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Original article

Osteonecrosis in stroke-prone spontaneously hypertensive rats: effect of glucocorticoid

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Abstract

Background. High-dose administration of a steroid hormone has been associated with a major risk of osteonecrosis. In this study we investigated the effects of a steroid hormone on the incidence of osteonecrosis of the femoral head in stroke-prone spontaneously hypertensive rats/Nagasaki (SHRSP/Ngsks).

Methods. A total of 71 SHRSP/Ngsks were divided into two groups: a control group (C group, $n = 40$) and a steroid hormone group (S group, $n = 31$) given 5 mg (about 20 mg/kg) of methylprednisolone acetate during the 17th week of age. We compared the groups' laboratory data, histological appearance, incidence of osteonecrosis, and expression of oxidative stress on immunohistochemical analysis using the monoclonal antibodies anti-4HNE and anti-8OHdG.

Results. The S group showed an increase in total cholesterol, with the amounts of high-density lipoprotein, low-density lipoprotein, and triglycerides all significantly higher than in the C group. Histological examination showed that the frequency of necrosis of the femoral head was significantly higher in the S group (95.2%) than in the C group (51.2%). Most of the histological features of the osteonecrosis demonstrated typical features of a similar sort in the two groups. However, the S group showed bone marrow spaces in the femoral head that were occupied by an increased number of adipocytes and that were swollen, partially degenerative, and necrotic. On immunohistochemical analysis, the stains of anti-4HNE and anti-8OHdG antibody were stronger in the S group than in the C group.

Conclusions. This study confirmed, to a remarkable degree, the suspicion that the administration of steroid hormone increases the number of adipocytes in marrow. Fat degeneration and necrosis, considered early signs of osteonecrosis, were also observed. It has been hypothesized that osteonecrosis is produced by the ischemic change accompanying compartment pressure load in marrow, where fat degeneration, necrosis, and endothelial cell injury might occur together with oxidative stress.

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Introduction

Several mechanisms have been suggested to cause idiopathic osteonecrosis of the femoral head[1]: thrombus formation in the hypercoagulable state[2,3]; intramedullary hemorrhage; microvessel destruction[4,5]; fat embolism due to abnormal lipid metabolism; and an intraosseous pressure rise with fat cell enlargement.[6–9]

Recently, high-dose administration of a steroid hormone has been associated with a major risk of osteonecrosis.[10,11] However, because the mechanism by which steroid hormones induce osteonecrosis is still unknown, prophylactic treatment cannot be undertaken. Most recently, apoptosis and oxidative stress have been the focus of attention as the mechanisms underlying steroid-induced osteonecrosis.[12–14]

In skeletal surveys of spontaneously hypertensive rats (SHRs), it has been observed that osteonecrosis occurs frequently in the epiphysis of the femoral head and that the histological findings closely resemble those in humans; therefore, we have used these rats as a model to investigate the etiology of osteonecrosis.[15] In addition, immune-complex rabbits and steroid-treated rabbits have been used for animal models of osteonecrosis.[16]

In this study we investigated the effects of a steroid hormone on osteonecrosis of the femoral head in stroke-prone spontaneously hypertensive rats/Nagasaki (SHRSP/Ngsks). We also looked at DNA oxidation injury occurring in the bone following steroid administration and focused on the relation between DNA oxidation injury and osteonecrosis in SHRSP/Ngsks.

Materials and methods

Animals

The rats used in this study were male SHRSP/Ngsks that were system-cost-bred at the Biomedical Research

Center, Division of Comparative Medical Center for Frontier Life Sciences, Nagasaki University. All the rats were housed under conditions of controlled temperature (24° ± 2°), humidity (55% ± 2%), and artificial light from 8:00 a.m. to 6 p.m. each day at the center.

There were 40 rats in the control group (C group) and 31 in the steroid hormone medication group (S group); both groups were kept on SP feed. At the age of 17 weeks, the S group had an intramuscular injection of methylprednisolone acetate 5 mg (about 20 mg/kg) in their backs.

