FULL PAPER *Pathology*

# Granulocyte Colony-Stimulating Factor Has No Adverse Effects on Atherosclerotic Lesions in High Cholesterol-Fed Miniature Swine

Hirotake TAKAI[1)]*, Akio MIYOSHI[1)], Masaki YAMAZAKI[1)], Kenji ADACHI[1)], Kouichi KATAGIRI[2)], Hitoshi ARAKAWA[2)], Kiyoka KATSUYAMA[2)], Tsuneo ITO[2)], Etsuko FUJII[1)], Shuji HAYASHI[1)], Atsuhiko KATO[1)] and Masami SUZUKI[1)]

*[1)]Department of Safety Assessment, Fuji Gotemba Research Laboratory, Chugai Pharmaceutical Co., Ltd., 1–135 Komakado, Gotemba-shi, Shizuoka 412–8513 and [2)]Chugai Research Institute for Medical Science, Inc., 1–135 Komakado, Gotemba-shi, Shizuoka 412–8513, Japan*

(Received 6 March 2008/Accepted 18 April 2008)

ABSTRACT. Granulocyte colony-stimulating factor (G-CSF) is widely used to mobilize peripheral blood stem cells, and expected to restore cardiac function for patients with coronary artery diseases as a consequence of progression of atherosclerosis. Safety issues related to the administration of G-CSF to these patients, however, are still under study. The animal model for atherosclerosis was produced by feeding miniature swine a high-cholesterol diet for 3 months. G-CSF (5 or 10 $\mu$g/kg/day) was given to the animal model by daily subcutaneous injections for 10 days and 20 main arteries were evaluated pathologically. In addition, the general toxicological effects were studied on clinical signs, body weight, hematology, blood chemistry and pathology. In the G-CSF-treated groups, a variety of changes related to the major pharmacological activity of G-CSF including an increase in white blood cell (WBC) counts were observed. In many arteries, atherosclerotic lesions similar to Type I-V of the proposed classification by the American Heart Association were observed. No effects of the G-CSF treatment were seen on the histopathological findings, incidence, severity or distribution of atherosclerotic lesions. In addition, no infiltration of neutrophils to the lesions was observed. These findings suggest that the administration of G-CSF causes neither exacerbation or modification of atherosclerotic lesions nor adverse changes despite that a sufficient increase in WBC counts could be achieved in the peripheral blood.

KEY WORDS: atherosclerosis, atherosclerotic lesions, G-CSF, high cholesterol diet, miniature swine.

*J. Vet. Med. Sci.* 70(9): 943–950, 2008

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that can act selectively on cells of the neutrophil lineage [1] and also facilitate to mobilize multipotent stem cells of bone marrow into peripheral blood [9, 11]. Stem cells, which may differentiate into myocardial cells or endothelial cells [4, 9, 12, 23], have been the target of intensive investigation for the preclinical and clinical use in regenerative medicine [3, 12, 19]. Mobilization of stem cells by G-CSF has been considered to become a potent therapy for patients with acutely or chronically damaged myocardium due to its easy and universal application.

However, it has been reported recently that in the clinical trial for coronary artery disease (CAD) patients performed by the National Institutes of Health (NIH) there occurred a serious adverse event which might be associated with G-CSF administration: 1 out of 16 patients experienced non-ST-segment elevation myocardial infarction (MI) and another patient died of MI [6]. This fact has caused anxiety concerning the safety of G-CSF among people engaging in regenerative medicine. The clinical concern about patients with CAD is that they have, more or less, attenuation of the coronary arteries as a consequence of progression of atherosclerosis [14]. There should be a variety of risk factors which have to be validated before safe and universal application of G-CSF to patients with cardiovascular system disorders. The present study was carried out to evaluate the effects of G-CSF on atherosclerotic lesions, using a miniature swine atherosclerosis model which has been reported to exhibit similarities to human in the distributions and properties of atherosclerotic lesions [22]. In addition, the general toxicological effects of G-CSF on this model were also evaluated.

* CORRESPONDENCE TO: TAKAI H., Department of Safety Assessment, Fuji Gotemba Research Laboratory, Chugai Pharmaceutical Co., Ltd., 1–135 Komakado, Gotemba-shi, Shizuoka 412–8513, Japan.
e-mail: takaihrt@chugai-pharm.co.jp

## MATERIALS AND METHODS

*Animals*: Nine 3-month-old Göttingen miniature swine (5 males and 4 females) were obtained from Chugai Research Institute for Medical Science, Inc. (Nagano, Japan). They were housed in cages (1 animal/cage) in an animal room maintained in a controlled environment of 22 ± 3°C, humidity of 35 to 70%, 10 to 15 air changes per hour, and a 12 hr light cycle (7:00 to 19:00). All swine were fed a 420 g cholesterol diet (High-cholesterol diet for miniature swine, Nisseiken Co., Ltd., Tokyo, Japan) once per day and water *ad libitum*. The high-cholesterol diet was continuously fed to them during the period of G-CSF administration.

*G-CSF*: Recombinant human G-CSF manufactured by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan) was used for this study. This agent was dissolved in phosphate buffer to final concentrations of 50 and 100 $\mu$g/m*l*.

