Onset of steroid-induced osteonecrosis in rabbits and its relationship to hyperlipaemia and increased free fatty acids


Objectives. To clarify the initial onset time of osteonecrosis after the start of steroid treatment and its relation to the onset of abnormal lipid metabolism.

Methods. Animal models were prepared by administering methylprednisolone to rabbits using five different steroid regimens.

Results. A single, acute ischaemic event suggested by the frequency, size or number of necrotic foci within the proximal femur was not different among the groups. Histological evidence of osteonecrosis first occurred 1–2 weeks after initial steroid administration. At the same time there were significantly abnormal elevations in serum lipids, which persisted for between 1 and 2 weeks after the initial corticoid treatment. Triglycerides, total cholesterol and free fatty acids were markedly elevated in all groups; these lipid abnormalities were significantly present in the rabbits with osteonecrosis but not in the rabbits without osteonecrosis.

Conclusions. This study shows that (i) osteonecrosis appears in rabbits shortly after corticoids are first administered, and (ii) osteonecrosis in rabbits is chronologically associated with the onset of hyperlipaemia and increased free fatty acids. This supports the occurrence of intraosseous fat embolism as a cause of osteonecrosis.

Key words: Steroid, Osteonecrosis, Animal model, Hyperlipaemia, Free fatty acids.

Osteonecrosis (ON) frequently occurs when steroids are administered as a treatment for collagen diseases or for immunosuppression after organ transplantation [1–4]. In order to deepen our understanding of the prevention, diagnosis and treatment of steroid-induced ON, it is important to clarify the time of disease onset after starting steroid treatment. It is also essential to clarify the relationship between the time when abnormal fat metabolism appears and the onset of ON, because abnormal fat metabolism is thought to be related to steroid-induced ON [5–9].

Animal models allow histological studies of early necrotic stages. Recent studies introduced animal models of methylprednisolone-induced ON [10, 11], and the present authors also succeeded in establishing a rabbit ON model with high reproducibility of the necrosis by using methylprednisolone [12]. In this model, the lesion of ON is not created on the epiphysis, but ON satisfies the definition of ON in man. The present study histopathologically investigated the occurrence of ON in rabbits under five steroid regimens, examined onset time, and we discuss the relationship between ON onset and changes in serum lipid levels.

Materials and methods

Eighty-five adult female Japanese White rabbits (mean body weight 3.5 kg, mean age 24 months) were used. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kanazawa University School of Medicine, Japan.

The basic dosage of steroid in the current study was determined as 4 mg/kg/week because the steroid dose for SLE, which is a major basic disease of ON in man, is usually 2–4 mg/kg/week, and we also confirmed the occurrence of necrosis in another rabbit study at the dose of 4 mg/kg/week methylprednisolone acetate (MP; UpJohn, Tokyo, Japan) [10]. Five administration protocols were prepared. In group A (n = 15) 4 mg/kg of MP was injected i.m. once and the rabbit was killed 3 days later. In group B (n = 15) 4 mg/kg of MP was injected i.m. once a week for 4 weeks and the rabbit was killed 1 week later. In group C (n = 15) 4 mg/kg of MP was injected i.m. once a week for 2 weeks and the rabbit was killed 1 week later. In group D (n = 15) 4 mg/kg of MP was injected i.m. four times (once a week for 4 weeks) and the rabbit was killed 1 week later. In group E (n = 15) 4 mg/kg of MP was injected i.m. eight times (once a week for 8 weeks) and the rabbit was killed 1 week later. As the control, 10 rabbits were fed under the same conditions but did not receive steroid injections.

The femora obtained were cut into coronal sections using a tissue cutter (Exakt, Hamburg, Germany), soaked in 10% formalin for about 1 week for fixation, decalcified using ethylene diamine tetraacetic acid solution, embedded in paraffin, sliced into 5 µm sections, stained with haematoxylin and eosin, and examined under a light microscope.

For each group, the frequency of ON, its location, the number of necrotic foci, the size of the ON area and its histology were examined. All specimens were read individually by three coauthors, who are experienced pathologists (T.K., S.Y. and M.K.), without giving information with regard to the treatment
groups. The authors defined ON as the diffuse presence of empty lacunae or osteocyte ghosts in the bone trabeculae, accompanied by surrounding bone marrow necrosis [10, 12]. The specimen was determined as having a necrotic focus when all three pathologists evaluated it as having necrosis.

The frequency of ON was obtained as the number of rabbits with ON divided by the total number in the group. The rabbit was evaluated as having ON when either side of the femora had a necrotic lesion that met the above-mentioned definition.

