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Granulocyte colony-stimulating factor induced reduction in pulpal necrosis

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Abstract

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Aim To examine the effect of recombinant granulocyte colony-stimulating factor (G-CSF) on the number and function of neutrophils and on the histopathology of pulpal inflammation in normal and neutropenic rats.

Methodology The effect of G-CSF on changes in pulpal tissue was investigated at 2, 4, 7, and 10 days after pulpal exposure of the mandibular first molar of normal rats and of those with methotrexate-induced neutropenia. The area of pulpal necrosis was measured. The neutrophil count in peripheral blood was determined, and their phagocytosis and chemotactic reaction were also examined. Statistical significance was examined by use of the two way analysis of variance.

Results In untreated rats, G-CSF significantly ($P < 0.05$) increased the number of peripheral neutrophils and their chemotactic reaction, but did not affect pulpal inflammation. In methotrexate-induced neutropenic rats, the phagocytosis and migration of neutrophils reduced, and the area of pulpal necrosis enlarged. After the G-CSF injection, the decreases in neutrophil count and their functions significantly ($P < 0.05$) reversed, and the enlargement of pulpal necrosis inhibited.

Conclusions These findings indicate that G-CSF prevented the reduction in neutrophil function and reduced the pulpal necrosis observed in the neutropenic rats, and suggest that neutrophils defend against bacterial invasion in pulpal tissue.

Keywords: chemotaxis, granulocyte colony-stimulating factor, methotrexate, neutrophil, pulpitis.

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Introduction

Neutrophils play a critical role in the host defense mechanism against various bacterial infections. They are highly motile phagocytic cells that constitute the first line of defense in the innate immune system. Such defense mechanisms are involved in the killing of invading microorganisms by the liberation of reactive oxygen species and lysosomal enzymes. Especially, neutral proteases in neutrophil granules, *e.g.* elastase, cathepsin G, gelatinase, collagenase, and others, are strong enzymes that can kill microbes (Segal 2005). However, the actions of this defense system may also result in tissue destruction. If the killing system of the neutrophil is out of control, it may attack the host tissue.

Although the relationship between neutrophils and development of pulpal inflammation has been investigated and these cells may play an important role (Warfvinge 1987, Kawashima *et al.* 2005), the role of the neutrophil has not been defined in terms of the pathology of pulpal inflammation. Upon bacterial invasion after pulpal exposure, the neutrophils defend the pulpal tissue against bacteria by migrating into it and phagocytizing them. However, neutrophilic degranulation of neutrophil can cause the destruction of pulpal tissue. Elastase, cathepsin G, and lactofferin have been detected in human inflamed pulp (Cootauco *et al.* 1993, Rauschenberger *et al.* 1994), as well as neutrophil collagenase (MMP-8) (Wahlgren *et al.* 2002, Tjäderhane *et al.* 2007).

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Recombinant granulocyte colony-stimulating factor (G-CSF) is currently used for the treatment of various types of neutropenia, as treatment with it enhances neutrophilic functions (Carulli 1997). G-CSF increases the number of neutrophils in the peripheral blood (Bensinger *et al.* 1993) and their functions, such as the production of superoxide (Kitagawa *et al.* 1987), chemotaxis (Yuo *et al.* 1989), and phagocytosis (Repp 1991). However, the correlation between G-CSF and pulpal inflammation has not yet been investigated.

Methotrexate is an immunosuppressive agent having the side effect of inhibiting neutrophil production in the bone marrow (Willoughby & Giroud 1969). Methotrexate-induced neutropenia caused periodontal inflammation in rats to worsen (Yoshinari *et al.* 1994). After pulpal exposure of rats injected with methotrexate, the decrease in neutrophil numbers favoured bacterial invasion and increased the extent of pulpal necrosis (Nakamura *et al.* 2002).

To determine the role of neutrophils, the effect of G-CSF on pulpal inflammation in neutropenic rat induced by methotrexate was examined.