*Study design*: Nine animal models for atherosclerosis

were produced by feeding a high cholesterol diet for 3 months. Groups of 3 swine each received daily subcutaneous injections of G-CSF in the cervix at a dose of 5 or 10 $\mu$g/kg/day for 10 days. Although the major concern of side effect of G-CSF application to the patients is an excessive elevation of WBC counts, the doses and treatment period of G-CSF in the present study were determined taking into consideration the upper limit of WBC counts acceptable to medical doctors of the regenerative medicine (approximately $300 \times 10^2/\mu l$) and the estimated clinical treatment period (4–6 days) [20]. The results of preliminary study confirmed the adequacy of the doses and treatment period selected for the present study. The injection volume was adjusted to 0.1 ml/kg. In addition to the G-CSF-treated groups, a vehicle control group was prepared. During the administration period, clinical signs of all animals were observed daily before and after the dosing. All animals were weighed at 10, 4 and 1 days before commencement of administration (day -10, day -4, and day -1) and at 3, 7, and 10 days of administration (day 3, day 7 and day 10). Blood samples were collected from each animal through the anterior sinus of the vena cava on days -10, -4 (measured as pretreatment values), 3, 7, and 10, and were subjected to hematology and/or blood chemistry. Electrocardiography was performed on days -4 and 10. All animals were necropsied the day after the final administration.

The present procedures of animal experiment were approved by the Ethical Committee for Treatment of Laboratory Animals at Chugai Pharmaceutical Co., Ltd.

*Hematology*: Blood samples for hematological analysis were collected in tubes treated with EDTA-2K or sodium citrate on days -10, -4, 3, 7, and 10. Citrated plasma was collected following centrifugation of samples at 4°C for 10 min at 1,900 × g. The following 19 hematological parameters were determined: Red blood cell count, white blood cell (WBC) count, platelet count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean cell hemoglobin, mean cell hemoglobin concentration, differential leukocyte ratio for stab cells (Stab), segmented cells (Seg), eosinophils (Eosin), basophils (Baso), monocytes (Mono), lymphocytes (Lymph), erythroblast count (Ebl), active partial thromboplastin time (APTT), prothrombin time (PT), and fibrinogen level (FIB). WBC counts were determined using a Bayer H1E Hematology analyzer (Bayer Diagnostics, Tarrytown, NY). APTT, PT and FIB were determined by means of an Amelung-Coagulometer KC 10 (Amelung GmbH, Lemgo, Germany). Stab, Seg, Eosin, Baso, Mono, Lymph, and Ebl were counted under light microscopy and the ratio of each differential leukocyte or erythroblast count to the total leukocyte count was calculated. All other parameters were analyzed using a Sysmex K-4500 automated hematology analyzer (Sysmex Co., Long Grove, Illinois, U.S.A.).

*Blood chemistry*: Blood samples for biochemical analysis were collected on days -10, -4, and 10. Samples were allowed to clot at room temperature for 60 min. Serum was separated from the samples by centrifugation at 4°C for 10 min at 1,900 × g and then frozen at –70°C prior to analysis. Serum levels of the following 23 biochemical parameters were determined: aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), alkaline phosphatase (Al-P), gamma-glutamyl transpeptidase (GGT), lactic dehydrogenase (LDH), creatinine-phosphotransferase (CPK), glucose (Glu), total bilirubin (T-Bil), total cholesterol (T-Cho), triglycerides (TG), low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL), blood urea nitrogen (BUN), creatinine (CRN), inorganic phosphorus (IP), calcium (Ca), sodium (Na), potassium (K), chloride (Cl), total protein (TP), albumin (Alb), and albumin:globulin ratio (A/G). Analyses of all other parameters were performed using an automatic analyzer (Hitachi 7170, Tokyo, Japan).

*Electrocardiography*: On days -4 and 10, all animals were initially preanesthetized by an intramuscular injection of 10 mg/kg ketamine hydrochloride and 0.05 mg/kg atropine sulfate and then anesthetized with isoflurane in a respired gas mixture. Electrocardiogram by standard limb lead was monitored under stable isoflurane anesthesia with a BIOVIEW1000 composite monitor (NEC Corporation, Tokyo, Japan).

*Pathology*: On the day following the final administration, all animals were preanesthetized by an intramuscular injection of 10 mg/kg ketamine hydrochloride and 0.05 mg/kg atropine sulfate and then sacrificed by exsanguination through the left axillary artery or vein. At necropsy, 20 arteries (common carotid, branchiocephalic trunk, subclavian, axillary, aortic arch, thoracic aorta, pulmonary, internal carotid, brachial, abdominal aorta, celiac, anterior cerebral, basilar, renal, cranial mesenteric, external iliac, internal iliac, femoral, right coronary, paraconal interventricular branch), hearts, livers, spleens, kidneys, lungs, brains, and bone marrow (femur) were removed from all animals. The hearts, livers, spleens, kidneys, and lungs were weighed and the relative organ weights/100g BW were calculated. All organs removed were fixed in 20% neutral buffered formalin (20% NBF), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). As to the brains, the cerebral cortex and medulla oblongata, which lie directly on the anterior cerebral artery and basilar artery respectively, were observed grossly and processed as described above. Some of the arterial sections were stained with elastica van Gieson (EVG) to assess in detail changes in the elastic layers and extracellular matrix (ECM), or were subjected to immunohistochemical analysis as mentioned below. After fixation in 20% NBF, additional samples of several arteries were immersed in 10% sucrose in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) for 4 hr at 4°C, followed by 15% sucrose in PBS for 4 hr at 4°C and 20% sucrose in PBS overnight at 4°C, embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), sectioned on a microtome in a cryostat, and stained with Oil red O to identify fat deposits in the arterial intima.

