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GM-CSF Stimulates Mouse Macrophages and Causes Inflammatory Effects in Vitro

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Abstract: Macrophages are involved at an early stage of the inflammatory reaction and have an important role in wound healing. Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates the proliferation and differentiation of macrophages. We investigated the secretion of tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) from mouse macrophages (RAW264.7) activated by GM-CSF. RAW264.7 cells were cultured on titanium (Ti) discs. Secretion of TNF-α and IL-4 was evaluated using enzyme-linked immunosorbent assay (ELISA) at 24 h and 48 h. Cell morphologies were observed using SEM, and cell viability was accessed by an MTT assay.

GM-CSF caused rough and irregular surface morphology on the macrophages and resulted in a significant difference in cell viability after 48 h (p<0.05). TNF-α secretion significantly decreased after 48 h without GM-CSF compared with that at 24 h (p<0.05). GM-CSF significantly increased the secretion of TNF-α after 24 h and 48 h (p<0.05). IL-4 secretion was significantly different with or without GM-CSF stimulation at 24 h and 48 h (p<0.05). There was a significant increase in IL-4 secretion 24 h and 48 h after GM-CSF stimulation (p<0.05).

These results suggest that macrophage stimulated GM-CSF may promote secretion of anti-inflammatory and pro-inflammatory cytokines on Ti.

Key words: GM-CSF, Macrophage, TNF-α, IL-4, Titanium

Introduction

Titanium (Ti) and Ti alloy have been widely used as dental implant materials because of their excellent biocompatibility and mechanical properties7. The tight bonding of titanium to the bone is known as osseointegration3.

The integration of dental implants into bone tissue evokes a series of responses directed against these foreign objects, which is known as a foreign body response. Absorption of proteins including extracellular matrix components is the first biological response at the implant-tissue interface, and then a cellular response is initiated2-4. The subsequent wound healing process consists of a series of stages including inflammation, tissue repair, and tissue remodeling5-7.

Macrophages have an important role in directing the early events in wound healing. The interaction of macrophages with the implant surface may be an important determination of the resultant material-tissue interface. Macrophages organize the activation of numerous cell types and regulate the intensity and duration of inflammatory responses. During the foreign body response, macrophages regulate the duration and intensity of different stages of the foreign body response8-10. Permanent activation of macrophages during the foreign body response may result in persistent inflammatory reactions.

Brodbeck et al. studied the role of biomaterial surface properties on the expression of cytokines by leukocytes2,3,11. They classified cytokines according to their roles in the foreign body response: anti-inflammation, pro-inflammation, anti-wound healing, or pro-wound healing. For example, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are pro-inflammation and anti-wound healing cytokines, whereas transforming growth factor beta-1 (TGF-β1) and interleukin-4 (IL-4) are anti-inflammation and pro-wound healing cytokines.

The surface topography of Ti affects the specific expression of bone morphogenetic protein 2 in adherent macrophages12. In contrast, the adhesion of macrophages on rough and smooth Ti substrates do not alter the expression of pro-inflammatory cytokines such as IL-613. Jeroen et al. studied macrophage behaviors on Ti coated with multilayered DNA and found that macrophages had decreased levels of the pro-inflammatory cytokine TNF-α, but no difference in the secretion of anti-inflammatory cytokines such as TGF-β114.

Simulating factors, such as granulocyte-colony stimulating factor (G-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF), cause macrophage proliferation and differentiation. GM-CSF promote proliferation and differentiation of granulocyte and macrophage by affecting hematopoietic progenitor cells, whereas G-CSF only affect neutrophil proliferation and differentiation. It is presumed that macrophage activated with GM-CSF promote wound healing process and can be potentially reduced healing period.

Here, we examined the secretion of a pro-wound healing cytokine IL-4 and a pro-inflammation cytokine TNF-α from macrophages in the presence or absence of GM-CSF to understand the relationship between inflammation and the bone healing process.
Materials and Methods

Titanium substrate

Commercially pure Ti disks (99.9% mass Ti, Grade 2, φ5 mm diameter x 0.5 mm thickness, φ15 mm diameter x 1 mm thickness, Furuuchi Chemical Co., Tokyo, Japan) were used. Ti samples were polished with #1200-grit water-proof paper, cleaned with acetone, and then dried in a desiccator at room temperature. Before cell culture, the specimens were sterilized with ethylene oxide gas.