Materials and methods

Animals and animal protocol

Sixty-four male Wistar rats, each weighing about 240 g, were divided into four groups. The rats in Group A received no injection. The animals in Group B received an intraperitoneal injection of 0.01 mg kg^{-1} body weight of G-CSF (Kirin, Tokyo, Japan) 12 h before pulpal exposure and every day after the exposure throughout the experiment. The animals in Group C received an intraperitoneal injection of 7.5 mg kg^{-1} body weight of methotrexate (Wyeth, Tokyo, Japan) once a day for 3 days before the pulpal exposure (Nakamura *et al.* 2002). Those in Group D received an injection of methotrexate in the same manner on Group C, then, they received the G-CSF in the same manner on Group B.

In the present study, pulpal inflammation was introduced by pulpal exposure of the rat mandibular first molar, as described in a previous study (Yamasaki *et al.* 1994). All animals were anaesthetized with diethyl ether. Then the pulpal tissue was exposed at the mesial portion of the occlusal surface of the molar. The exposed area remained open to the oral environment throughout the experiment.

The principles of laboratory animal care according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the animal protocol institutionally approved by the Ethics Committee of the School of Dentistry, Aichi-Gakuin University were followed.

Measurement of total leukocytes and neutrophils in the blood

Four animals of each group were sacrificed at 2, 4, 7, and 10 days after the exposure. Before sacrifice, the peripheral blood was sampled, and the numbers of total leukocytes and neutrophils were counted.

Histology

The mandible of each animal was removed and fixed in periodate-lysine-paraformaldehyde fixative. Then, the mandible was decalcified by being soaked in 5% EDTA solution for approximate 60 days, embedded in paraffin, and sectioned serially at 5 μm in the mesiodistal plane. The sections were stained with haematoxylin and eosin. The mesial root of mandibular first molar was investigated histologically.

The presence of neutrophils that had infiltrated the pulpal tissue was examined immunohistochemically. A rabbit polyclonal antiserum (AD51140) reactive with rat polymorphonuclear cells was obtained from Inter-Cell Technologies Inc. (Hopewell, NJ, USA). Cells reactive with this antibody were detected by use of an ABC kit (PK-4001: Vector Laboratories, Buringame, CA, USA). For the final chromogenic reaction, the slides were exposed to freshly prepared substrate solution that consisted of diaminobenzidine tetrahydrochloride and hydrogen peroxide. Then they were counterstained with Meyer's haematoxylin, and the pulpal and periapical tissues of mesial root were examined.

Histometry

In the histological sections, the areas of pulpal necrosis and apical periodontal ligament were measured histometrically. In the area of pulpal necrosis, there was no cellular tissue in the root canal. The area of the apical periodontal ligament between the root apex and the alveolar bone in the mesial root was determined (Yamasaki *et al.* 1994, Nakamura *et al.* 2002). It was measured by using an image-processing system that consisted of a light microscope (BX50: Olympus Co., Tokyo, Japan), colour camera (C-4040: Olympus Co., Tokyo, Japan), and personal computer (Power Book, Apple Computer Inc., CA, USA). The average areas

were determined for each mandible, and the average values were calculated for each period.

In the immunohistochemical sections, the antibody-reactive neutrophils were counted per unit area in the residual pulpal tissue. An area 0.06 mm square in the residual pulpal tissue of the mesial root was examined, and the number of antibody-reactive cells per unit area (cells/mm^2) was calculated (Yamasaki *et al.* 2006).