Immunohistochemical staining was performed according to the labeled streptavidin-biotin (LSAB) method with a

Table 1. Classification of atherosclerotic lesions of miniature swine in this study

| Findings | Grade 1 | Grade 2 | Grade 3 |
|---|---|---|---|
| intimal thickening | **P** | **P** | **P** |
| infiltration of foamy cells | **P** | **P** | **P** |
| destruction of elastic layers | **P** | **P** | **P** |
| increase of spindle cells | | **P** | **P** |
| increase of extracellular matrix | | **P** | **P** |
| calcification | | **P** | **P** |
| formation of fibrous cap | | | **P** |

Abbreviation: P, present.

Dako LSAB-2 kit (Dako, Carpinteria, CA, U.S.A.). Antibody against α-smooth muscle actin (α-SMA) (clone 1A4, Dako Cytomation Co., Ltd., Kyoto, Japan) was applied as the primary antibody. The immunoreaction was visualized by peroxidase-diaminobenzidine reaction. The sections were finally counterstained with hematoxylin.

The severity of the atherosclerotic lesions was graded into 3 classes (Table 1) as follows: Grade 1; intimal thickening and destruction of elastic layers with infiltration of foamy cells into the intima (Fig. 1a and 1b), Grade 2 (intermediate); all components of Grade 1 lesions with an increase in intimal spindle-shaped cells accompanied by an increase in ECM and occasional calcification (Fig. 1d), Grade 3; a prominent build-up of fibrous cap with substantial increases in ECM elaborated by dense distribution of collagen and elastic fibers and remarkable calcification (Fig. 1f, 1h and 1i).

## RESULTS

*Clinical signs and body weights*: No abnormal clinical signs were observed in any group throughout the study period. All animals of both control and treated groups showed normal body weight gain.

*Hematological findings*: In G-CSF-treated groups, an increase in WBC (mainly segmented neutrophils) counts was observed (Fig. 2). WBC counts gradually increased during the time course of G-CSF administration, reaching approximately $300 \times 10^2/\mu l$ (range, $233–388 \times 10^2/\mu l$) on day 3, 400 to $700 \times 10^2/\mu l$ on day 7 and 400 to $900 \times 10^2/\mu l$ on day 10. No definite dose response was observed between the dose levels of 5 and 10 $\mu$g/kg. In addition, no clear differences in WBC count elevation profiles were observed between either sex. No other changes of the treatment were noted in hematology.

*Blood chemistry findings*: None of the parameters examined showed any distinct difference between the control and G-CSF-treated groups.

However, the levels of T-Cho, HDL, LDL, and GGT in all swine of the present study were higher than those of the baseline data of the swine fed on ordinary diets [21, 24] (Table 2 shows data extracted from the results of blood-chemical analysis on day -4).

*Electrocardiography*: No abnormal electrocardiographic patterns were observed in any animal on both days -4 and 10.

*Pathological findings of atherosclerotic lesions*: In many arteries, areas of yellow-white discoloration were grossly observed on the inner surface, some of which were seen as raised plaques (especially remarkable in abdominal arteries). There were no differences in gross findings between the control and G-CSF-treated groups.

Histopathologically, intimal thickening, infiltration of foamy cells into the intima, increase of ECM, increase of spindle-shaped cells, calcification, cholesterol clefts, and build-up of fibrous cap were observed. Foamy cells accumulating in the intima were positive for Oil red O staining (Fig. 1c), indicating that they were lipid-laden cells. Moreover, in the lesions of Grade 2 and 3, extracellular space also contained Oil red O positive droplets that frequently formed into small dispersed pools or larger and confluent cores (lipid cores) (Fig. 1g). The immunohistochemical staining revealed spindle-shaped cells that were positive for α-SMA, indicating that they derived from smooth muscle cells (Fig. 1e). In the comparison of the control group with the G-CSF-treated groups, no changes in histopathological findings and no increase in incidence of each finding following G-CSF administration were observed (Table 3). In addition, no infiltration of neutrophils to the lesions was seen in any of the groups.

No clear differences in gross and histopathological findings of arteries were observed between either sex within the same group.

*Incidence and severity of atherosclerotic lesions*: Incidence and severity of atherosclerotic lesions are shown in Table 4. Grade 1–3 lesions were observed in the control group with incidence highest in Grade 1, average in Grade 2, and lowest in Grade 3. These results were similar to those of the G-CSF-treated groups. No differences in incidence and severity of the lesions were seen in the two G-CSF-treated groups.

*Distribution of atherosclerotic lesions*: Atherosclerotic lesions were frequently distributed in the abdominal aorta, aortic arch, celiac artery, right coronary artery, and paraconal interventricular branch. No definite differences were observed in distribution of the lesions between the control and G-CSF-treated groups (Table 5).

*Pathological findings and organ weights of spleens, livers, hearts, lungs, brains and kidneys*: In the G-CSF-treated groups, the enlargement and increased weights of spleen, increase in the number of granulocytic cells in bone marrow, and increased extramedullary hematopoiesis in liver and spleen were observed (data not shown).

