# Vascular Endothelial Growth Factor in Rabbits During Development of Corticosteroid-Induced Osteonecrosis: A Controlled Experiment

TAMON KABATA, TADAMI MATSUMOTO, SHINICHI YAGISHITA, TOMOHIKO WAKAYAMA, SHOICHI ISEKI, and KATSURO TOMITA

***ABSTRACT.*** ***Objective.*** Vascular endothelial growth factor (VEGF) is an angiogenic promoter that is rapidly induced as a response to local hypoxia. We investigated VEGF expression in rabbits in a controlled experiment to clarify the onset of ischemic events in corticosteroid-induced osteonecrosis (ON).

***Methods.*** Ninety-nine mature Japanese white rabbits were divided into 6 treatment groups and an untreated control group. The treatment groups received a single intramuscular injection of 4 mg/kg methylprednisolone acetate; they were euthanized at different times, and tissue samples were obtained from their femora. We examined the development of ON and the expression of VEGF using histopathology, immunohistochemistry, Northern blot analysis, and Western blot analysis.

***Results.*** On histopathological examination, the earliest indication of ON was 5 days after the corticosteroid treatment. The frequency of ON occurrence reached a plateau at or after Week 1. VEGF expression was accompanied by the development of ON. VEGF-positive cells detected by immunohistochemistry were found among bone marrow cells, frequently located in the area surrounding ON, suggesting that VEGF production was switched on as a result of the ischemic events that cause ON. The level of VEGF-mRNA expression indicated by Northern blot analysis peaked at 3 days after the corticosteroid treatment and decreased gradually to the levels present in the control group at 7 days after treatment. Western blot analysis revealed VEGF protein production at 3 days after the corticosteroid treatments. Levels of VEGF expression 2 weeks or more after the corticosteroid treatment were almost the same as in the control group.

***Conclusion.*** We observed early expression of VEGF in the cells around the corticosteroid-induced ON lesions in rabbits. These results suggest that the ischemic events that cause ON begin soon after the initial corticosteroid treatment. (First Release Nov 1 2008; J Rheumatol 2008;35:2383–90; doi:10.3899/ jrheum.070838)

*Key Indexing Terms:*
OSTEONECROSIS CORTICOSTEROIDS RABBITS ISCHEMIC EVENT
VASCULAR ENDOTHELIAL GROWTH FACTOR DISEASE ONSET

It is thought that corticosteroid-associated osteonecrosis (ON) is caused by blood flow impairment in the femoral head after the systemic administration of corticosteroids. However, the timing of the occurrence of ischemia of the femoral head after starting the corticosteroid treatment and the mechanism that causes ischemia are not well understood[1-3]. ON is asymptomatic at the initial stage, and becomes symptomatic when collapse occurs. Therefore, clinical pathology in the early disease stage is difficult to investigate; animal experiments are required in order to elucidate these unclear points.

A previous study introduced animal models of methylprednisolone-induced ON[4], and we also have established a similar rabbit model of ON with high reproducibility of the necrosis by using methylprednisolone[5,6]. In these methylprednisolone-induced ON models, the ON lesion is not created on the epiphysis, but the ON satisfies the definition of ON in humans. Currently, several research studies of corticosteroid-induced ON are being conducted using methylprednisolone-induced rabbit ON models[7,8].

Vascular endothelial growth factor (VEGF) is a protein that specifically acts on vascular endothelial cells and accelerates their growth. One of its properties is that it is induced in reaction to the hypoxic condition in tissues. Numerous

*From the Department of Orthopaedic Surgery, Kanazawa University School of Medicine, Kanazawa; Department of Orthopaedic Surgery, Kanazawa Medical University, Uchinada; and Department of Anatomy (I), Kanazawa University School of Medicine, Kanazawa, Japan.*

*Supported by the Japanese Investigation Committee for Osteonecrosis of Femoral Head, under the auspices of the Ministry of Health and Welfare of Japan.*

*T. Kabata, MD, PhD, Assistant Professor, Department of Orthopaedic Surgery, Kanazawa University School of Medicine; T. Matsumoto, MD, PhD, Professor, Department of Orthopaedic Surgery, Kanazawa Medical University; S. Yagishita, MD, PhD, Department of Orthopaedic Surgery, Kanazawa University School of Medicine; T. Wakayama, MD, PhD, Associate Professor; S. Iseki, MD, PhD, Associate Professor, Department of Anatomy (I), Kanazawa University School of Medicine; K. Tomita, MD, PhD, Professor, Department of Orthopaedic Surgery, Kanazawa University School of Medicine.*

*Address reprint requests to Dr. T. Kabata, Department of Orthopaedic Surgery, Kanazawa University School of Medicine, 13-1, Takaramachi, Kanazawa, 920-8641, Japan. E-mail: tamonkabata@aol.com*

*Accepted for publication July 10, 2008.*

experimental studies have shown that VEGF expression is elevated in ischemic lesions in animal models[9-13]. Based on this property, it will be possible to further clarify the relationship between ischemia and corticosteroid-induced ON by investigating the expression of intraosseous VEGF in the clinical state of corticosteroid-induced ON of the femoral head.

In our study, a methylprednisolone-induced ON model was used to investigate the expression of intraosseous VEGF after corticosteroid administration and to determine its relationship with the development of corticosteroid-induced ON, along with the period of occurrence of ischemic episodes.