Preparation of neutrophils

Before sacrifice at 4 days after the pulpal exposure, heparinized whole blood (6 mL) was obtained from each rat in each group. The blood was mixed with Plasmagel (Woods & Proffitt 1987), and the mixture was stood at 37 °C for 30 min to sediment red blood cells by gravity. The red blood cell-poor supernatant fluid was collected and washed twice with RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan). The washed cells were resuspended into 5 mL of RPMI-1640, layered on Ficoll-Paque PLUS (Pharmacia, Uppsala, Sweden) and centrifuged at 800 *g* at 4 °C for 10 min. After centrifugation, the pellet was resuspended into 5 mL of RPMI-1640 with 12.5% foetal bovine serum (FBS; Sigma Chemical, St Louis, MO, USA), layered on Ficoll-Paque PLUS and centrifuged at 90 *g* at 4 °C for 15 min. The supernatant fluid with neutrophils was collected and washed twice with RPMI-1640. Then, the cells in the pellet were counted by staining with trypan blue (Sigma Chemical, St Louis, MO, USA). The concentration of neutrophils was adjusted to 1.0×10^6 cells/mL for use in the neutrophil function assay.

Chemotaxis assay

This assay was performed in a 48-well modified Boyden chamber (Neuro Probe Inc., Bethesda, MD, USA) with a 5-μm pore size PVP-free polycarbonate filter (Nuclepore: Costar Co., Cambridge, MA, USA). N-formylmethionyl-leucyl-phenylalinesolution (fMLP) was purchased from Signa Chemical (St. Louis, MA, USA) as a chemoattractant. The fMLP were dissolved in RPMI-1640.

The bottom wells were filled with 30 μL of fMLP in RPMI-1640. Control wells were filled with RPMI-1640 alone. A polycarbonate filter sheet (25 mm × 80 mm) was placed on top of the wells in the bottom plate and the gasket and top plate were then fixed in place. A 50 μL volume of neutrophil suspension in RPMI-1640 was added to the upper wells. The chamber was incubated for 100 min at 37 °C in humidified air with 5% CO_2. After incubation, the top plate, gasket, and filter was removed. The filter was air-dried and stained with Diff-Quick (International Reagents, Tokyo, Japan).

The number of cells that had migrated through to the underside of the filters were counted in 20 high powerfields (40× objective) as an index of chemotactic response.

Phagocytosis

Phagocytosis by neutrophil was determined by using a flow cytometry assay. Fluorescent fluid-phase (1.0 μM; Polysciences, Warrington, PA, USA) was diluted in RPMI-1640 with 10% FBS. A 1.5 mL aliquot of neutrophil suspension in RPMI-1640 and 1.5 mL of fluorescent fluid-phase in RPMI-1640 with 10% FBS were mixed by centrifugation and placed at 37 °C for 1 h. Next, they were collected and washed three times with RPMI-1640. The samples were then washed twice in PBS by centrifugation at 800 *g* for 5 min. After washed, the samples were fixed with 10% formalin. Finally, the cells were analyzed by flow cytometry (Becton-Dickinson FACScan with Cellquest software, San Jose, CA, USA). The percentage of phagocytosing neutrophils was determined by gating the neutrophil population in a linear diagram. and its fluorescence histogram was analyzed.

Statistical analysis

All measurements were made on four serial sections from each animal. The average values were determined for each animal, and the average values were calculated for each group. Data were expressed as the mean ± standard deviation of the mean (SD). Statistical analysis was performed by using the two way analysis of variance.

Results

Blood findings

The numbers of total leukocytes and neutrophils in the peripheral blood of all groups are shown in Fig. 1. In Group A, the numbers of total leukocytes and neutrophils did not show any changes during the entire experimental period. In G-CSF-treated Group B, their numbers increased and were statistically ($P < 0.05$) higher than those of Group A throughout the experimental period. In methotrexate-treated Group C, both