In all groups including the control group, whitish yellow livers and pale red spleens were grossly observed. Microscopically, fatty change in hepatocytes, aggregations of foamy kupffer cells in liver sinusoid, and aggregations of foamy cells in red pulps were observed.

No abnormal pathological changes were observed in the hearts, lungs, brains, and kidneys of any animals.

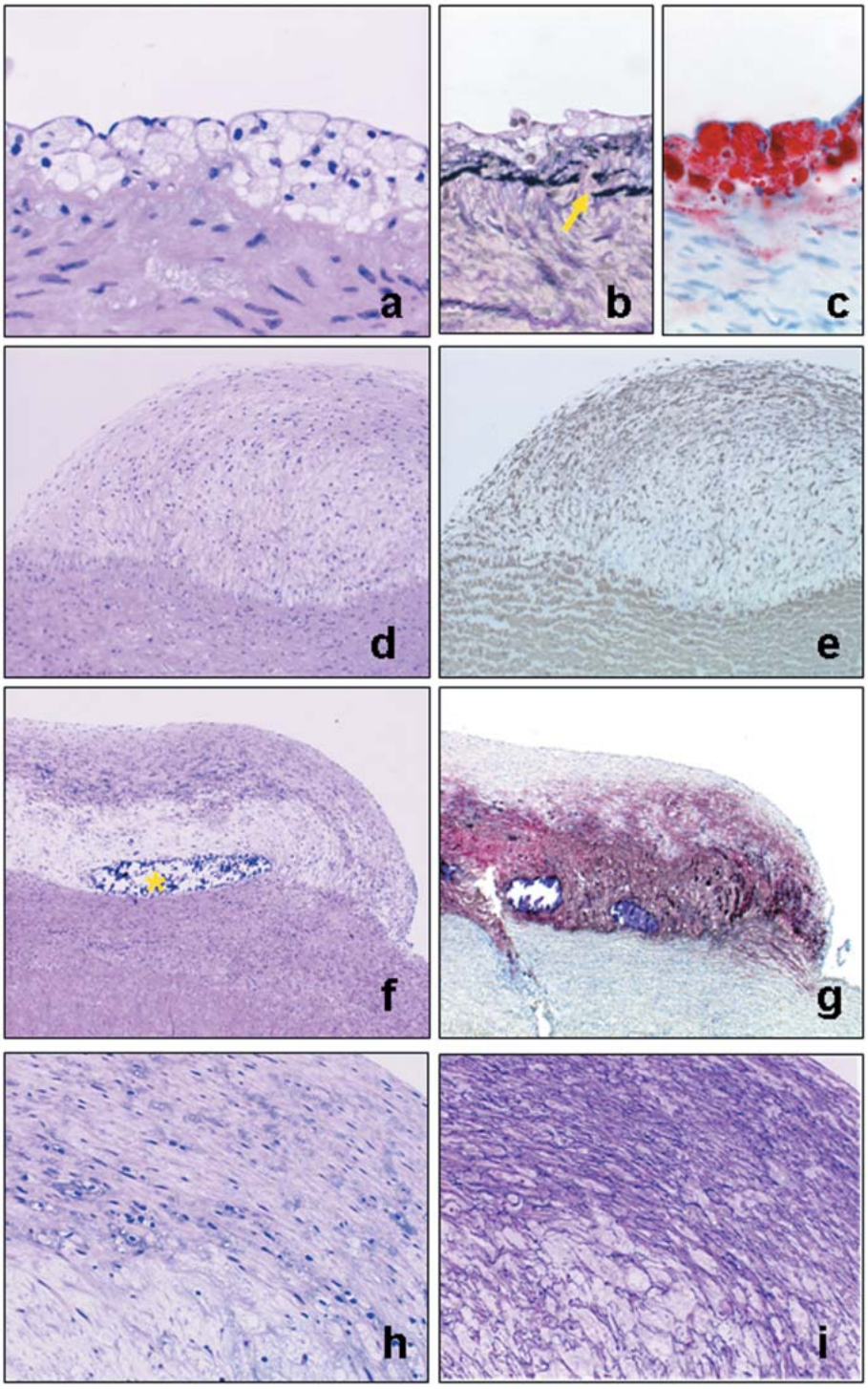


Fig. 1. Grade 1 lesions in the right coronary artery (a-c), grade 2 lesions in the abdominal artery (d, e) and grade 3 lesions in the abdominal artery (f-i) of a high cholesterol-fed miniature swine. a) Intimal thickening and infiltration of foamy cells into intima are seen. HE b) Destruction of elastic layers are found (arrow). EVG. c) Foamy cells in the intima are positive for Oil red O staining. d) In addition to the findings found in grade 1 lesions, a marked increase in spindle-shaped cells and a slight increase in ECM are seen. The central area of the plaque has less cell density than the endothelial surface and has an accumulation of extracellular foamy substance. HE. e) Spindle-shaped cells are positive for α-SMA. f) A prominent fibrous cap was formed in the surface of plaque and calcification found (asterisk). In the central area of the plaque, there is a marked accumulation of extracellular foamy substance, which formed a confluent core. HE. g) The core contains numerous Oil red O-positive droplets. h) High magnification of the fibrous cap of Fig. 1f. The fibrous cap is composed of spindle-shaped cells (smooth muscle cells), ECM, and foamy cells. HE. i) The major constituents of the ECM include collagen and elastic fibers. EVG. Bar=50 $\mu$m.