## MATERIALS AND METHODS

*Animals*. Ninety-nine adult female Japanese White rabbits (mean body weight 3.5 kg, mean age 24 mo) were used. The 99 rabbits were divided into 6 treatment groups of 13 to 15 rabbits each and an untreated control group of 15 rabbits. Treatment groups received a single intramuscular injection of 4 mg/kg methylprednisolone acetate (Upjohn, Tokyo, Japan), and were euthanized at 1 day (1-day group), 3 days (3-days group), 5 days (5-days group), 1 week (1-week group), 2 weeks (2-weeks group), and 4 weeks (4-weeks group) after the initial corticosteroid treatment. The 1-day, 2-week, and 4-week groups consisted of 13 rabbits each, and the 3-day, 5-day, and 1-week groups 15 rabbits each. The control group of 15 rabbits was fed under the same conditions and did not receive corticosteroid injections. After corticosteroid treatment, tissue samples were obtained by dissection and removal of the femora from both sides of the animals.

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kanazawa University School of Medicine.

*Treatments. Light microscopic examinations*. The femora were cut into coronal sections using a tissue cutter (Exakt, Hamburg, Germany), soaked in 10% formalin for about 1 week for fixation, decalcified using EDTA solution, embedded into paraffin, sliced into 4-$\mu$m sections, and stained with hematoxylin and eosin (H&E) for histopathologic examination. Ten rabbits were used for each group. The frequency of ON and its location and histology were examined for each group. According to the criteria established by Yamamoto, *et al*[4], we defined ON as the diffuse presence of empty lacunae or osteocyte ghosts in the bone trabeculae, accompanied by necrosis in the surrounding bone marrow. Frequency of ON was calculated as the number of rabbits with ON divided by the total number in the group. A rabbit was evaluated as having ON when either side had a necrotic lesion that met the above definition. The ON location was evaluated in terms of 6 areas: 2 epiphyseal regions (femoral head and trochanteric region), 2 metaphyseal regions (medial and lateral), and 2 shaft regions (medial and lateral).

*Immunohistochemistry*. Immunohistochemistry was used to localize VEGF protein in the rabbit femora. After preparation using standard immunohistochemistry techniques (deparaffinization, rehydration, inhibition of endogenous peroxidase activity, and elimination of nonspecific antibody bindings), the 4-$\mu$m sections, which were serial sections of the H&E staining samples, were incubated 16 h with mouse monoclonal antibodies against VEGF (NeoMarkers, Fremont, CA, USA) diluted 1:100. Sections were then treated with 5% biotinylated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) and peroxidase-conjugated streptavidin (Dako). After detection of the bound peroxidase by immersing the sections in a mixture of 0.05% diaminobenzidine (Dojin, Kumamoto, Japan), the slides were counterstained with hematoxylin, dehydrated, and mounted. As a control, alternate sections were incubated without a primary antibody.

If the cytoplasm was darkly stained, we considered that the cell had expressed VEGF, and if 10 or more of the cells expressing VEGF had aggregated, we defined this state as "VEGF-positive." The VEGF-positive rate was calculated as the number of rabbits with VEGF-positive results divided by the total number in the group. Location of the VEGF-positive area was evaluated in terms of the same 6 areas used for the ON evaluation.

*Northern blot analysis*. Northern blot analysis was performed to detect and compare the levels of VEGF mRNA in the 6 corticosteroid-treated groups and the control group. Three rabbits were used for each group.

Production of a 570-bp cDNA probe for rat VEGF was as described[14]. Although the sequence for rabbit VEGF cDNA has not been published, we confirmed in a preliminary examination that the rat cDNA probe hybridized with rabbit VEGF mRNA was the same size as that of rat VEGF mRNA (data not shown). Therefore, in this study, we used the rat VEGF-cDNA probe for Northern blot analysis.

Rabbit bone marrows from one-third of the proximal part of the femur were removed and frozen immediately with liquid nitrogen, then stored at –80°C until analysis. Total RNA (20 $\mu$g) was extracted from the frozen rabbit bone marrows with the guanidine-phenol-chloroform method, and used for Northern blot. Briefly, 20 $\mu$g aliquots of the RNA samples were denatured with glyoxal and electrophoresed in 1% agarose gel. An RNA ladder (Life Technologies, Rockville, MD, USA) was used as a molecular size marker. Samples were then blotted onto nylon membranes (Pall BioSupport; East Hills, NY, USA) and cross-linked by ultraviolet irradiation. The membranes were first prehybridized at 65°C for 2 h in 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 10× Denhardt's solution, 0.1% Sarkosyl, 10 mM EDTA, and 250 $\mu$g/ml denatured salmon sperm DNA, and then hybridized in the same solution with $^{32}$P-labeled VEGF cDNA probe. After incubation at 65°C overnight, the membranes were washed extensively in 6× SSC (1× SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0) containing 0.1% Sarkosyl at 65°C. They were then exposed to Kodak Biomax MS film (Kodak, Rochester, NY, USA) with intensifying screen at –80°C for autoradiography. The gels were dried and analyzed with a BAS imaging plate scanner (BAS-2000; Fujifilm, Tokyo, Japan). The radioactivity associated with VEGF-mRNA was quantified using Image Gauge software (version 3.41; Fujifilm).