The location of ON was evaluated in terms of six areas, i.e. two epiphysial regions (femoral head and trochanteric region), two metaphysial regions (medial and lateral) and two shaft regions (medial and lateral).

The number of necrotic foci was counted on each femur, and group means were compared.

In order to measure ON area, light microscopic images were captured in a personal computer (Power Macintosh 8100/80; Apple Computer), and processed using the NIH Image 1.61 software program (Bethesda, U.S.).

Blood samples were obtained before treatment (all groups), 1 and 3 days after the initial steroid i.m. injection (all groups), 1 week (groups B, C, D and E), 2 weeks (groups C, D and E), 4 weeks (groups D and E) and 8 weeks (group E) after the initial i.m. injection. The samples were examined for changes in biochemical factors in the lipid system, i.e. total cholesterol, triglycerides, free fatty acids and high-density lipoprotein (HDL) cholesterol.

Statistics used were Fisher’s exact test for the frequency of ON, Kruskal–Wallis’ rank sum test for the number of necrotic foci and the size of ON lesions, and two-way analysis of variance for biochemical test results. For multiple comparisons when a significant group difference was obtained, Scheffe’s F test was used. Comparison between the groups with or without ON was done with the Mann–Whitney U-test. P values less than 0.05 were considered to be significantly different.

Results

Onset and frequency of ON

ON did not occur in the controls and group A. ON was detected in 7 rabbits of group B (47%), 9 of group C (60%), 11 of group D (73%) and 10 of group E (67%). ON appeared 1 week after steroid administration; its frequency increased until the 2nd week, and then reached a plateau. There was no significant group difference among groups B, C, D and E, which received one to eight steroid injections.

Location of ON

Among the six areas examined, ON was concentrated on the medial side of the metaphysial region and shaft region, but there was no group difference in the location.

Number and size of necrotic foci

The number and size of necrotic foci were not significantly different among the groups (Kruskal–Wallis test). Frequency was 0 in the controls and group A; 1.38 ± 0.51 in group B, 1.27 ± 0.47 in group C, 1.46 ± 0.66 in group D, and 1.23 ± 0.44 in group E. Size (mm²) was 0 in the controls and group A; 5.58 ± 1.79 in group B, 3.80 ± 2.64 in group C, 5.11 ± 5.04 in group D and 5.12 ± 5.23 in group E.

Histopathological findings

In the controls and group A, there were no changes suggesting ON. Histological findings in groups B, C, D and E are summarized in Fig. 1.

In the cases that had ON lesions affecting both femurs, or that had two or more necrotic foci within one specimen, the histopathological finding of each lesion showed no chronological difference between similar-appearing lesions.

Haematological findings

In the lipid system, total cholesterol, triglycerides and free fatty acids markedly increased in the period between 1 and 2 weeks after the initial steroid administration, and these high levels were maintained during the observation periods. On the other hand, a sharp decrease in HDL cholesterol was found in the period between 3 days and 1 week after the initial steroid injection. No abnormality was found in the data of the control group (Fig. 2a, i–iv).

In the comparison between cases with and without ON, total cholesterol, triglycerides and free fatty acids in cases with bone necrosis increased significantly in the period between 1 and 2 weeks after the initial steroid injection. On the other hand, there were no differences in HDL cholesterol levels between cases with and without ON (Fig. 2b, i–iv).

There was no significant relationship between the changes in lipid levels or area under the curve for the lipid levels and the number of osteonecrotic foci (data not shown).

Discussion

A recent study by Yamamoto et al. in rabbits demonstrated histologically that a single dose of methylprednisolone (20 mg/kg) can induce multifocal ON [10]. We, in a previous study, established a similar rabbit model by administering methylprednisolone at 4 mg/kg/week, in which ON can be produced in the femur and humerus with high reproducibility [12]. ON lesions in these rabbit models present histological characteristics similar to human ON, i.e. osteocytic death surrounded by necrotic bone marrow with or without repair tissue [13].

However, the rabbit models are different from human ON in that (i) their ON regions are not on the epiphysis but on the medial side of the metaphysis and diaphysis, and (ii) their ON lesions develop very often but do not collapse [10, 14, 15]. These differences may be due to metabolic differences between humans and rabbits, mechanical differences between quadrupeds and bipeds, or other reasons. Although the mechanism of development of ON is not completely the same as in humans, there may be several common pathways which lead to ON development. To investigate the denominators of ON in human and rabbits may help us to understand developmental mechanism of ON. We believe these animal models are useful to investigate development mechanisms of steroid-induced ON in human.