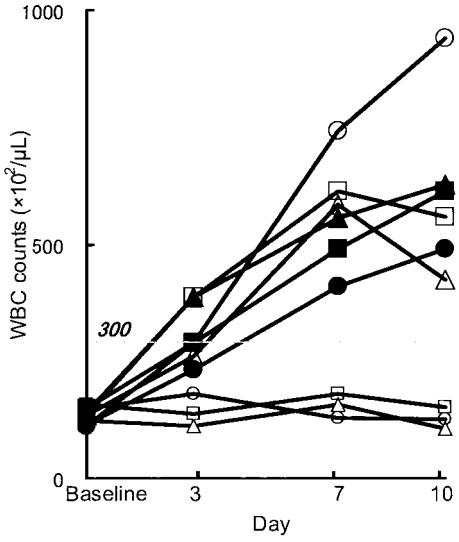


Fig. 2. WBC counts of miniature swine injected with G-CSF at dosages of 0 $\mu$g/kg/day (small open circle, triangle, and square: animal Nos. 1–3, respectively), 5 $\mu$g/kg/day (large closed circle, triangle and square: animal Nos. 4–6, respectively), and 10 $\mu$g/kg/day (large white circle, triangle and square: animal Nos. 7–9, respectively). The values at baseline represent the mean between the values on day -10 and day -4. The WBC count of $300 \times 10^2/\mu l$ represents the estimated upper limit among medical doctors using G-CSF as a regenerative medicine.

## DISCUSSION

In this study, we evaluate the effects of G-CSF on atherosclerotic lesions as well as on the general toxicology in an animal model for atherosclerosis produced by feeding miniature swine a high-cholesterol diet for 3 months.

In all animals, increased levels of lipid parameters such as T-Cho, HDL and LDL, and lipid deposition in organs and tissues as demonstrated by areas of yellow-white discoloration and raised plaques on the inner surface of many arteries, whitish-yellow livers, pale red spleens, fatty change of hepatocytes, aggregations of foamy Kupffer cells in liver sinusoid, and aggregations of foamy cells in red pulps were observed. These changes are considered a consequence of feeding a high-cholesterol diet for a given period of time [22] and ensured that all animals had features sufficient to be a model for atherosclerosis. In addition, increased levels of GGT were found in all animals, which were deemed secondary effects due to lipid deposition in organs or progression of atherosclerotic lesions [22].

In the G-CSF-treated groups, a significant increase in WBC counts was observed, reaching approximately $300 \times 10^2/\mu l$ on day 3, $400–700 \times 10^2/\mu l$ on day 7, and $400–900 \times 10^2/\mu l$ on day 10. These values were nearly equal to or far greater than the acceptable range of WBC counts (within approximately $300 \times 10^2/\mu l$) for medical doctors of the regenerative medicine. In addition to an increase in WBC counts, the enlargement and increased weights of spleens, increased number of granulocytic cells in bone marrow, and increased extramedullary hematopoiesis in liver and spleen, that are also related to the major pharmacological activity of G-CSF were observed in the G-CSF-treated groups. However, no adverse changes were observed in any of the tests carried out in this study.

The atherosclerotic lesions seen in this model were similar to Types I-V of the proposed classification by the American Heart Association (AHA) [16–18] (Table 6). Grade 1 lesions consisting primarily of foamy cells were similar to Type I or II of the AHA classification grossly designated as fatty streaks. Grade 2 lesions, containing increased spindle-shaped cells (derived from smooth muscle cells) and accumulations of extracellular lipid droplets as well as infiltration of foamy cells, were close to Type III (preatheroma) or IV (atheroma). However, at this grade, the accumulation of extracellular lipid was not as remarkable as in human Type IV lesions, characterized by a large, confluent, and disruptive core of extracellular lipids including cholesterol crystals. In contrast, in this study Grade 2 lesions seemed to have a more remarkable increase in smooth muscle cells and ECM than Type III or IV. This increase is possibly promoted at an earlier stage during the course of the disease in miniature swine compared with human atherosclerotic lesions. In fact, it is known that the increase in smooth muscle cells or collagenous fibers is modest in Type III or IV

Table 2. Comparison of T-Cho, HDL, LDL and GGT levels between high cholesterol-fed and normal miniature swine

| Group | T-Cho (mg/d*l*) | HDL (mg/d*l*) | LDL (mg/d*l*) | GGT (IU/*l*) |
|---|---|---|---|---|
| high cholesterol-fed[a)] | 449 ± 95.2 (9) | 106 ± 7 (9) | 189 ± 67 (9) | 85.8 ± 19.7 (9) |
| normal[b)] | 81.5 ± 7.1[c)] (6) | 29 ± 9[d)] (14) | 17 ± 8[d)] (14) | 37.8 ± 6.0[c)] (6) |

Each value represents the mean ± SD.

Parentheses indicates the number of miniature swine examined.

a) The values for high cholesterol-fed miniature swine were extracted from the results of blood-chemical analysis on day -4.

b) Extracted data from references/baseline data collected at Chugai Reserch Institute for Medical Science, Inc (CMS): c), the data from an experiment manual for miniature swine[21]; d), the data from Cardiovascular Research 2002; 56(1): 135–144[24].