The rats were raised in regular rat cages. They were weighed regularly, and their systemic blood pressure was measured indirectly with an electrosphygmomanometer (TK370A; Ynicom, Chiba, Japan) after prewarming the tails for 10 min at 38°C to dilate the caudal artery. The rats of both groups were sacrificed under ethyl ether anesthesia at 19 weeks of age. The experimental protocol was approved by the Guidelines for Animal Experimentation, Nagasaki University.

Blood tests

Blood samples were collected via intracardiac aspiration to measure the number of leukocytes, erythrocytes, and blood platelets and the hemoglobin level using an ACE counter FLC-240 (Fukuda Densi, Tokyo, Japan). For the biological tests, total protein, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG) were measured at Mitsubishi Kagaku Bio-Chemical Laboratories, Tokyo, Japan.

Histological examination

Both proximal femora were fixed in 10% buffered formalin solution for 24 h after extraction; they were then prepared for paraffin embedding after decalcification with the following agent, which was originated in the Okayama University Oral Pathology Division: a mixture of citric acid 29 g, trisodium citrate dihydrate 18 g, formic acid (HCOOH) 99% 100 ml, and distilled water 900 ml. Subsequently, thin normal sections through the teres ligament were stained with hematoxylin-eosin (H&E), and the histological changes were examined by light microscopy.

Osteonecrosis was determined according to the criteria of Arlet et al.[17]: degeneration, necrosis, and disappearance of marrow cells as well as the nuclear disappearance and hypochromasia of osteocytes of the trabeculae.

Oxidative stress analysis

We investigated DNA oxidation injury by the following immunohistochemical method: Primary antibodies (anti-4HNE monoclonal antibody and anti-8OHdG monoclonal antibody) were purchased from Nihon-Yusi Corporation (Tokyo, Japan), and we followed standard immunohistochemical protocol using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA).

Statistical analysis

All data were expressed as the mean ± standard error of mean (SEM). Differences between groups were examined for statistical significance using Fisher's exact probability test and the Mann-Whitney U-test. $P < 0.05$ denoted a statistically significant difference.

Results

Systolic blood pressure and body weight

Although blood pressure rose with age, a statistical difference was not seen between the groups. Body weight, however, differed significantly at the time of sacrifice for the S group (230.4 ± 26.3 g) compared to the C group (291.1 ± 23.3 g) ($P < 0.001$). Importantly, there was a dramatic loss of weight in the S group between the time of medication and their sacrifice: 17 weeks of age (286.1 ± 29.4 g) compared to 19 weeks of age (230.4 ± 26.3 g).

Blood count evaluation

The number of leukocytes and erythrocytes and the hemoglobin levels were not significantly different between the groups. The number of blood platelets in the S group ($290.0 \pm 96.8 \times 10^3$) was lower than that of the C group ($344.8 \pm 35.5 \times 10^3$) ($P$ 0.0019). The total protein level of the S group (5.09 ± 0.54 g/dl) was significantly lower than that of the C group (5.74 ± 0.46 g/dl) ($P <$ 0.0001). The total cholesterol level of the S group (82.1 ± 16.5 mg/dl) was significantly higher than that of the C group (55.8 ± 7.4 m/dl) ($P < 0.0001$). The HDL level of the S group (38.0 ± 6.7 mg/dl) was significantly higher than that of the C group (25.7 ± 4.1 mg/dl) ($P < 0.0001$). The LDL level of the S group (9.74 ± 2.13 mg/dl) was significantly higher than that of the C group (7.95 ± 1.78 mg/dl) ($P < 0.0001$). The TG level of the S group (92.2 ± 19.1 mg/dl) was significantly higher than that of the C group (79.4 ± 25.3 mg/dl) (P 0.022) (Fig. 1).