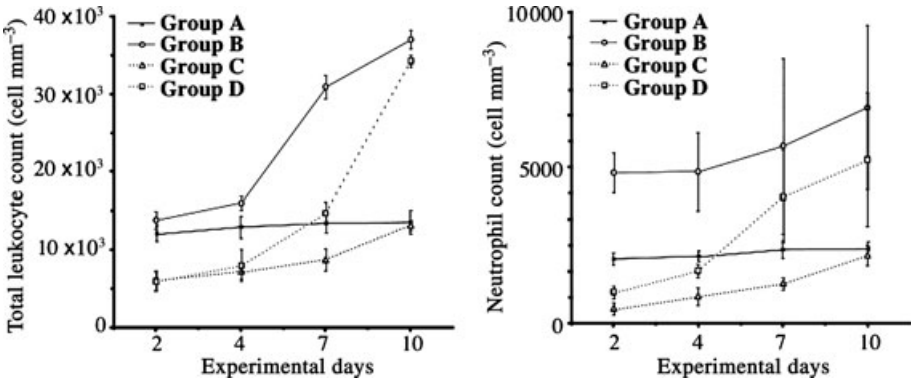

Figure 1 Numbers of total leukocytes and neutrophils in peripheral blood (cell/mm^3; mean ± SD): In Group B, total leukocyte and neutrophil counts statistically increased compared with those of Group A throughout the experimental period. In Group C from 2 to 7 days, both numbers significantly decreased compared with those of Group A. In Group D, their numbers statistically increased compared with those of Group A at 2 and 4 days, whereas they increased at 10 days.

counts decreased and were statistically ($P < 0.05$) less than those of Group A from 2 to 7 days. However, there was no significant difference at 10 days, between those of Groups A and C. In Group D, their counts decreased and were statistically ($P < 0.05$) less than those of Group A at 2 and 4 days. At 7 days, there was no significant difference between those of Groups A and D. At 10 days, the number of neutrophils increased and was statistically ($P < 0.05$) higher than that of Group A.

Histological findings

In Group A at 2 and 4 days, evidence of abscess formation due to the pulpal exposure was noted and evidence of slight inflammation around the abscesses were seen. Until 7 days, a small abscess and necrosis were found. The inflammation in the residual pulpal and periapical tissues were investigated as well as alveolar bone resorption. By 10 days, the necrosis had spread to over half of the pulpal tissue. The inflammation in the residual pulpal and periapical tissues had become stronger, and signs of alveolar bone resorption were evident. During all experimental periods, the histological changes in Group B were similar to those of Group A. In Group C, the histological changes at 2 and 4 days were similar to those of Group A. However, by 7 days, most of the pulpal tissue had become necrotic. Inflammatory changes in the residual pulpal and periapical tissues and resorption of the alveolar bone were seen (Fig. 2a). At day 10, the pulpal tissue was completely necrotic, and a small abscess was present around the root apex. Signs of severe inflammation and alveolar bone resorption were observed in the periapical tissue (Fig. 2b). In Group D, the histological changes were similar to those of Groups A and B. The pulpal necrosis and periapical inflammation were less as compared with those of Group C (Fig. 2c,d).

Immunohistochemical findings

In Group A at 2 and 4 days, some antibody-reactive neutrophils had infiltrated the pulp, and they especially accumulated in and around the abscess due to the pulpal exposure. By 7 and 10 days, the antibody-reactive cells had spread toward the periapical tissue (Fig. 3a). In Group B, immunolocalization of neutrophils was the same as that for Group A. In Group C at 2 and 4 days, only a few neutrophils had infiltrated in the pulp, and they accumulated in and around the abscess. At 7 days, few neutrophils were found in the residual pulpal tissue or were scattered in the periapical tissue (Fig. 3b). By 10 days, they had accumulated around the apical abscess. In Group D, immunolocalization of neutrophils was the same as that for Groups A and B.

Histometrical measurements

The area of pulpal necrosis in Group A gradually enlarged toward the root apex from 2 to 10 days. This area in Group B also enlarged to the same extent as in Group A. In Group C, this area was greatly enlarged. At 7 and 10 days, it was significantly ($P < 0.05$) larger than that in Group A. Group D showed the same area of pulpal necrosis as Group A (Fig. 4).