Table 3. Histopathological findings and incidence of each finding in arteries of high cholesterol-fed miniature swine injected with G-CSF for 10 days

| Dose ($\mu$g/kg/day) | 0 | | | | 5 | | | | 10 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal No. | 1 | 2 | 3 | | 4 | 5 | 6 | | 7 | 8 | 9 | |
| Sex | M | M | F | | M | F | F | | M | M | F | |
| Findings Total no. of arteries examined | 20 | 20 | 20 | total | 20 | 20 | 20 | total | 20 | 20 | 20 | total |
| intimal thickening | 6[a)] | 10 | 9 | **25** | 12 | 9 | 11 | **32** | 3 | 8 | 9 | **20** |
| infiltration of foamy cells | 5 | 10 | 7 | **22** | 12 | 9 | 11 | **32** | 2 | 8 | 9 | **19** |
| destruction of elastic layers | 6 | 10 | 5 | **21** | 11 | 8 | 11 | **30** | 2 | 4 | 8 | **14** |
| increase of extracellular matrix | 2 | 3 | 3 | **8** | 4 | 2 | 6 | **12** | 0 | 2 | 4 | **6** |
| increase of spindle-shaped cells | 1 | 3 | 5 | **9** | 3 | 2 | 5 | **10** | 0 | 1 | 5 | **6** |
| calcification | 2 | 4 | 1 | **7** | 2 | 3 | 4 | **9** | 0 | 2 | 4 | **6** |
| cholesterol cleft | 0 | 0 | 0 | **0** | 0 | 1 | 0 | **1** | 0 | 0 | 0 | **0** |
| formation of fibrous cap | 0 | 1 | 0 | **1** | 1 | 0 | 0 | **1** | 0 | 0 | 0 | **0** |
| infiltration of neutrophils | 0 | 0 | 0 | **0** | 0 | 0 | 0 | **0** | 0 | 0 | 0 | **0** |

Abbreviations: M, male; F, female.
a) Number of arteries with the finding.

Table 4. Incidence and severity of atherosclerotic lesions in high cholesterol-fed miniature swine injected with G-CSF for 10 days

| Dose ($\mu$g/kg/day) | Animal No. | No. of arteries examined | Incidence | Severity | | |
|---|---|---|---|---|---|---|
| | | | | grade 1 | grade 2 | grade 3 |
| 0 | 1 | 20 | 6[a)] | 5 | 1 | 0 |
| | 2 | 20 | 10 | 8 | 1 | 1 |
| | 3 | 20 | 9 | 8 | 1 | 0 |
| | | | Total 25 | 21 | 3 | 1 |
| 5 | 4 | 20 | 12 | 9 | 2 | 1 |
| | 5 | 20 | 9 | 7 | 2 | 0 |
| | 6 | 20 | 11 | 7 | 4 | 0 |
| | | | Total 32 | 23 | 8 | 1 |
| 10 | 7 | 20 | 3 | 3 | 0 | 0 |
| | 8 | 20 | 8 | 7 | 1 | 0 |
| | 9 | 20 | 9 | 7 | 2 | 0 |
| | | | Total 20 | 17 | 3 | 0 |

a) Number of arteries with lesions.

lesions [17]. Grade 3 lesions, characterized by build-up of fibrous cap and distinct lipid core, were similar to Type V lesions, referred to as fibroatheroma. Grade 3 was the most advanced stage found in this study. However, there were no disruption of the lesions, hematoma or hemorrhage, or thrombotic deposits (lesions corresponding to Type VI) in any of the arteries examined.

In this study, no clear sex differences were observed in reactivity against G-CSF administration (such as WBC count elevation profiles) as well as in gross and histopathological findings of arteries. Accordingly, it can be reasonable to put the results of both sexes together and evaluate their findings as a relevant group.

As to the effect of G-CSF on atherosclerotic lesions, no differences were seen in histopathological findings, incidence, severity, or distribution between the control and the G-CSF-treated groups. Moreover, G-CSF treatment did not become a trigger of neutrophil infiltration to the lesions. These results strongly suggest that the administration of G-CSF does not lead to exacerbation or modification of atherosclerotic lesions despite that a sufficient increase in WBC counts could be achieved in the peripheral blood.

On the other hand, in the clinical trials performed on patients with CAD, 2 out of the 16 patients injected with G-CSF experienced serious cardiac events [6]. Another study reported that the rate of in-stent restenosis in the G-CSF-treated group was unexpectedly increased, raising the specter of harmful effects in clinical scenarios in which vessel injury exists [8]. Moreover, the preclinical study in apolipoprotein E-deficient mice, which were maintained on a high-fat diet, demonstrated an exacerbation of the atherosclerotic lesions after treated with G-CSF and GM-CSF [5]. In contrast, there have been some reports in support of our data which indicate no adverse effects of G-CSF on atherosclerotic lesions. In the study with 23 acute MI patients in a nonrandomized trial, no severe side effects of G-CSF were observed [10]. In fact, it was revealed that treatment with G-CSF significantly improved regional wall motion, myocardial perfusion, and ejection fraction at 3 months follow-up. Two additional randomized placebo-controlled studies

