone and periodontal tissue regeneration, such as IGF plus platelet-derived growth factor (PDGF) [3], VEGF plus PDGF [25], and VEGF plus BMP [26]. However, these approaches also require high doses of each factor for adequate regeneration. In this regard, the novelty

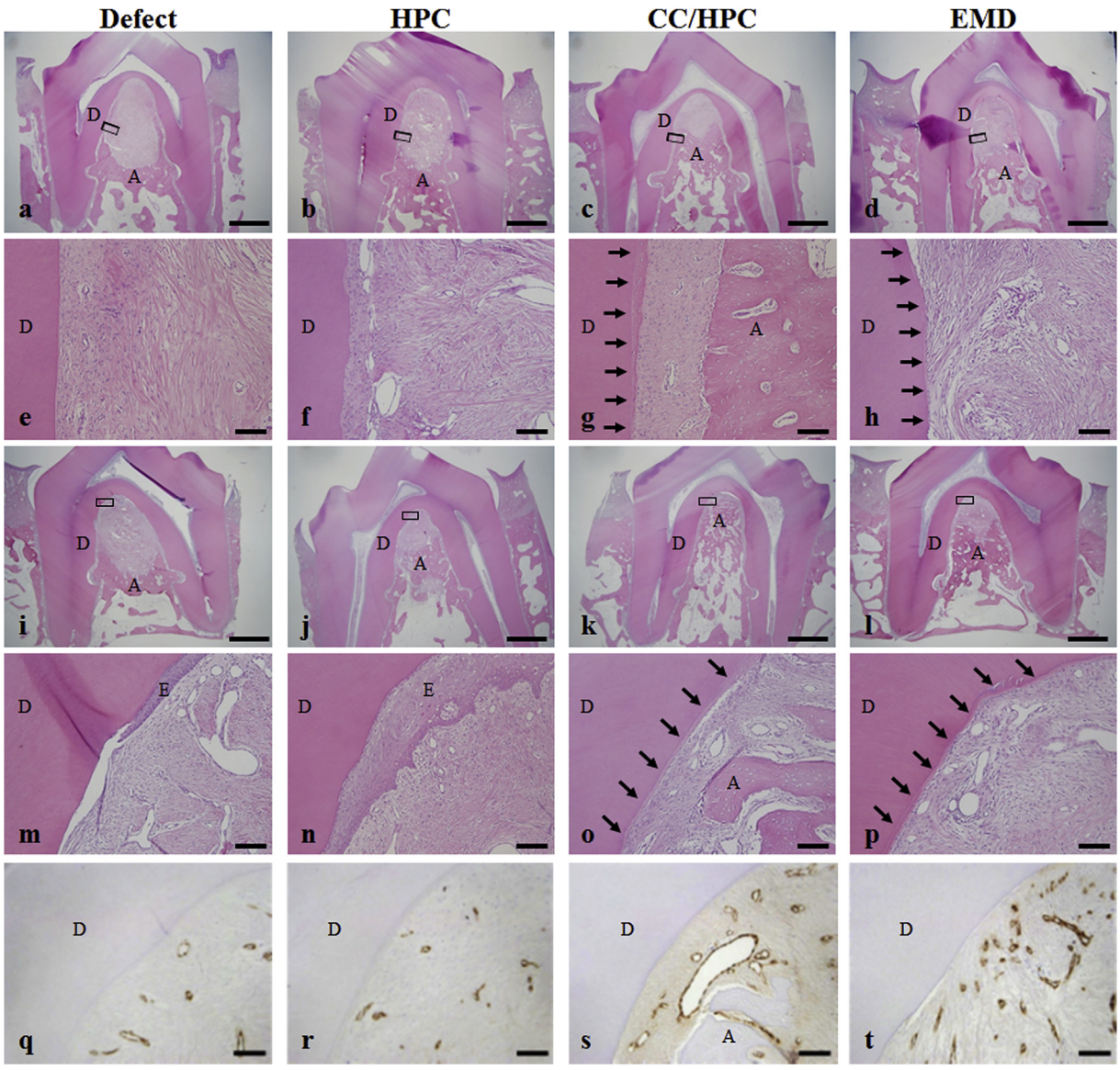

Fig. 3. Histological and immunohistochemical evaluations. (a–p) Mesiodistal sections stained with hematoxylin and eosin. Overviews of periodontal defect sites at the buccal sight (a–d) and the center (i–l). (e–h and m-p) Highly magnified sections of the framed areas in (a–d and i–l), respectively. (q–t) The upper parts of defects immunohistochemically stained with vWF. Scale bar: a–d and i-l 2 mm, e–h and m–t 100 μm. E, epithelium; D, dental root; A, alveolar bone; Arrow, cementum.

and advantage of CC is the low cytokine concentrations required to achieve regeneration.