The area of the apical periodontal ligament in Group A gradually enlarged throughout the entire experimental period. This area in Group B was the same as

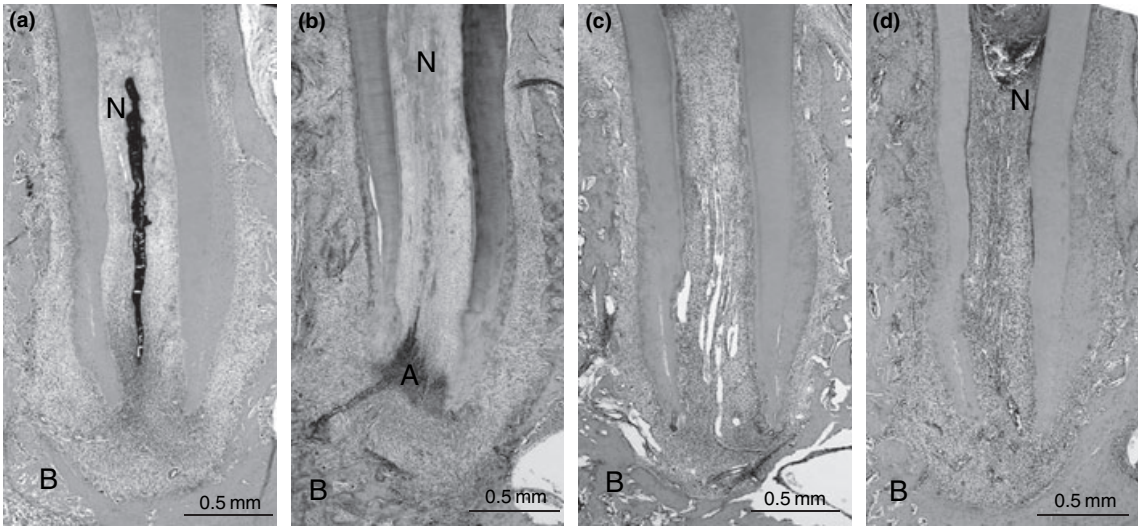

Figure 2 Histopathology of pulpal and periapical tissues in the mesial root of a mandibular first molar showing: Group C at 7 days (a), Group C at 10 days (b), Group D at 7 days (c), and Group D at 10 days (d). A-abscess, B-alveolar bone, and N-pulpal necrosis. In Group C at 7 days (a), the pulpal tissue is almost necrotic. Evidence of severe inflammation in the residual pulpal and periapical tissues and alveolar bone resorption is seen. At 10 days (b), the pulpal tissue is completely necrotic, and a small abscess is present around the root apex. Signs of severe inflammation and alveolar bone resorption are observed in the periapical tissue. In Group D at 7 days (c), a small abscess and necrosis are found at the site of pulpal exposure. Signs of inflammation in the residual pulpal and periapical tissues, as well as alveolar bone resorption, are evident. At 10 days (d), over half of the pulpal tissue is necrotic. The inflammation in the residual pulpal and periapical tissues are stronger than that at 7 days, and signs of alveolar bone resorption are seen.

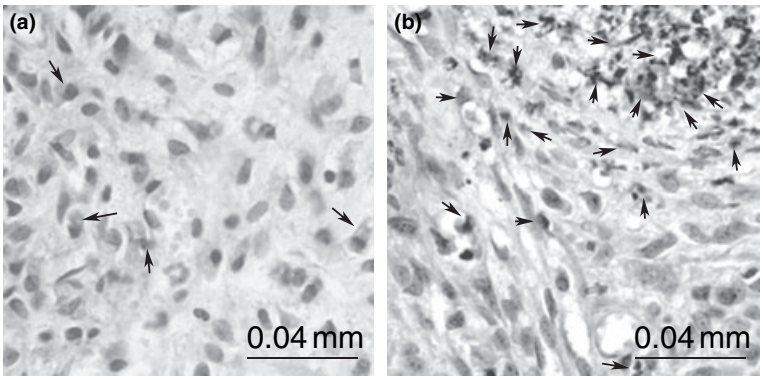

Figure 3 Immunohistology of pulpal tissue in the mesial root of a mandibular first molar showing: Group C (a) and Group D (b) at 7 days, Arrows-point to neutrophils. There are few neutrophils in the residual pulpal tissue of Group C (a), whereas many antibody-reactive cells are present in the pulpal abscess and have spread toward the root apex in Group D (b).

that in Group A. At 10 days, this area in Group C was statistically ($P < 0.05$) larger than that in Group A. This area in Group D was the same as that in Group A (Fig. 5).