Table 5. Distribution of atherosclerotic lesions in high cholesterol-fed miniature swine injected with G-CSF for 10 days

| Site | Dose ($\mu$g/kg/day) | | |
|---|---|---|---|
| | 0 | 5 | 10 |
| Abdominal aorta | 3[a](3) | 3 (3) | 2 (2) |
| Aortic arch | 3 | 3 | 3 |
| Celiac artery | 2 | 3 (1) | 3 |
| Paraconal interventricular branch (heart) | 1 (1) | 3 (1) | 2 (1) |
| Right coronary artery | 2 | 3 | 1 |
| External iliac artery | 2 | 2 (2) | 2 |
| Cranial mesenteric artery | 2 | 3 | 1 |
| Pulmonary artery | 3 | 2 | 0 |
| Subclavian artery | 2 | 1 | 1 |
| Femoral artery | 1 | 1 (1) | 1 |
| Thoracic aorta | 1 | 1 | 1 |
| Anterior cerebral artery | 0 | 2 | 1 |
| Brachiocephalic artery | 1 | 1 | 0 |
| Internal carotid artery | 0 | 2 | 0 |
| Basilar artery | 1 | 0 | 1 |
| Internal iliac artery | 0 | 1 (1) | 1 |
| Common carotid artery | 1 | 0 | 0 |
| Axillary artery | 0 | 1 | 0 |
| Brachial artery | 0 | 0 | 0 |
| Renal artery | 0 | 0 | 0 |

a) Number of swine with lesions.
Parentheses indicates the number of swine with lesions more than grade 2.

in which G-CSF was administered to the patients after MI and successful percutaneous intervention demonstrated neither benefit nor adverse effects [7, 13, 25]. In the previous animal studies, Shindo *et al*. reported that a long-term administration of GM-CSF for 7 months in a rabbit model of atherosclerosis had no adverse effect but rather a protective effect [15]. Similarly, a recent publication demonstrated that knocking out the GM-CSF gene in mice exacerbated atherosclerosis [2]. Thus, results from clinical and preclinical studies are conflicting in terms of both potential benefit and adverse outcomes. In our study, although no adverse effects of G-CSF administration on atherosclerotic lesions were observed, the terminal stange of atherosclerosis (Type VI) could not be obtained in the animals. Therefore, it is unclear whether or not G-CSF administration or the increase in WBC counts may affect the feature of Type VI lesions. To determine the clinical effects of G-CSF administration and the increase in WBC counts on atherosclerotic lesions, further study and the accumulation of clinical data are indispensable.

Collectively, the present study demonstrates that the administration of G-CSF or an increase in WBC counts does not exacerbate or modify atherosclerotic lesions and nothing other than the efficacy of G-CSF was observed in the G-CSF-treated groups. However, since serious adverse events that might be associated with G-CSF administration have been reported in the CAD patients, the administration of G-CSF to patients with serious cardiovascular system disorders should be avoided or carried out under the careful monitoring of cardiovascular and inflammatory parameters.

## REFERENCES


1. Demetri, G.D. and Griffin, J.D. 1991. Granulocyte colony-stimulating factor and its receptor. *Blood* **78**: 2791–2808.
2. Ditiatkovski, M., Toh, B.H. and Bobik, A. 2006. GM-CSF deficiency reduces macrophage PPAR-gamma expression and aggravates atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb. Vasc. Biol.* **26**: 2337–2344.
3. Forrester, J.S., Price, M.J. and Makkar, R.R. 2003. Stem cell repair of infarcted myocardium: an overview for clinicians. *Circulation* **108**: 1139–1145.
4. Graf, T. 2002. Differentiation plasticity of hematopoietic cells. *Blood* **99**: 3089–3101.
5. Haghighat, A., Weiss, D., Whalin, M.K., Cowan, D.P. and Taylor, W.R. 2007. Granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor exacerbate atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **115**: 2049–2054.
6. Hill, J.M., Syed, M.A., Arai, A.F., Powell, T.M., Paul, J.D., Zalos, G., Read, E.J., Khuu, H.M., Leitman, S.F., Horne, M., Csako, G., Dunbar, C.E., Waclawiw, M.A. and Cannon, R.O. 3rd. 2005. Outcomes and risks of granulocyte colony-stimulating factor in patients with coronary artery disease. *J. Am. Coll. Cardiol.* **46**: 1643–1648.
7. Jorgensen, E., Ripa, R.S., Helqvist, S., Wang, Y., Johnsen, H.E., Grande, P. and Kastrup, J. 2006. In-stent neo-intimal hyperplasia after stem cell mobilization by granulocyte-colony stimulating factor: preliminary intracoronary ultrasound results from a double-blind randomized placebo-controlled study of patients treated with percutaneous coronary intervention for


Table 6. Comparison of the classification of atherosclerotic lesions between human and miniature swine

| Definition and Classification of the AHA | | Classification in this study | |
|---|---|---|---|
| Type | Main histology | Grade | Main histology |
| I<br>II<br>(fatty streak) | isolated macrophage foam cells<br>mainly intracellullar lipid accumulation | 1 | Lesions like Type I-II |
| III<br>(Preatheroma) | Type II changes and small extracellular lipid | 2 | Lesions like Type III-IV plus increase of smooth muscle cells and ECM |
| IV<br>(atheroma) | Type II changes and core of extracellular lipid | | |
| V<br>(fibroatheroma) | lipid core and fibrotic layer | 3 | Lesions like Type V |
| VI | Hemorrhage and thrombus | | |

ST-elevation myocardial infarction (STEMMI trial). *Int. J. Cardiol.* **111**: 174–177.