*Western blot analysis*. Western blot analysis was performed to detect and compare the levels of VEGF protein in the 3-day, 5-day, 1-week, and control groups. Two rabbits were used for each group.

Rabbit bone marrows from one-third of the proximal part of the femur were removed and frozen immediately by liquid nitrogen, then stored at –80°C until analysis. Four thousand milligrams of the bone marrow sample were homogenized on ice in 2 ml of RIPA lysis buffer. After centrifugation at 3000 rpm, the supernatants were examined for protein concentration using a BCA protein assay kit (Pierce, Rockford, IL, USA) and used as cell lysates. Aliquots of cell lysates at 45 $\mu$g protein/lane were separated by electrophoresis in 12% polyacrylamide gel in the presence of 0.1% SDS and then transferred to PVDF membranes (BioRad, Hercules, CA, USA). After treatment with 5% nonfat skim milk in phosphate buffered saline (PBS) with 0.05% Tween20, the membranes were incubated with mouse monoclonal antibodies against VEGF (NeoMarkers) diluted 1:200 in 1% bovine serum albumin in PBS for 16 h at 4°C. After washing, the membranes were incubated with horseradish peroxidase–conjugated goat anti-mouse IgG antibody (Dako) for 1 h at room temperature and rinsed in PBS. Peroxidase activity was visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Uppsala, Sweden).

*Statistical analysis*. Statistics were calculated using Fisher's exact test for frequency of ON and the VEGF-positive rate. For radioactivity associated with VEGF-mRNA, all data were expressed as the mean ± standard deviation (SD), and were compared among the 7 groups by a 1-factor ANOVA with Scheffe's F test for post-hoc comparisons. The level of significance was set at $p < 0.05$.

## RESULTS

*Light microscopy examinations*. No special changes were observed in the control group, 1-day group, and 3-day

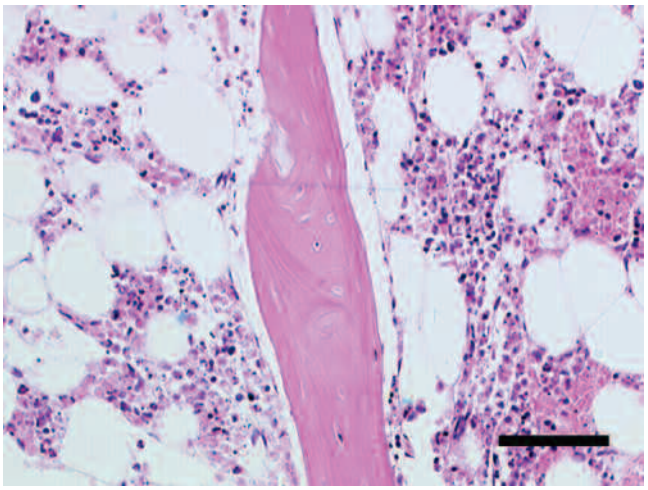
group. ON was observed in the 5-day, 1-week, 2-week, and 4-week groups on the medial side of the metaphyseal and shaft region.

In the ON observed in the 5-day and 1-week groups, the necrotic area showed increased eosinophilic changes, and became clearly distinguishable from the surrounding normal bone marrow. Bone trabeculae showed empty lacunae or osteocyte ghosts. Hemopoietic cells showed necrotic changes and degenerative changes. Adipocytes also became necrotic; however, the cytoarchitecture was relatively maintained (Figure 1A).

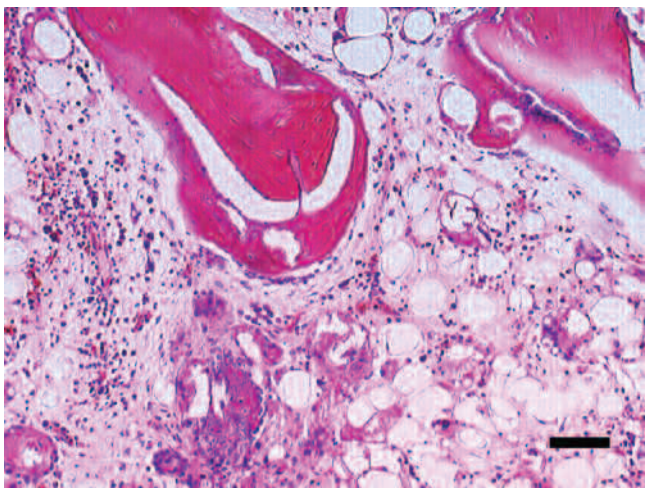
In the 2-week group, the eosinophilic property was elevated in parts with ON compared to the 5-day and 1-week group. Necrotic trabeculae had empty osteocyte lacunae, and hematopoietic cells around the trabeculae became completely necrotic. Additionally, necrotic adipocytes lost their cytoarchitecture and collapsed.

In the ON observed in the 4-week group, necrotic bone tissue was surrounded by fibrotic granulation tissue that included marked accumulations of macrophages and foreign-body giant cells. The repair process progressed from the periphery. Active appositional bone formation was observed around necrotic bone trabeculae (Figure 1B).

