DNA oxidation injury in bone early after steroid administration is involved in the pathogenesis of steroid-induced osteonecrosis

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Objective. Using a rabbit model, we investigated the DNA oxidation injury occurring in bone following steroid administration and focused on the relation between DNA oxidation injury and osteonecrosis.

Methods. Japanese white rabbits weighing about 3.5 kg were injected with a single intramuscular dose of methylprednisolone 4 mg/kg and divided into groups consisting of 10 rabbits each, which were killed after 3, 5 and 14 days (groups A, B and C respectively). As a control, five untreated rabbits (group N) were also studied. An immunohistochemical study of the diaphysis of the proximal femur was conducted using the monoclonal antibody N45.1, which is a highly specific antibody against 8-hydroxy-2'-deoxyguanosine, an index of DNA oxidation injury. Also, using NIH Image freeware, the positive area (8-OHdG %PA) of each group was calculated and the four groups were compared.

Results. Osteonecrosis was detected only in group C (70%). N45.1 positivity was noted in bone marrow haematopoietic cells and was particularly marked in groups B and C. 8-OHdG %PA was 1.6 ± 0.2% in group N, 2.2 ± 0.4% in group A, 4.8 ± 0.4% in group B and 5.1 ± 0.5% in group C, with significantly greater oxidation injury found in groups B and C (P<0.001).

Conclusion. Oxidative injury was demonstrated soon after the administration of methylprednisolone in a rabbit model prior to the development of osteonecrosis. This finding may suggest new strategies to prevent steroid-induced osteonecrosis, such as the optimally timed (early) administration of antioxidant agents.

KEY WORDS: Steroid-induced osteonecrosis, Oxidation, 8-OHdG.

There is a general consensus that circulatory disturbances occurring within bone underlie the development of steroid-induced osteonecrosis [1]. Recently, in vivo oxidative stress, which has been implicated in numerous pathological conditions [2, 3], including vascular injury [4], has been reported to play a role in the pathogenesis of steroid-induced osteonecrosis as well [5]. Apoptosis has also been suggested to be involved [6], and is known to be induced also by tissue oxidative injury. When tissue DNA sustains oxidative injury, 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is recognized as a major marker of oxidative injury, is produced [7]. The present study, using a rabbit model, was undertaken to investigate the presence/absence of DNA oxidative injury in the diaphysis of the proximal femur, which is the site most susceptible to the development of steroid-induced osteonecrosis in the rabbit model [5], early after steroid administration, and to clarify the relation between osteonecrosis and DNA oxidative injury.

Materials and methods

Adult female Japanese white rabbits (mean body weight 3.5 kg) were injected once with methylprednisolone at 4 mg/kg body weight into the right gluteal muscle, and 10 animals each were killed 3, 5 and 14 days after steroid administration. They were designated as groups A, B and C respectively, and compared with group N, which consisted of five rabbits that were fed under the same conditions but not injected with steroid.

Immediately after the animals had been killed the bilateral femurs were isolated and fixed in 10% formalin for 1 week, and the specimens were decalcified in 10% EDTA. The specimens were then embedded in paraffin, and cut into 4 μm sections.

All protocols in this study were performed in accordance with the guidelines of the Animal Research Committee of Kanazawa Medical University.

Histopatology

Necrosis of bone and marrow tissues was examined in haematoxylin–eosin-stained preparations by light microscopy. Osteonecrosis was judged to be present when necrosis of medullary haematopoietic cells or fat cells or empty lacunae or condensed nuclei in osteocytes was noted. Osteonecrosis was judged to be present when osteonecrosis was identified in either isolated femur [8]. The rate of development of osteonecrosis was calculated as the ratio of the number of rabbits with osteonecrosis to the total number of rabbits used.

Immunohistochemistry

To examine the development of DNA oxidative injury in bone, the femur was stained immunohistochemically with monoclonal antibody N45.1. Briefly, after deparaffinization, the sections were...
treated with 0.3% H$_2$O$_2$ in methanol for 30 min at room temperature, and with 0.1% trypsin for 15 min at 37°C. Then the sections were reacted with N45.1 monoclonal antibody (10 g/ml) for 1 h at room temperature in a humidity chamber, followed by incubation with Dako EnVision/HRP system (Dako, Tokyo, Japan) for 30 min at room temperature. Sections were then treated with DAB for 5 min, and counterstaining was carried out with haematoxylin–eosin for 10 min.