Cell culture

The mouse macrophage cell line RAW264.7 (DS Pharma biomedical, Japan) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; DS Pharma Biomedical Co., LTD, Osaka, Japan) containing 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were passaged every seventh day, and confluent cells were removed by scraping and subsequently split in a ratio of 1:3-1:5.

Cells were seeded on Ti disks (φ5 mm) in tissue culture polystyrene (TCPS) 96-well plates at a density of 3.35 x 10⁵ cells/well, and cultured for 24 h and 48 h. For stimulation of macrophages, cells were cultured in the medium with 250 ng/ml GM-CSF (Sigma-Aldrich Co., USA), and medium with GM-CSF was changed at 12 h.

Cell morphology

Cells were seeded on Ti disks (φ15 mm) in 24-well plates at a density of 4.3 x 10⁵ cells/well and subsequently cultured for 24 h and 48 h. Cells were rinsed twice with phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS), and subsequently fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffered solution for 15 min. Fixed cells were rinsed twice with cacodylate buffered solution and dehydrated using a graded series of ethanol. Finally, fixed cells were dried with tetramethylsilane.

The cell samples were sputter coated with gold, and the morphologies of attached cells were observed using a scanning electron microscope (SEM, JSM-5600LV, JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 10 kV.

Cell viability

Cells were seeded on Ti disks (φ5 mm) in 96-well plates at a density of 3.35 x 10⁵ cells/well and subsequently cultured for 24 h and 48 h. For stimulation of macrophages, cells were cultured in medium with 250 ng/ml GM-CSF (Sigma-Aldrich Co., USA), and medium with GM-CSF was changed every 12 h.

Cell viability was assessed using the commercially available tetrazolium salt ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], MTT) kit (Sigma-Aldrich Co., USA). At 24 and 48 h, culture medium was replaced by a 10% MTT-DMEM solution, and cells were incubated at 37°C for 4 h. A stop solution was added to each well, and the absorbance at 570 nm wavelength was measured using a microplate reader (Molecular Devices: EmaxPlus). The reaction solution was transferred to new plate immediately in triplicate after the addition of stop solution before measuring.

Cytokine secretion

The secretion of two cytokines, TNF-α and IL-4, which are a pro-inflammation cytokine and a pro-wound healing cytokine, respectively, was measured in culture media supernatant after a 24 h and 48 h incubation by an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., USA). An equal amount of assay diluent and medium from cultured RAW264.7 cells was added to 96-well ELISA plates coated with an anti-TNF-α or anti-IL-4 monoclonal antibody, and then incubated at room temperature for 2 h. After washing with wash buffer, HRP-conjugated detection antibody was added to each well and incubated at room temperature for 2 h. After washing, substrate solution was added to each well and incubated at room temperature under light shielding for 30 min.

The reaction was stopped by the addition of stop solution and measured by a microplate reader at 450 nm (Molecular Devices, CA, USA).

Statistical analysis

Data from cell cultures were analyzed by one way ANOVA and the post hoc Tukey’s test for multiple comparisons among means at p<0.05 using Origin Pro 8J (Origin Lab Corp., MA, USA). The obtained values are represented as mean ± standard deviation.

Results

Cell morphologies

Fig. 1 shows the cell morphologies by SEM observation. RAW264.7 macrophages cultured on Ti discs had a round shape, which is a typical
GM-CSF stimulation increased IL-4 secretion at both 24 h and 48 h stimulation after 48 h compared with that at 24 h (p<0.05). In addition, there was a significant increase in the secretion of TNF-α after 24 h and 48 h with GM-CSF stimulation compared with the level at 24 h (p<0.05). There was a significant increase in IL-4 without GM-CSF compared with that at 24 h (p<0.05). In addition, GM-CSF stimulation increased IL-4 secretion at both 24 h and 48 h (p<0.05).