The antibody-reactive neutrophils in the residual pulpal tissue in Group A gradually increased in number during the entire experimental period. Their number in Group B was similar to that in Group A. In Group C, these cells were not present in the residual pulpal tissue, and thus there was a significant difference ($P < 0.05$) in their number between Groups A and C. Groups D and A were similar in terms of number of antibody-reactive cells (Fig. 6).

Migration of neutrophil

The chemotactic response of all groups to fMLP was shown in Fig. 7. Most neutrophils of all groups migrated at a concentration of 10^{-7} M fMLP. At concentrations from 10^{-8} M to 10^{-5} M fMLP, the

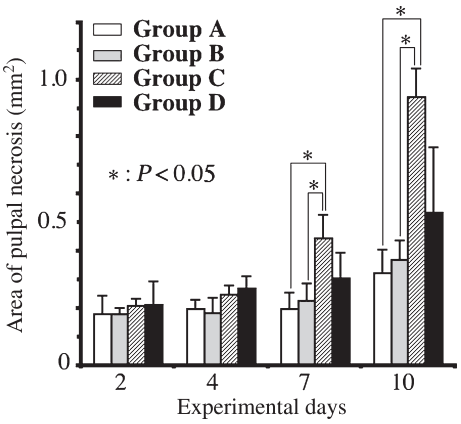

Figure 4 Area of pulpal necrosis (mm^2; mean ± SD): The pulpal necrosis in Group A gradually enlarges throughout the experiment. This area in Group B is similar to that in Group A. The area in Group C at 7 and 10 days is statistically ($P < 0.05$) larger than that in Group A or B. This area in Group D is similar to that in Group A.

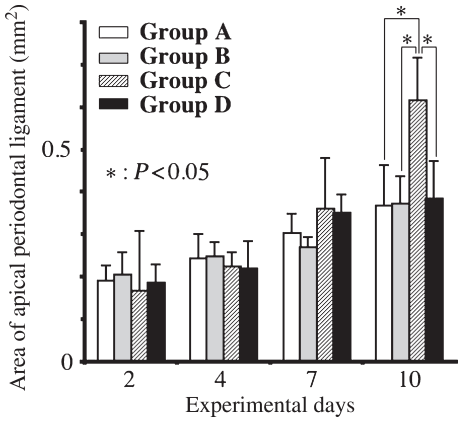

Figure 5 Area of apical periodontal ligament (mm^2; mean ± SD): The area occupied by the apical periodontal ligament in Group A gradually enlarges throughout the experimental period, with similar enlargement for Group B. In Group C, this area at 10 days is statistically ($P < 0.05$) greater than that in Group A or B. This area in Group D is similar to that in Group A.

neutrophil migration of Group B was significantly ($P < 0.05$) greater than that of Group A. At concentrations from 10^{-9} M to 10^{-5} M fMLP, the migration of Group C was statistically ($P < 0.05$) weaker than that of Group A. The neutrophils of Group D migrated similarly as those of Group A, with no significant difference.