ON occurred in 2 of 10 rabbits in the 5-day group (20%), 5 of 10 rabbits in the 1-week group (50%), 6 of 10 rabbits in the 2-week group (60%), and 6 of 10 rabbits in the 4-week group (60%). The occurrence frequency of ON was significantly higher in the 1-, 2-, and 4-week groups than in the control, 1-day, and 3-day groups. There was no significant

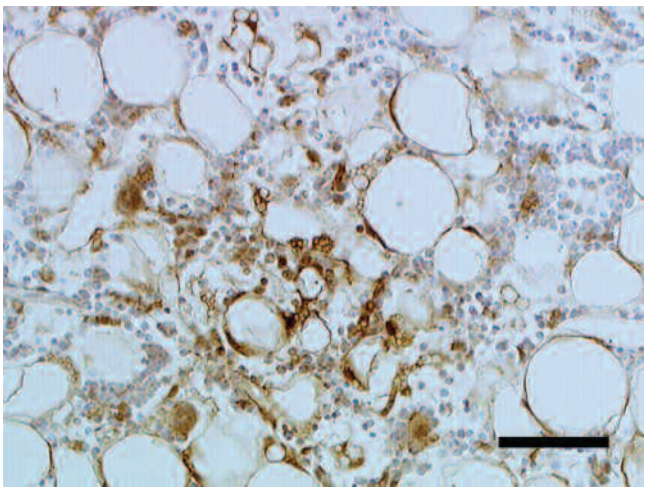


A

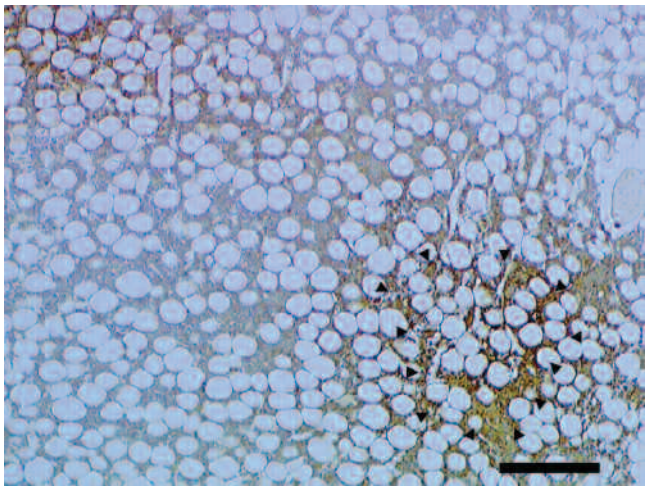


B

*Figure 1*. Histopathologic findings of osteonecrosis. (A) 5-day group. Necrotic area showed accumulation of degenerative or necrotic marrow and fat cells, and bone trabeculae showed either empty lacunae or osteocyte ghosts. (B) 4-week group. Necrotic bone trabeculae showing empty lacunae were surrounded by appositional bone formation that was associated with granulation tissue in the area surrounding necrotic bone marrow tissue. H&E staining; original magnification (A) ×200, (B) ×100. Scale bars (A) 20 $\mu$m, (B) 20 $\mu$m.



A



B

*Figure 2*. Immunohistochemical findings of VEGF in the 3-day group. (A) VEGF-expressing cells were aggregated and found among the bone marrow cells, e.g., interstitial and myeloid cells. (B) Larger aggregation of VEGF-expressing cells (arrowheads). Original magnification (A) ×200, (B) ×20. Scale bars (A) 20 $\mu$m, (B) 200 $\mu$m.

difference among the 5-day, 1-week, 2-week, and 4-week groups (Table 1).

*Immunohistochemistry*. VEGF-expressing cells were observed sporadically in the control and 1-day groups, but there was no marked accumulation. Accumulation of VEGF-expressing cells was observed in the 3-day, 5-day, and 1-week groups (Figure 2A, 2B). VEGF occurred in various types of cells, such as adipocytes, macrophages, vascular endothelial cells, and hematopoietic cells irrespective of cell species. Additionally, no VEGF-expressing cells were observed in sites where ON existed. VEGF-expressing cells were scattered around the ON in the 2-week and 4-week groups. However, no marked accumulation was observed.

With regard to the VEGF-positive rate, 1 of 10 rabbits in the control group (10%), 2 of 10 in the 1-day group (20%), 5 of 10 in the 3-day group (50%), 6 of 10 in the 5-day group (60%), 5 of 10 in the 1-week group (50%), 2 of 10 in the 2-week group (20%), and 2 of 10 in the 4-week group (20%) were positive. The positive rate was higher in the 3-day, 5-day, and 1-week groups than in the control, 1-day, 2-week, and 4-week groups; however, there was no significant difference (Table 2).

To clarify the results of the histopathological examinations including H&E staining and immunohistochemistry, all specimens that received histological examinations were divided into 4 groups: (1) controls, (2) ON-positive specimens, (3) ON-negative specimens, (4) specimens with unknown ON occurrence. Because the corticosteroid-induced rabbit model of ON we used showed immature ON about 1 week after the corticosteroid administration, and mature ON 2 weeks after administration[6,8], the 2-week and 4-week groups were divided into ON-positive specimens and ON-negative specimens, while the 1-day, 3-day, 5-day, and 1-week groups were divided into ON-positive specimens and specimens with unknown ON occurrence. In the control group, only one specimen was VEGF-positive. In the 1-day group, none of the specimens showed ON (therefore denoted unknown ON occurrence), and 2 specimens were VEGF-positive. In the 3-day group, there were no ON-positive specimens, although 50% of the specimens were VEGF-positive. In the 5-day group, all specimens that showed ON were VEGF-positive, but 4 that did not show ON (unknown ON occurrence) were also VEGF-positive. In the 1-week group, all the ON-positive specimens were VEGF-positive, while all the specimens that did not show ON (unknown ON occurrence) were VEGF-negative. On the other hand, only one-third of the ON-positive specimens were VEGF-positive in the 2-week and 4-week groups. These results are depicted in Figure 3. In the 3-day, 5-day, and 1-week groups, the VEGF-positive cells often occurred in the medial area of the metaphyseal and shaft regions, which are areas with a predilection for ON (Figure 4). No marked trend was observed in the control, 1-day, 2-week, and 4-week groups.