Histological findings

The histological findings of osteonecrosis of the femoral head (ONFH) were defined generally as follows: The dead trabeculae exhibited empty lacunae, showed appositional bone formation, or both. Because ONFH has

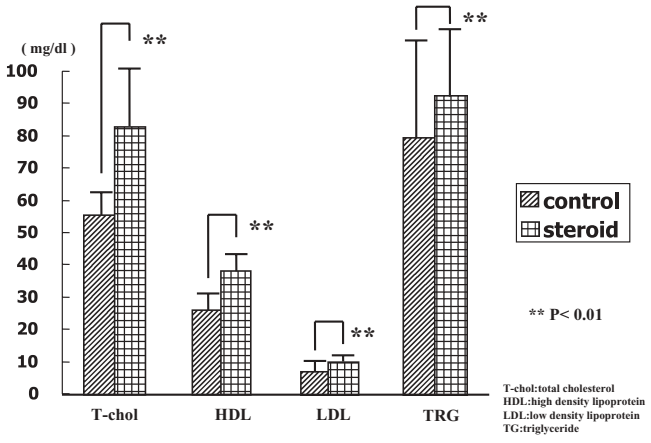

Fig. 1. Laboratory data of stroke-prone spontaneously hypertensive rats/Nagasaki (SHRSP/Ngsk)

various stages, it was essential for this study to detect the early stage clearly, so we adopted the criteria of Arlet et al.[17] (Fig. 2) and defined early osteonecrosis as a condition in which the marrow cell had disappeared or had degenerated, although trabecular osteocytes were still alive (Fig. 3).

Histologically, the frequency of ONFH in the S group (95.2%, 59/62 femoral heads) was significantly higher than that of the C group (51.2%, 42/80 femoral heads) ($P < 0.0001$). In the C group, the histological findings of the femoral head were typical ONFN in that the trabeculae bone exhibited empty lacunae and normal bone marrow cells had disappeared. In the S group, 58.1% (36/62 femoral heads) showed the histological findings of ONFH similar to that in the C group. However, 37.1% (23/62 femoral heads) demonstrated ONFH dur-

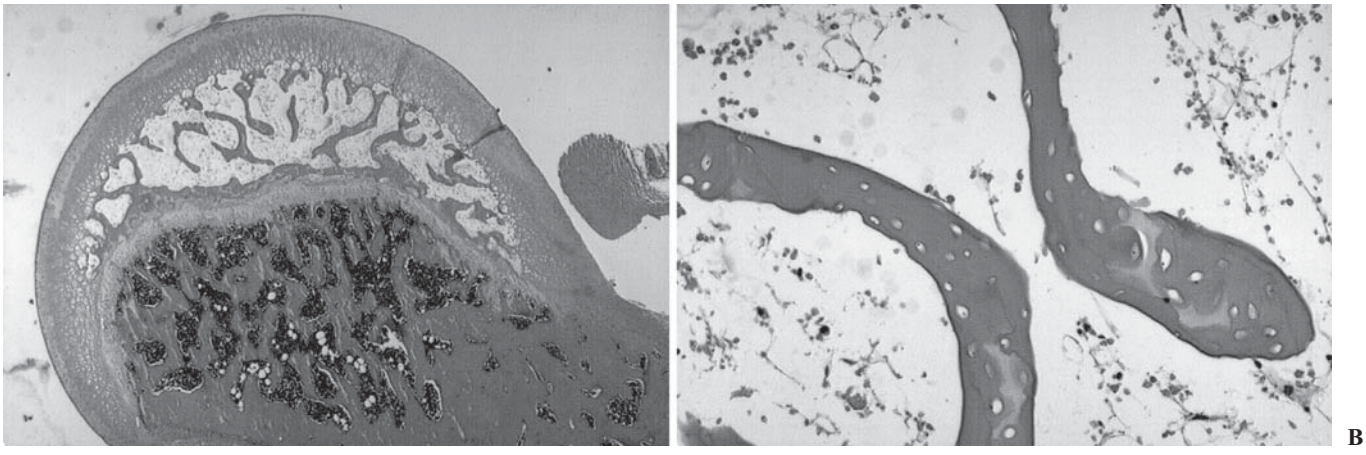

Fig. 2. Typical osteonecrosis. Photomicrography of femoral head necrosis observed in SHRSP/Ngsks at 19 weeks of age. **A** Bone trabecular pattern was destroyed, with wide cavities filled with necrotic debris. H&E ×20. **B** Magnification of the section indicated in **A**. Loss of osteocyte core in the lacunae of trabecular bone (empty lacunae). H&E ×200