Corticosteroid treatment is considered to be an important risk factor for ON [2–4]. However, the time when ON occurs after starting steroid administration has not been clarified. In the present study, ON was not found 3 days after steroids were initiated, but it began to be detected after 1 week and was obvious at 2 weeks. Furthermore, the frequency, number and size of necrotic foci appearing at between 1 and 8 weeks was not significantly different. Despite the histological changes of necrosis, there was limited evidence of reparative revascularization and reossification. In the cases with multiple necrotic foci, each focus had the same histological changes and there was no sequential enlargement of the necrotic foci or evidence of reinfarction. Based on these findings, ON in this animal model was thought to occur around 1 week after the initial steroid administration. The present study showed that ON can occur at a quite short time after steroid administration, and our present findings support other clinical information [16, 17].

Could episodes that induce ON repeatedly occur when steroid is administered continuously? This question is still under
FIG. 1. (a) Histopathological findings of ON in group B. The necrotic area showed accumulation of degenerative or necrotic marrow and fat cells, and bone trabeculae showed either empty lacunae or osteocyte ghosts. Though the fat cells showed degenerative or necrotic changes, their architecture was not collapsed. Haematoxylin–eosin staining. Original magnification ×200. (b) Histopathological findings of ON in group C. Necrotic area showed accumulation of cell debris, completely narcotized haematopoietic and fat cells, and loss of the architecture of these cells, i.e. collapse. Bone trabeculae also underwent necrotic changes. Haematoxylin–eosin staining. Original magnification ×200. (c, d) Histopathological findings of osteonecrosis in group D. Bone marrow cells showed cytolysis, karyorrhexis and karyolysis, while necrotic fat cells were clearly collapsed. On the border area, fibroblasts and/or histiocytes were found, but appositional bone formation around the necrotic bone trabeculae was not observed. Haematoxylin–eosin staining. Original magnification: c, ×100; d, ×200. (e) Histopathological findings of ON in group E. Necrotic trabeculae were devoid of osteocytes within lacunae, and degeneration of adipocytes and cytolysis of haematopoietic cells were observed. At the border area, many fibroblasts and histiocyte were found, and appositional bone formation around the necrotic bone trabeculae was observed (arrows). Haematoxylin–eosin staining. Original magnification ×150.
FIG. 2. (a) Changes in plasma lipid levels. (i) Total cholesterol; (ii) triglycerides; (iii) free fatty acids; (iv) HDL cholesterol. *P < 0.05; **P < 0.01; †P < 0.05; ††P < 0.01 vs values before steroid treatment. Figures represent mean ± s.d. (b) Changes in plasma lipid levels in rabbits with and without ON. (i) Total cholesterol; (ii) triglycerides; (iii) free fatty acids; (iv) HDL cholesterol. Circles, rabbits with ON. Squares, rabbits without ON. Figures represent mean ± s.d. *P < 0.05, **P < 0.01.
discussion [18, 19]. In the present study, the frequency of ON in the 1- to 8-week treatment groups was not significantly different, and there was no sign of the development of new ON or the expansion of the necrotic area. Some clinical studies support our findings [16, 17, 20]. These findings indicate that steroid-induced ON is an acute ischaemic disease similar to cardiac and cerebral infarctions.

In our haematological examination, serum lipid levels increased sharply 1 week after the initial steroid administration, and this is the time when ON was thought to occur in this model. This point suggests that ON is closely related to serum lipid levels. Previous studies suggested that abnormal lipid metabolism is related to steroid-induced ON of the femoral head [5–9], and hyperlipaemia and increased free fatty acids are considered to be important risk factors. However, there has been no report showing that ON and lipid level changes occur at the same time, and the present study for the first time confirmed the simultaneous occurrence.

Jones et al. [6, 7] proposed a fat embolization theory whereby hyperlipidaemia and associated abnormalities in the blood coagulation system are a possible mechanism of ON. In the present study, lipid levels in our model animals increased to 5–30 times higher than in normal rabbits, and we regard these levels as high enough to accelerate the coagulation system. The hypothesis of Jones et al. explains the current haematological findings sufficiently, i.e. the blood coagulation system could be activated about 1 week after steroid administration, when fat levels increase sharply, then thrombi are formed within the blood vessels of the bone, and this finally induces ON. The significant difference in the plasma lipid levels of rabbits with and without ON 1 week after the initial steroid administration may also suggest changes in fat embolism as a cause of ON. In addition, no significant relationship between the number of osteonecrotic foci and the steroid dose or duration in the present study suggests that the occurrence of ON does not depend on steroid dosage but is related to lipid metabolism in the early treatment period.

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### Key messages

- Osteonecrosis appears shortly after corticoids are first administered.
- Osteonecrosis is chronologically associated with the onset of hyperlipaemia and increased free fatty acids.

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The authors have declared no conflicts of interest.

### References