*Table 1*. Frequency of osteonecrosis (ON).

| Group | No. of Examined Rabbits | No. with ON |
|---|---|---|
| Control | 10 | 0 |
| 1-day | 10 | 0 |
| 3-day | 10 | 0 |
| 5-day | 10 | 2 |
| 1-week | 10 | 5* |
| 2-week | 10 | 6* |
| 4-week | 10 | 6* |

* Statistically significant compared to controls, Fisher's exact test, $p < 0.05$.

*Table 2*. Frequency of VEGF expression.

| Group | No. of Examined Rabbits | No. of VEGF-positive Rabbits |
|---|---|---|
| Control | 10 | 1 NS |
| 1-day | 10 | 2 NS |
| 3-day | 10 | 5 NS |
| 5-day | 10 | 6 NS |
| 1-week | 10 | 5 NS |
| 2-week | 10 | 2 NS |
| 4-week | 10 | 2 NS |

NS: not statistically significant, Fisher's exact test.

*Northern blot analysis*. A band indicating 3.9 kb of VEGF-mRNA was observed in all groups (Figure 5). The same quantity of RNA was used for each group, so the amount of mRNA representation was proportional to the concentration of the band. Therefore, the peak of VEGF-mRNA expression occurred 3 days after the corticosteroid treatments; in the 3-day group the amount of VEGF-mRNA expression was 2.1-times greater than in the untreated control group. This increase was statistically significant ($p < 0.05$). After that the value decreased, showing a plateau from the 1-week group to the 4-week group, and the representation was approximately the same as in the control group (Figure 6).

*Western blot analysis*. A band considered to be the expression of VEGF was observed in the 3-day group. The molecular weight was 46 kDa. The other groups exhibited no clear band indicating VEGF expression (Figure 7).

## DISCUSSION

The pathogenetic mechanism of corticosteroid-associated ON of the femoral head is uncertain[1-3]. It has a histopathological resemblance to the traumatic ON of the femoral head that occurs after nutrient vessels have been damaged. Therefore, there is a consensus that it is finally caused by intraosseous ischemia. Premature histopathological tissue samples have rarely been obtained immediately after the occurrence of ON because we do not know exactly when an ischemic episode occurs in the femoral head after the administration of a corticosteroid. This may be one of the factors impeding elucidation of its pathophysiology. Thus,

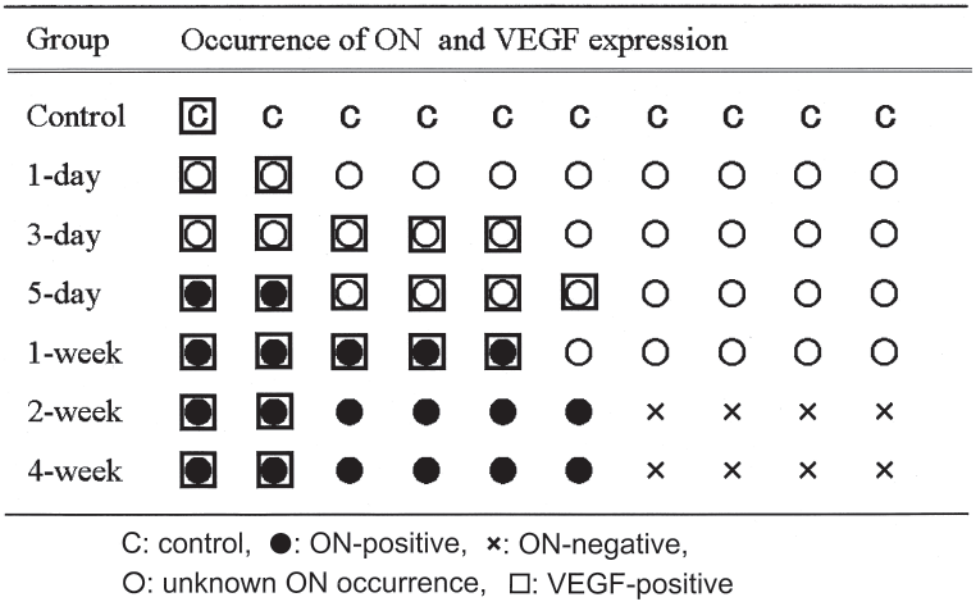



*Figure 3*. Relationship between the occurrence of ON and the expression of VEGF.