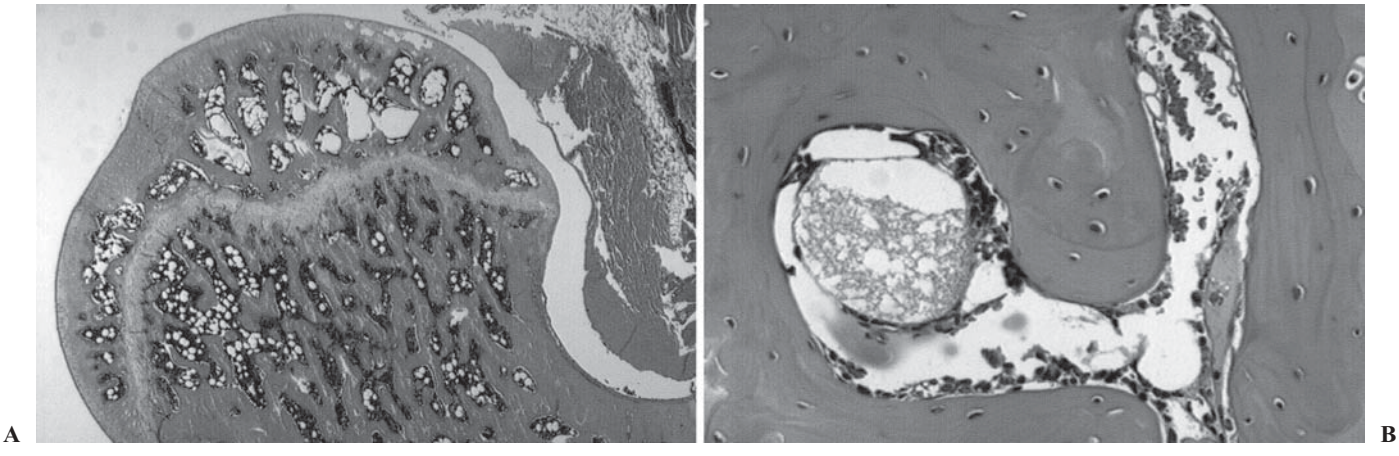

Fig. 3. Osteonecrosis at an early stage. Photomicrography of the femoral head observed in SHRSP/Ngsks at 19 weeks of age with medicated methylprednisolone acetate (MPSL) (about 20 mg/kg). **A** Bone marrow spaces are filled with adipocytes, and normal marrow cells (e.g., granulocytes, myelocytes) are decreased. H&E ×20. **B** Magnification of the section indicated in **B**. Bone marrow spaces are filled with adipocytes that exhibit swelling, partial degenerative change, and necrosis but live trabecular osteocytes. H&E ×200