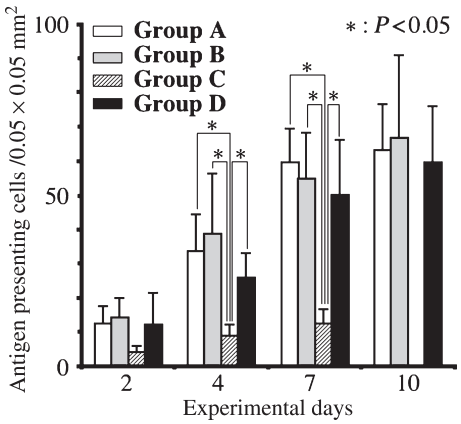

Figure 6 Antibody-reactive neutrophils in the residual pulpal tissue (cells/mm^2; mean ± SD): The number of neutrophils in Group A gradually increases during the entire experimental period. Their number in Group B is similar to that in Group A. In Group C, these cells are rarely present in the residual pulpal tissue from 2 to 7 days, and there are significant differences ($P < 0.05$) between Group A or B and at 7 days. However, no cell is detectable in the residual pulpal tissue at 10 days (ND). The number of these cells in Group D is similar to that in Group A.

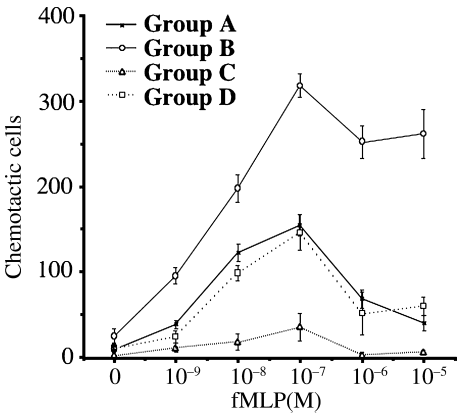

Figure 7 Neutrophil migration: The neutrophils of Group A migrate dose-dependently at concentrations of fMLP from 10^{-9} M to 10^{-7} M, and then the migration decreases from 10^{-6} M to 10^{-5} M fMLP. The neutrophils of Group B migrate dose-dependently from 10^{-9} M to 10^{-7} M fMLP, with the migration becoming slightly weaker at 10^{-6} M and 10^{-5} M fMLP. From 10^{-8} M to 10^{-5} M fMLP, the chemotaxis of Group B is significantly ($P < 0.05$) greater than that of Group A. The neutrophils of Group C slightly migrate to only 10^{-7} M fMLP, whereas they do not react to the other doses. At concentrations of fMLP from 10^{-9} M to 10^{-5} M, the chemotaxis of Group C is statistically ($P < 0.05$) weaker than that of Group A. The neutrophils of Group D migrated in a dose-dependent fashion as similar to that of Group A.

Phagocytic activity of neutrophils

The phagocytic index of Group A was 77.9 ± 9.3% (mean ± SD). After G-CSF injection, the phagocytic activity of Group B raise to 85.8 ± 10.8%, but there was no significant difference between groups A and B. In Group C, after the methotrexate injections, this activity (52.7 ± 14.7%) was significantly ($P < 0.05$) less than that of Group A. In Group D, the index was 75.2 ± 9.6%, and G-CSF thus prevented the reduction in phagocytosis due to neutropenia, There was a significant difference ($P < 0.05$) between groups C and D, but not between groups A and D.

Discussion

In the present study, the histology of pulpal inflammation and the function of neutrophils in rats were demonstrated. Pulpal inflammation is known to be induced by bacterial invasion after pulpal exposure in rat molars. Migration and phagocytosis assay are the most commonly used to examine neutrophil function. The results indicate that neutrophils play an important role in defending against bacterial invasion of the pulpal tissue.

Granulocyte colony-stimulating factor strongly enhances the production of neutrophils in bone marrow (Bensinger *et al.* 1993) and up-regulates neutrophil migration (Yuo *et al.* 1989). After the injections of G-CSF, the neutrophil count in the peripheral blood and their migration increased in the present study. Rat neutrophils are the most sensitive to fMLP among various chemotactants and the optimal chemotactic concentration is 10^{-7} fMLP (Sugawara *et al.* 1995). The fMLP was demonstrated that most neutrophils migrated at 10^{-7} fMLP. G-CSF is also known to enhance phagocytosis by neutrophils (Repp 1991). In this study, phagocytic activity increased after G-CSF injections, but there was no significant difference between normal and G-CSF treated rats. The assay of neutrophil function was carried out at 4 days after pulpal exposure, because G-CSF significantly increased the number of peripheral neutrophils and prevented the decrease in their count in neutropenic rats at 4 days. G-CSF was not sufficient to enhance the phagocytic activity at this time, but it may significantly increase it after 4 days. On the other hand, it did not change histologically the pulpal inflammation throughout the experimental periods. The most common uses of G-CSF include treatment for drug-induced neutropenia, neutropenia associated with chemotherapy and radiotherapy, congential neutropenia, and acceleration of neutrophil recovery after bone marrow transplantation (Carulli 1997). The main effect of G-CSF is recovery from neutropenia, but it may not cause increased immunity and the inhibition of the inflammation in pulp exposed rats without neutropenia.