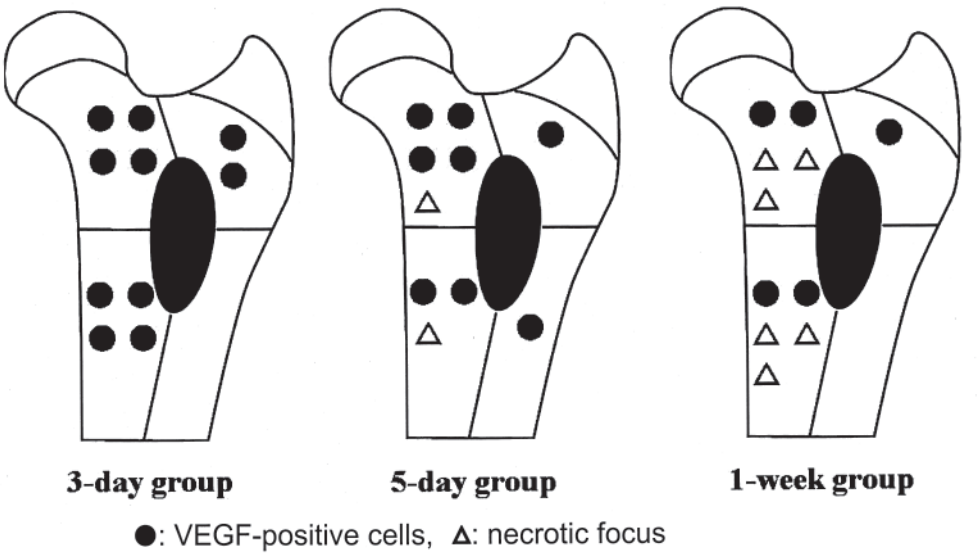



*Figure 4*. Location of VEGF-positive cells and osteonecrotic areas in the proximal femur.

we felt it would be valuable to investigate the occurrence mechanism and clinical condition of corticosteroid-induced ON of the femoral head using effective animal models.

Recently, Yamamoto, *et al* reported for the first time that administration of a large dose (20 mg/kg) of methylprednisolone acetate to rabbits caused ON that was histopathologically similar to human ON, from the metaphysis to the diaphysis of the long shaft bone[4]. We prepared a rabbit model by administering 4 mg/kg methylprednisolone acetate once a week, and reported that ON occurred in 73%[6]. These models differ from human ON in the following terms: (1) ON lesions are developed between the metaphysis and diaphysis of the long bone (femur and humerus); and (2) necrotic ON lesions do not collapse. However, because the rabbit ON presents histopathologic characteristics similar to human ON, these animal models are useful for investigating the development mechanisms of corticosteroid-induced ON in humans. In the model by Yamamoto, *et al*[4] rabbits often died because the dose of corticosteroid was too large, while our method (weekly administration of corticosteroid) was not appropriate for observing the occurrence time of ischemic episodes. Thus, in this study, we examined rabbits that received 4 mg/kg methylprednisolone acetate only once. As a result, the frequency of ON was 60% two weeks

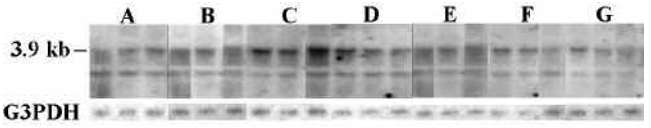



*Figure 5*. Northern blot detection of mRNA for VEGF and G3PDH in rabbit femur. 20 $\mu$g of total RNA was loaded in each line. VEGF-mRNA is localized as a band at 3.9 kb. (A) control group, (B) 1-day group, (C) 3-day group, (D) 5-day group, (E) 1-week group, (F) 2-week group, (G) 4-week group.

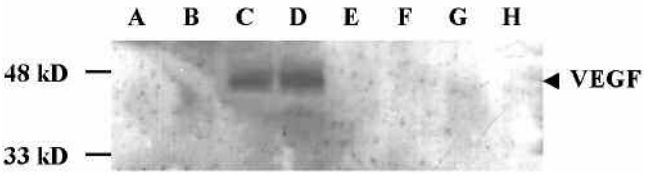



*Figure 7*. Western blot detection of VEGF protein in rabbit femur. VEGF is localized as a band near 46 kDa. (A) and (B): controls, (C) and (D) 3-day group, (E) and (F) 5-day group, (G) and (H) 1-week group.

after administering the corticosteroid. It has been confirmed that reproducible ON was produced in rabbits administered with 4 mg/kg methylprednisolone acetate only once, and that the rabbit is an appropriate corticosteroid-induced ON animal model.

It is said that the hypoxic condition is the most powerful factor among those that induce the expression of VEGF[15]. Thus, it has been suggested that the elevated expression of VEGF in tissues reflects the ischemic condition of the surrounding tissues. In animal studies, the elevated expression of VEGF in tissues has been shown in transient ischemia models of brain or spinal cord, and in models grown in a hypoxic condition[9-13]. On the other hand, corticosteroids are known to modulate some growth factors, including VEGF. Recently, several animal studies have indicated that corticosteroids strongly suppress VEGF expression[16-19]. Theoretically, then, high doses of methylprednisolone administered systematically in rabbits should suppress VEGF expression. However, our results showed that VEGF expression in the proximal femur was temporally increased. This suggests that systematic administration of a corticosteroid caused ischemic events in the proximal femur.