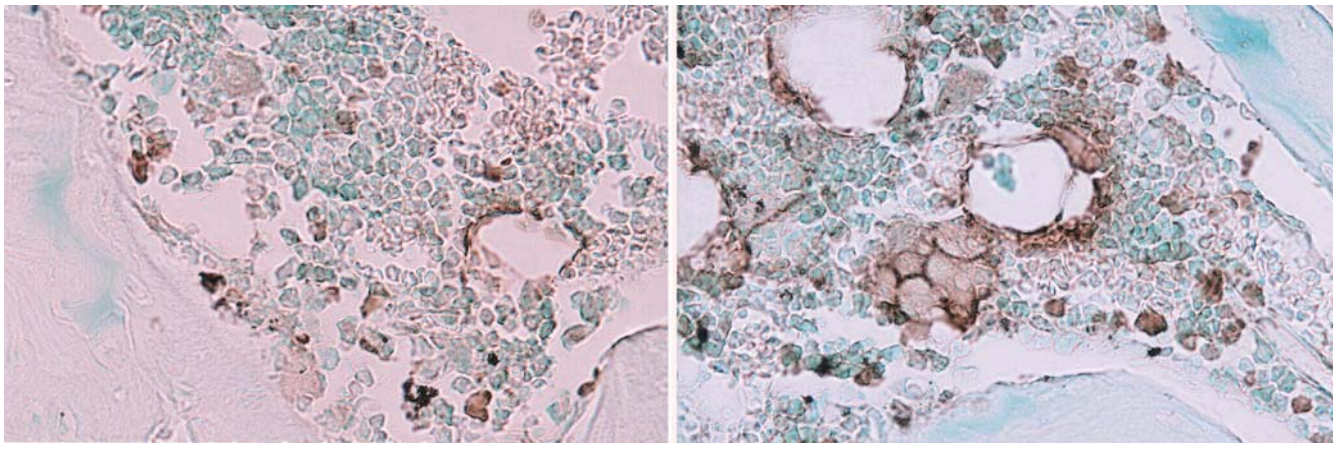

Fig. 4. Immunohistochemical appearance. **A** Photomicrography of the epiphysis of femur immunostained with anti-4HNE monoclonal antibody in an SHRSP/Ngsk (control). Counterstain methyl green. ×400. **B** Photomicrography of a membrane of an adipocyte in the epiphysis of the femur immunostained with anti-4HNE monoclonal antibody in an SHRSP/Ngsk with medicated MPSL. Counterstain methyl green ×400

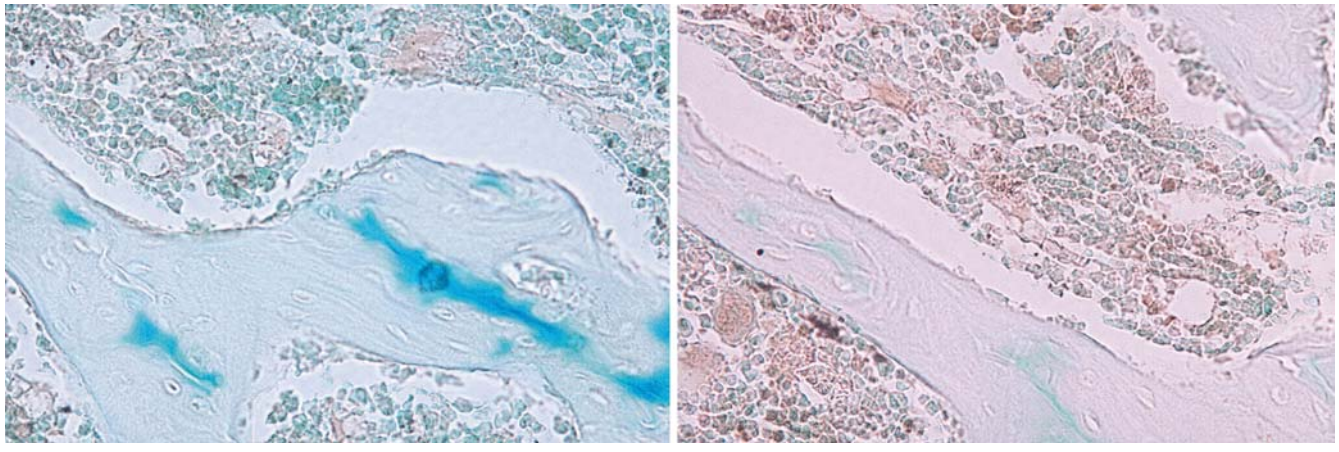

Fig. 5. Immunohistochemical appearance. **A** Photomicrography of the epiphysis of femur immunostained with anti-8OhdG monoclonal antibody in an SHRSP/Ngsk (control). **B** Photomicrography of marrow cell of the epiphysis of femur immunostained with anti-8OhdG monoclonal antibody in SHRSP/Ngsk with medicated MPSL. **A, B** Counterstain methyl green ×400

ing the early stages in which bone marrow spaces were filled with swollen adipocytes involving partial degenerative changes or necrosis. The femoral heads, which had osteonecrosis, had hyperplasia of adipocytes in metaphysis.

Oxidative stress

On immunohistochemical analysis, both antibodies stained weakly in the C group. The positive staining for anti-4HNE monoclonal antibody was obviously stronger on the rim of adipocytes of the epiphysis and metaphysis of the femur in the S group than in the C group (Fig. 4). Positive staining for anti-8OHdG monoclonal antibody was stronger in the bone marrow cells of the epiphysis and metaphysis of the femur in the S group than in the C group (Fig. 5).