**Discussion**

We evaluated the secretion of a pro-wound healing cytokine IL-4 and a pro-inflammation cytokine TNF-α from macrophages in the presence or absence of GM-CSF on Ti. Cell morphologies and viabilities were also evaluated.

Macrophages have a major role in normal wound healing and the reparative process around implants. Previous studies evaluated the biocompatibility of biomaterials used in orthopedics and dentistry using macrophages. For example, Petit et al. reported that ultra-high-molecular-weight polyethylene and alumina particles induced TNF-α release from murine J774 macrophages and claimed that implantation failure may be associated with changes in the production of cytokines from the immune system. The cascade of events behind a lost implant includes the release of pro-inflammatory cytokines by macrophages.

There are only a few reports about macrophage behavior on Ti. We evaluated cytokine secretion by macrophages with or without the stimulating factor GM-CSF. GM-CSF is critical for the proper maintenance of steady-state macrophage development and is essential for proper alveolar macrophage maturation. GM-CSF is often used in the presence of a co-stimulus, such as IL-4, to generate dendritic cell populations, and GM-CSF induces cells with dendritic cell properties.

We found that the viability of macrophages on Ti in the absence of GM-CSF increased with time, which was similar to the result by Sunarso et al. that found that GM-CSF stimulation did not influence cell viability after 48 h.

Among cytokines, numerous studies related with TNF-α as pro-inflammation cytokine, IL-4 and IL-10 as anti-wound healing cytokine, were reported as secreted cytokines from activated macrophage. Brodbeck et al. classified IL-4 as pro-wound healing group. van der Beucken et al. found that macrophages showed decreased secretion level of TNF-α on DNA-coated titanium implants. Thus, we focused the secretion of TNF-α and IL-4 on titanium.

TNF-α secretion is stimulated by lipopolysaccharide (LPS) on Ti. We found that TNF-α secretion on Ti was enhanced by GM-CSF stimulation, similar to LPS stimulation. Secretion of IL-4 on Ti was also enhanced by GM-CSF stimulation. TNF-α is a pro-inflammation and anti-wound healing cytokine, and IL-4 is an anti-inflammation and pro-wound healing cytokine. Although the detailed mechanism is unclear, GM-CSF stimulation of macrophages may induce an inflammatory response and healing process.

SEM observation indicated that GM-CSF stimulation increased the roughness and irregularity of the macrophage surface. It was reported that macrophage phenotype is classified M1 (classically activated macrophages) and M2 (alternatively activated macrophages). The difference between M1 and M2 has a possibility to introduce the different morphologies of macrophages with and without GM-CSF stimulation. However, it is difficult to make clear the relationship between the macrophage morphologies and cell viability or secretion level of TNF-α and that of IL-4. The detailed studies for secretin of other cytokines will elucidate the influence of the morphologies of macrophages.

Reifai et al. and Gretzer et al. reported that the topography of the Ti surface modulates the expression of pro-inflammatory and anti-inflammatory cytokines, such as TNF-α and IL-10, by macrophages. The influence of surface conditions on the secretion of inflammatory cytokines from macrophages should be investigated further.

Kamada et al. stimulated macrophages with GM-CSF at a concentration of 20 ng/ml. We used a GM-CSF concentration of 250 ng/ml.
Differences in GM-CSF concentration may influence the secretion of inflammatory cytokines\(^{20}\).

Stimulation of macrophages by GM-CSF did not cause any changes in cell viability and proliferation. However, increase in the number of viable cells without GM-CSF stimulation. As mentioned above, GM-CSF progresses the differentiation of macrophages. Thus, differentiation of macrophages on titanium substrate with GM-CSF stimulation should be further evaluated.

The secretion of TNF-\(\alpha\) and IL-4 was significantly increased by GM-CSF stimulation. It is presumed that GM-CSF stimulation promote inflammatory and wound-healing process. Secretion of other cytokines such as IL-1, IL-6 or TGF-\(\beta\) will be elucidate this assumption.

In conclusion, GM-CSF stimulation of macrophages promoted the secretion anti-inflammatory and pro-inflammatory cytokines on Ti. The results suggest that GM-CSF may promote a wound healing process after Ti implantation.

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**Conflict of Interest**

The authors have declared that no COI exists.

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