After injections of methotrexate in rats, The effect of neutropenia on chemotaxis and phagocytosis was examined. Methotrexate is known to result in a decreased neutrophil count (Wallace *et al.* 1990) and inhibition of neutrophil migration (Kraan *et al.* 2000, Schwanke *et al.* 2005), which leads to the initiation of bacterial invasion in pulpal tissue. Neutropenia induced bacterial invasion into pulpal tissue and subsequent pulpal necrosis (Nakamura *et al.* 2002). The immunohistological findings at 7 days revealed that many neutrophils migrated into the exposed pulp in normal rats, whereas few of them migrated in the neutropenic rats. Thus, an enlarged area of pulpal necrosis was seen in the latter. Methotrexate-induced neutropenia reduces the neutrophil infiltration at the initial stage of inflammation (Rylander 1974). The inhibited migration of neutrophils following bacterial invasion into the pulpal tissue may heighten the destruction of the pulpal tissue. Migration of neutrophils into the pulp is associated with pulpal inflammation due to various bacteria (Bergenholtz & Warfvinge 1982, Iwama *et al.* 2006). The histological findings at 10 days in normal animals indicated the resorption of alveolar bone in the periapical area. The bacterial invasion induced by neutropenia may have reached the periapical tissue earlier, as alveolar bone resorption was seen at day 7 in Group C. Thus, the investigations suggest that neutrophils may be necessary to defend against bacterial infection in pulpal tissue.

When G-CSF was injected into the neutropenic rat, the decreases in neutrophil count, their migration, and their phagocytosis were less. As shown immunohistometrically, neutrophilic infiltration into the pulpal tissue increased in those rats as compared with that in the neutropenic rat without G-CSF treatment. These findings indicate that G-CSF recovered the functions of the neutrophil, and thereby inhibited the bacterial invasion. G-CSF was shown earlier to prevent the inhibition of neutrophil migration seen in neutropenia (Siena *et al.* 1991) Also, PGG glucan, which has the biological effects of increases of neutrophil production and up-regulating of their phagocytosis inhibits pulpal necrosis and periapical bone resorption (Stashenko *et al.* 1995). In the present study, G-CSF up-regulated the number and functions of neutrophils, but it did not

inhibit the pulpal inflammation with necrosis, in normal rats. It inhibited only the enlargement of pulpal necrosis induced by neutropenia. G-CSF increased the number and function of neutrophils to kill the bacteria, and then the enlargement of pulpal necrosis and bone resorption were inhibited. These results suggest that treatment with G-CSF is necessary to prevent the rapid destruction of pulpal tissue in the case of neutropenia.

Neutropenia induced by methotrexate decreased neutrophil functions and resulted in the enlargement of pulpal necrosis with bacterial invasion. Thus in normal rats, G-CSF increased neutrophil number and functions, but did not change the histology, whereas in neutropenic rat, it inhibited the enlargement of pulpal necrosis of pulpal tissue.

Conclusions

Granulocyte colony-stimulating factor inhibited the decrease in the neutrophil count in the peripheral blood, the reduction in the chemotactic reaction, and the enlargement of necrosis seen in the methotrexate-treated neutropenic rat. This study shows that the neutrophil plays an important role to defend against bacterial invasion into the pulpal tissue.

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