Discussion

Osteonecrosis is a debilitating disease for patients who have to take a high-dose steroid hormone for the treatment of other conditions such as collagen diseases including systemic lupus erythematosus, rheumatoid arthritis, kidney disease, and after organ transplantation.[11] Several possible causes of idiopathic ONFH have been suggested, such as thrombus formation in the hypercoagulable state,[2,3] intramedullary hemorrhage, microvessel destruction,[4,5] fat embolism due to abnormal

lipid metabolism, and an intraosseous pressure rise with fat cell enlargement.[6–9] However, the precise mechanism by which steroid hormones induce osteonecrosis is still unknown, so prophylactic measures cannot be undertaken. Although it has often been reported that administration of steroid hormone might induce the adipocytes in marrow to cause hyperplasia, there have not yet been any reports of ONFH generating directly.

In skeletal surveys of SHR, Iwasaki et al. incidentally observed widespread osteonecrosis that occurred frequently in the epiphysis of the femoral head and found that the histological findings closely resembled those in humans, so we have been using these rats as models to investigate the etiology of ONFH.[15] Unlike other animal models, rats can be purebred, so we think they are reliable for studying etiology. Also, rats have not been widely used for the study of ONFH, and there have been no reports about a study of osteonecrosis connected with steroid hormone administration in rats. In this study we investigated the effects of steroid hormone on the incidence of ONFH in SHRSP/Ngsks.

There have been many histological evaluations of osteonecrosis.[18–20] Arlet et al. claimed that the lesions the appear in the histology of osteonecrosis can be classified into four types according to their phases: type 1, spots of fibrosis, necrosis, stasis, and hemorrhage surrounded by areas of apparently normal fatty marrow; type 2, extensive necrosis of the hematopoietic and fatty tissues of the bone marrow, occupying most of the spaces; type 3, the aforementioned marrow lesions are accompanied by trabeculae deprived of their osteocytes — microscopically observed emptying of the lacunae is the first clear sign of osteocytic necrosis; and type 4, signs of repair similar to those observed in the transitional zone underneath the sequestrum in advanced cases.[17]

Taking a different approach, Bauer and Stulberg argued that, histologically, lesions of osteonecrosis should be classified into three phases. The first reliable signs of osteonecrosis are hemorrage, loss of hematopoietic elements, loss of adipocyte nuclei, and a microvesicular fatty change of marrow adipocytes. The ruptured adipocytes produce round cavities that may be surrounded by histiocytes.[21] It has also been shown that processes that generate heat, such as the cutting of a saw, can create reactions that resemble type 1 lesions in Arlet's classification (spots of fibrosis, necrosis, stasis, and hemorrhage). Therefore, we obtained our study's histological samples after decalcification without heat. Our histological findings indicated massive adipocyte proliferation in the marrow space of the femoral head of SHRSP/Ngsks, and osteonecrosis significantly increased after steroid hormone administration. We found early osteonecrosis of Arlet's type 2 in 37.1% (23/62 femoral heads) in the S group.

Yamamoto et al. reported on two experimental rabbit models for osteonecrosis. One was produced by combining the Shwartzman reaction and a high-dose corticosteroid injection; and the other was produced by a single high-dose injection of corticosteroid. Hyperlipemia and hypercoagulability were recognized in these rabbit models.[16] In our study, blood tests indicated extreme hyperlipemia in the S group. Histological examination of the steroid-administered rats showed that the proliferation of adipocytes in the head and shaft of the femur was extreme, and fatty livers were also observed. As for hypercoagulability, the number of platelets was significantly lower, but thrombus formation was not recognized in the femoral head or shaft in the S group. In general, histologically, cardiac infarction, thrombus formation, and arterial sclerosis were not recognized in other organs, except for partial glomerular necrosis of the kidney in the S group. The important result of this study was the strong indication that steroid medication caused hyperlipemia and proliferation, degeneration, and necrosis of adipocytes in SHRSP/Ngsks. The fact that the rabbits seldom suffer osteonecrosis under normal conditions and that SHRSPs are more prone to the condition may be a factor in the different reaction to steroid hormone.