Each cytokine in CC was previously reported to contribute to bone and periodontal tissue regeneration, as follows. IGF-1 promotes the recruitment and osteogenic differentiation of MSCs and PDLCs [27,28]. VEGF-A promotes the recruitment and tube formation of endothelial cells, and induces angiogenesis and vascular permeability [29–32]. TGF-β has multiple functions depending on the particular kind of cell in which it is expressed. TGF-β1 promotes osteogenic differentiation of MSCs especially at low concentrations [33] and induces the synthesis and deposition of extracellular matrices [34–37]. TGF-β also exerts its anti-inflammatory effect through the induction of Foxp3-expressing regulatory T cells [38].

Cooperative effects which these cytokines exert on each other have also been reported. IGF-1 upregulates VEGF expression via hypoxia-inducible factor-2a [39]. TGF-β increases the amount of IGF available via the downregulation of IGF-binding protein expression [40]. TGF-β upregulates the synthesis of VEGF in endothelial cells [41]. In our prior study using a rat calvarial bone defect model, we demonstrated that CC promoted migration of stem cells and osteogenesis, compared with a single cytokine or the combination of two cytokines in CC (data not shown). Based on these findings, we assume the cytokines in CC may effectively activate the multiple stages of bone and periodontal tissue regeneration, individually or in cooperation with each other.

This paper is the first report on a defined cytokine cocktail mimicking MSC-CM for periodontal tissue regeneration. Such a defined cytokine cocktail can be used as standardized formulation, although cells or cell-derived products produced from human MSCs are difficult to be standardized. Moreover, it can be directly used during surgery without pre-culture patient-MSCs before use. There is not even an ethical problem to use a product secreted by a foreign donor in patient. From these points, CC is considered to be superior to non-standardized cells and cell-derived products. CC may be promising for periodontal tissue regeneration.

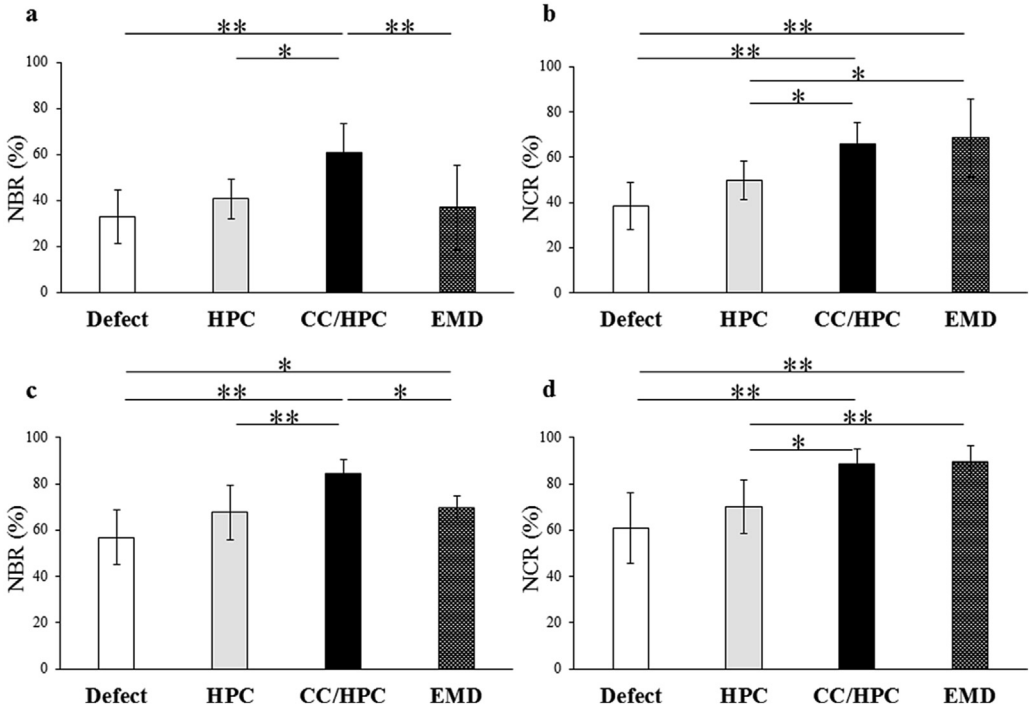

Fig. 4. Histomorphometric analysis. The NBRs and NCRs were evaluated at the buccal sight (a and b) and at the center (c and d). $^{*}p < 0.05$; $^{**}p < 0.01$. n = 8.

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