In this study, we investigated the expression of VEGF in the femur of a rabbit administered corticosteroid, and the relationship between the development of corticosteroid-induced ON and the occurrence period of ischemic episodes was examined. Based on the results of the experiment using immunohistochemistry, VEGF expression in this model can be characterized as follows: (1) VEGF-expressing cells are often localized; (2) VEGF occurs not only in interstitial cells but also in various cells such as marrow blood cells; (3) the positive rate of VEGF is higher at Day 3, Day 5, and Week 1 after corticosteroid administration, often occuring in the medial of the metaphyseal and shaft regions, which are areas of predilection for ON; and (4) no elevated expression was observed in tissues surrounding ON in Weeks 2 and 4 after corticosteroid administration. Based on characteristics (1) and (2) above, it was estimated that an ischemic episode occurred very locally after corticosteroid administration, and local tissues expressed VEGF temporarily in response to it. Meanwhile, ON was observed in H&E-stained speci-

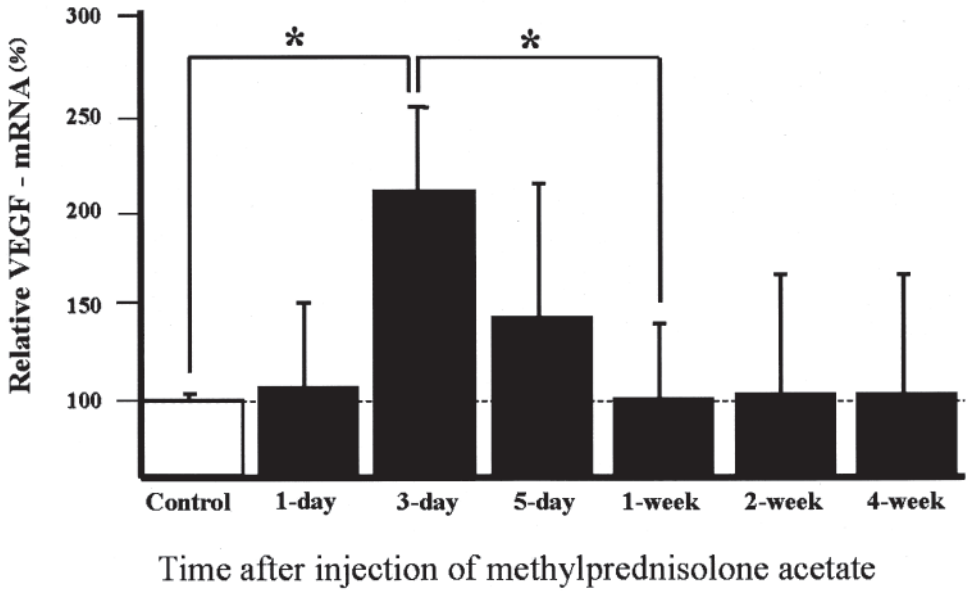



*Figure 6*. Relative levels of VEGF-mRNA expression (%) in rabbit femur. Increased expression of the 3.9-kb band was determined in semiquantitative fashion by densitometric scanning of a representative gel. The experiment was repeated 3 times with similar results. *$p < 0.05$ by Scheffe F test. Values are expressed as mean ± SD.

mens 5 days or more after administration of the corticosteroid. The occurrence frequency of ON in Week 1 or later showed a plateau. Based on characteristics (3) and (4), VEGF-positive cells develop in a limited period slightly earlier than the period when ON is observed in H&E-stained specimens and the development may be reduced after maturation of ON.

In our histopathological results, VEGF-positive specimens were not always ON-positive upon H&E staining. In the same way, the ON-positive specimens confirmed by H&E staining were not always VEGF-positive. In the 3-day group, there were no ON-positive specimens, although 50% of the specimens were VEGF-positive. In the 5-day group, 2 specimens that showed ON were VEGF-positive, but 4 that did not show ON were also VEGF-positive. In the 1-week group, all the ON-positive specimens were VEGF-positive, and all the specimens that did not show ON were VEGF-negative (Figure 3). To understand these results, it is necessary to remember that there is a time lag between the ischemic attack caused by methylprednisolone and the histological appearance of ON that can be confirmed by H&E staining. When an ischemic attack occurs, VEGF is usually expressed within several hours. On the other hand, H&E staining cannot confirm the histological reaction after ischemia for at least several days. For these reasons, we speculate that the VEGF-positive specimens in the 3-day and 5-day groups may have had ON even though H&E staining did not indicate its existence. The limitation of this study is that H&E staining of the specimens is the only way to evaluate the occurrence of ON.

Some reports of animal experiments have described development of ischemia, VEGF-mRNA, and VEGF protein. Plate, *et al* reported that VEGF-mRNA developed 3 hours or later after arterial occlusion occurred, peaked after 24 hours, and continued for 1 week in a rat middle cerebral artery occlusion model[9]. Similarly, Jin, *et al* reported that in a rat transient global cerebral ischemia model, VEGF-mRNA developed 8 hours after ischemia, and peaked after 24 hours; VEGF protein also reached a peak after 24 hours[12]. Hayashi, *et al* reported that in a rabbit model of reperfusion after spinal ischemia, the VEGF protein peak was observed 8 hours after the reperfusion[10]. Cherwek, *et al*[20] and Annex, *et al*[21] both reported the molecular weight of rabbit VEGF protein was 46 kDa in Western blot analysis. In our model, the development of VEGF-mRNA peaked on Day 3 after administration of the corticosteroid. Meanwhile, VEGF protein developed on Day 3 and the molecular weight was 46 kDa. In this model, based on our results and past reports, we estimate that the ischemic episode occurred on Day 2 at the earliest, and before and after Day 3 at the latest after administration of the corticosteroid. This supports the fact that ON was first observed in H&E-stained specimens 5 days after administration of the corticosteroid, the occurrence frequency of ON reached a plateau at Week 1 or later, and the VEGF-positive rate by immunohistochemistry rose at Day 3, Day 5, and Week 1 after administration of corticosteroid.