Drescher et al. reported that treatment with high-dose methylprednisolone reduced blood flow in both cortical and cancellous bone selectively in the pig. In the rigid intraosseous compartment, growth of fat cells might be the cause of a rise in intraosseous pressure and compresses the thin-walled sinusoids, with a subsequent decrease in blood flow.[22] Miyanishi et al. reported that the size of bone marrow fat cells increased and intraosseous pressure rose while the blood flow rate fell in the steroid-induced osteonecrosis rabbit model.[23] Because we did not measure the intraosseous pressure of the bone marrow, we could only speculate that adipocyte proliferation and injury of the endothelial cells of the vessels in the marrow might have caused the intraosseous pressure to rise, subsequently causing degeneration and necrosis of the adipocytes and marrow cells and, in the end, necrosis of trabecular osteocytes. In addition, the growth plate remains for a long time in rats, so we speculate that osteonecrosis occurred in the epiphysis, (it's) closed space.

Glueck et al. reported that hyperlipemia was linked to hypercoagulability and hypofibrinolysis, which were further associated with a tendency to venous thrombosis.[3] Wang and Cui reported that glucocorticoids not only stimulated but also regulated the processes of adipogenesis in cells from bone marrow stroma based on the results of a study of a multipotential bone marrow cell line treated with dexamethasone in vitro. Moreover, they suggested that concomitant use of lipid-clearing agents with steroids could be beneficial in

preventing the progression of ischemia that was induced in the femoral head by steroid administration.[10] Motomura et al. reported that treatment with anticoagulant plus a lipid-lowering agent prevented steroid-induced osteonecrosis in their experimental rabbit models.[24] We are seeking some agents for the prevention of hyperlipemia and ONFH using SHRSP/Ngsks with steroid hormones.

Furthermore, apoptosis has recently been suggested to be one of the mechanisms of osteonecrosis, and tissue oxidation injury is known to induce apoptosis.[12–14] Calder et al. demonstrated evidence of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) expression in bone and evidence of apoptosis in osteoblasts, osteocytes, and marrow cells from the femoral heads of patients with osteonecrosis.[12] Weinstein et al. demonstrated a possible mechanism that inhibited osteoblastogenesis and promoted apoptosis of osteoblasts and osteocytes in murine glucocorticoid-induced osteoporosis.[25] Ichiseki et al. reported that oxidative stress developed and vascular permeability was observed in the steroid-induced osteonecrosis rabbit model and suggested the possibility of prevention by the suppressing oxidative stress.[14] We used anti-4HNE monoclonal antibody and anti-8OHdG monoclonal antibody to detect oxidative stress. 4HNE is an aldehyde made from the oxidation of unsaturated fatty acid such as arachidonic acid. 8OHdG is the product of oxidation of 2-deoxyguanosine in DNA on oxidative stress. Using both antibodies, we obtained reliable data using the intravital and metabolic approaches.

In this study, the anti-4HNE monoclonal antibody expression was clearly stronger on the membranes of adipocytes of the epiphysis and metaphysis of the femur, and the anti-8OHdG monoclonal antibody expression was stronger in the S group than the C group in the bone marrow cells of the epiphysis and metaphysis of the femur. This was evidence of oxidative stress due to steroid administration, suggesting a relation with osteonecrosis.

In this study, osteonecrosis occurred only in the epiphysis, but oxidative stress was detected in the epiphysis and metaphysis of the femur. The pathophysiology of osteonecrosis is generally thought to be multifactorial. Because one of the factors, oxidative stress, was observed in the femoral head and neck, we speculate that the incidence of osteonecrosis was caused locally, possibly by microcirculation or intraosseous pressure (or both).

Conclusions

We have shown that the frequency of osteonecrosis was significantly higher in the group of SHRSP/Ngsks that underwent high-dose steroid administration. Also, it seems evident that oxidative stress was induced by steroid administration as part of the development of the steroid-induced osteonecrosis.

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