8. Kang, H.J., Kim, H.S., Zhang, S.Y., Park, K.W., Cho, H.J., Koo, B.K., Kim, Y.J., Soo, L.D., Sohn, D.W., Han, K.S., Oh, B.H., Lee, M.M. and Park, Y.B. 2004. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. *Lancet* **363**: 751–756.
9. Kocher, A.A., Schuster, M.D., Szabolcs, M.J., Takuma, S., Wang, B.J., Homma, S., Edwards, N.M. and Itescu, S. 2001. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nature Med.* **7**: 430–436.
10. Kuethe, F., Figulla, H.R., Herzau, M., Voth, M., Fritzenwanger, M., Opfermann, T., Pachmann, K., Krack, A., Sayer, H.G., Gottschild, D. and Werner, G.S. 2005. Treatment with granulocyte colony-stimulating factor for mobilization of bone marrow cells in patients with acute myocardial infarction. *Am. Heart. J.* **150**: 115.
11. Matsunaga, T., Sakamaki, S., Kohgo, Y., Ohi, S., Hirayama, Y. and Niitsu, Y. 1993. Recombinant human granulocyte colony-stimulating factor can mobilize sufficient amounts of peripheral blood stem cells in healthy volunteers for allogeneic transplantation. *Bone. Marrow Transplant.* **11**: 103–108.
12. Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S.M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bondine, D.M., Leri, A. and Anversa, P. 2001. Bone marrow cells regenerate infracted myocardium. *Nature* **410**: 701–705.
13. Ripa, R.S., Jorgensen, E., Wang, Y., Thune, J.J., Nilsson, J.C., Sondergaard, L., Johnsen, H.E., Kober, L., Grande, P. and Kastrup, J. 2006. Stem cell mobilization induced by subcutaneous granulocyte-colony stimulating factor to improve cardiac regeneration after acute ST-elevation myocardial infarction: result of the double-blind, randomized, placebo-controlled stem cells in myocardial infarction (STEMMI) trial. *Circulation* **113**: 1983–1992.
14. Roberts, W.C. 1990. Diffuse extent of coronary atherosclerosis in fatal coronary artery disease. *Am. J. Cardiol.* **65**: 2F–6F.
15. Shindo, J., Ishibashi, T., Yokoyama, K., Nakazato, K., Ohwada, T., Shiomi, M. and Maruyama, Y. 1999. Granulocyte-macrophage colony-stimulating factor prevents the progression of atherosclerosis via changes in the cellular and extracellular composition of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits. *Circulation* **99**: 2150–2156.
16. Stary, H.C., Blankenhorn, D.H., Chandler, A.B., Glagov, S., Insull, W. Jr., Richardson, M., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J. and Wagner, W.D. et al. 1992. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler. Thromb.* **12**: 120–134.
17. Stary, H.C., Chandler, A.B., Dinsmore, R.E., Fuster, V., Glagov, S., Insull, W. Jr., Rosenfeld, M.E., Schwartz, C.J., Wagner, W.D. and Wissler, R.W. 1995. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* **92**: 1355–1374.
18. Stary, H.C., Chandler, A.B., Glagov, S., Guyton, J.R., Insull, W. Jr., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J., Wagner, W.D. and Wissler, R.W. 1994. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* **89**: 2462–2478.
19. Strauer, B.E., Brehm, M., Zeus, T., Kostering, M., Hernandez, A., Sorg, R.V., Kogler, G. and Wernet, P. 2002. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* **106**: 1913–1918.
20. Takano, H., Ueda, K., Hasegawa, H. and Komuro, I. 2007. G-CSF therapy for acute myocardial infarction. *Trends Pharmacol. Sci.* **28**: 512–517.
21. Tanigawa, M. 2000. Background data of miniature swine. pp. 18–21. *In*: An Experiment Manual for Miniature Swine (Tanigawa M *et al.* eds). SLA Laboratory Co., Ltd. (in Japanese).
22. Tanigawa, M., Akaike, I., Adachi, J., Shinkai, H., Tokoi, K., Uchiyama, T., Ibaraki, T. and Mochizuki, K. 1986. Gottingen miniature swine as a model for diet-induced atherosclerosis. *Jikken Dobutsu*. **35**: 47–57.
23. Tomita, S., Li, R.K., Weisel, R.D., Mickle, D.A.G., Kim, E.J., Sakai, T. and Jia, Z.Q. 1999. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation* **10**: 247–256.
24. Verhamme, P., Quarck, R., Hao, H., Knaapen, M., Dymarkowski, S., Bernar, H., Van, Cleemput, J., Janssens, S., Vermylen, J., Gabbiani, G., Kockx, M. and Holvoet, P. 2002. Dietary cholesterol withdrawal reduces vascular inflammation and induces coronary plaque stabilization in miniature pigs. *Cardiovasc. Res.* **56**: 135–144.
25. Zohlnhofer, D., Ott, I., Mehilli, J., Schomig, K., Michalk, F., Ibrahim, T., Meisetschlager, G., von, W.J., Bollwein, H., Seyfarth, M., Dirschinger, J., Schmitt, C., Schwaiger, M., Kastrati, A. and Schomig, A. 2006. Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial. *JAMA*. **295**: 1003–1010.