VEGF expression in a rabbit corticosteroid-induced ON model was investigated to clarify the timing of the onset of ischemic events. VEGF expression was accompanied by the development of ON. Levels of VEGF expression increased around 3 to 5 days after the corticosteroid treatments when ON was immature, then decreased at 2 weeks or later when ON matured. The onset of the ischemic event that might cause ON occurred roughly 2 days after the initial corticosteroid treatment. These results suggest that the ischemic events that cause ON begin to occur soon after the initial corticosteroid treatment.

## REFERENCES


1. Mont MA, Jones LC, Hungerford DS. Nontraumatic osteonecrosis of the femoral head: ten years later. J Bone Joint Surg Am 2006;88:1117-32.
2. Mankin HJ. Nontraumatic necrosis of bone (osteonecrosis). New Engl J Med 1992;326:1473-79.
3. Chang CC, Greenspan A, Gershwin ME. Osteonecrosis: current perspectives on pathogenesis and treatment. Semin Arthritis Rheum 1993;23:47-69.
4. Yamamoto T, Irisa T, Sugioka Y, Sueishi K. Effects of pulse methylprednisolone on bone and marrow tissues: Corticosteroid-induced osteonecrosis in rabbits. Arthritis Rheum 1997;40:2055-64.
5. Kabata T, Kubo T, Matsumoto T, et al. Apoptotic cell death in steroid induced osteonecrosis: an experimental study in rabbits. J Rheumatol 2000;27:2166-71.
6. Kabata T, Kubo T, Matsumoto T, et al. Onset of steroid-induced osteonecrosis in rabbits and its relationship to hyperlipaemia and increased free fatty acids. Rheumatology Oxford 2005;44:1233-7.
7. Miyanishi K, Yamamoto T, Irisa T, et al. Effects of cyclosporin A on the development of osteonecrosis in rabbits. Acta Orthop 2006;77:813-9.
8. Ichiseki T, Kaneuji A, Katsuda S, Ueda Y, Sugimori T, Matsumoto T. DNA oxidation injury in bone early after steroid administration is involved in the pathogenesis of steroid-induced osteonecrosis. Rheumatology Oxford 2005;44:456-60.
9. Plate KH, Beck H, Danner S, Allegrini PR, Wiessner C. Cell type specific upregulation of vascular endothelial growth factor in an MCA-occlusion model of cerebral infarct. J Neuropathol Exp Neurol 1999;58:654-66.
10. Hayashi T, Sakurai M, Abe K, Sadahiro M, Tabayashi K, Itoyama Y. Expression of angiogenic factors in rabbit spinal cord after transient ischaemia. Neuropathol Appl Neurobiol 1999;25:63-71.
11. Minchenko A, Bauer T, Salceda S, Caro J. Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. Lab Invest 1994;71:374-79.
12. Jin KL, Mao XO, Nagayama T, Goldsmith PC, Greenberg DA. Induction of vascular endothelial growth factor and hypoxia-inducible factor-1-alpha by global ischemia in rat brain. Neuroscience 2000;99:577-85.
13. Marti HJ, Bernaudin M, Bellail A, et al. Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. Am J Pathol 2000;156:965-76.
14. Fan L, Wakayama T, Yokoyama S, Amano O, Iseki S. Downregulation of vascular endothelial growth factor and its receptors in the kidney in rats with puromycin aminonucleoside nephrosis. Nephron 2002;90:95-102.

15. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992;359:843-45.
16. Ha IS, Um EY, Jung HR, Park HW, Cheong HI, Choi Y. Glucocorticoid diminishes vascular endothelial growth factor and exacerbates proteinuria in rats with mesangial proliferative glomerulonephritis. Am J Kidney Dis 2002;39:1001-10.
17. Koedam JA, Smink JJ, van Buul-Offers SC. Glucocorticoids inhibit vascular endothelial growth factor expression in growth plate chondrocytes. Mol Cell Endocrinol 2002;197:35-44.
18. Pufe T, Scholz-Ahrens KE, Franke AT, et al. The role of vascular endothelial growth factor in glucocorticoid-induced bone loss: evaluation in a minipig model. Bone 2003;33:869-76.
19. Edelman JL, Lutz D, Castro MR. Corticosteroids inhibit VEGF-induced vascular leakage in a rabbit model of blood-retinal and blood-aqueous barrier breakdown. Exp Eye Res 2005;80:249-58.
20. Cherwek DH, Hopkins MB, Thompson MJ, Annex BH, Taylor DA. Fiber type-specific differential expression of angiogenic factors in response to chronic hindlimb ischemia. Am J Physiol Heart Circ Physiol 2000;279:932-38.
21. Annex BH, Torgan CE, Lin P, et al. Induction and maintenance of increased VEGF protein by chronic motor nerve stimulation in skeletal muscle. Am J Physiol 1998;274:860-67.