Quantification of immunohistological data (8-OHdG %PA)

Immunohistological data were quantified using NIH Image freeware and calculated as the positive area (%PA) per visual field. Briefly, colour slides (35 mm) of five appropriate locations, which were focused on the proximal femur, were taken of each specimen at a magnification of 40×5, covering an area of approximately 335×220 μm. Colour images were obtained as PICT files with a slide scanner connected to a computer running Windows 2000. The brightness and contrast of each image file were uniformly enhanced with Adobe Photoshop version 5.0J, followed by image analysis with NIH Image freeware. Oxidative injury was evaluated by calculating the percentage occupied by positive cells relative to the total area in five randomly selected visual fields. Specimens from the four groups (groups N, A, B and C) were analysed. The mean of the data obtained from five independent fields was used as a representative value for each animal.

Statistical analysis

Statistical analysis of 8-OHdG %PA of each group was performed by one-way analysis of variance. Differences were considered significant at $P < 0.05$.

Results

Histopathology

There was no necrosis of the bone or bone marrow in groups N, A or B, whereas osteonecrosis was observed in seven of 10 rabbits (70%) in group C (Fig. 1a). Necrotic areas in group C were clearly demarcated from the surrounding normal tissue. Empty lacunae were found in bone trabeculae, and the surrounding bone marrow tissue also showed necrotic changes (haematopoietic cell and fat cell necrosis).

Immunohistochemistry (Fig. 1b, c)

Positive findings on immunohistochemical study were noted in each of the groups in haematopoietic cells in the proximal femur. However, there were only a few, sporadic positive cells in groups N and A, whereas marrow in groups B and C exhibited clusters of haematopoietic cells with oxidative injury. These clusters were observed in the diaphysis of the proximal femur, which in the present model is the site where osteonecrosis most frequently occurred.

Fig. 1. Histopathological and immunohistochemical studies in femurs of rabbits treated with steroid. (a) Group C (haematoxylin–eosin staining); (b) group N (N45.1 staining); (c) group B (N45.1 staining). (a) Histopathologically, osteocytes in the stained bone contain achromatic nuclei or empty lacunae, showing typical features of osteonecrosis. Medullary haematopoietic cells around the site of osteonecrosis are mixed with necrotic and degenerated cells. (b) In group N, only a few sporadic positive cells are seen. (c) In group B, clusters of positive haematopoietic cells were detected, in contrast to group N. These clusters were observed in the diaphysis of the proximal femur.
develops. Furthermore, 8-OHdG %PA was 1.6 ± 0.2% in group N, 2.2 ± 0.4% in group A, 4.8 ± 0.4 in group B and 5.1 ± 0.5 in group C, representing a significant increase in groups B and C compared with groups N and A (P < 0.001) (Fig. 2). On the other hand, the difference between groups B and C was not significant, despite the development of osteonecrosis in 70% of the animals in the latter group.

Discussion
Knowledge of the timing of steroid-induced osteonecrosis development would be extremely useful in elucidating the pathogenetic mechanisms and devising prophylactic countermeasures.

Kubo et al. [9] and Sugano et al. [10] used MRI to investigate the period of development of steroid-induced osteonecrosis in man and found that in early cases it developed within a few weeks after the administration of large doses of steroids. In our model, at 5 days after steroid administration, when histopathologically osteonecrosis was not apparent, haematopoietic cells in bone marrow already showed evidence of marked oxidative injury. This is consistent with the period of enhanced vascular permeability occurring in the proximal femur in this model [5]. These findings suggest that quite early after steroid administration ischaemic events occur in bone, creating an environment that favours the development of osteonecrosis. Moreover, the fact that there was no significant difference in the development of oxidative injury between groups B and C despite the 70% incidence of osteonecrosis in the latter group suggests that haematopoietic cell oxidative injury developing before osteonecrosis becomes a cause rather than a result of this condition.

In general, mitochondrial DNA is particularly susceptible to oxidative injury [11], and the following series of events is known to occur. First, the mitochondrial membrane potential decreases, permeability transition pores that are present on the membrane expand, and cytochrome C is released, resulting in activation of apoptosis and cell death [12, 13]. This is consistent with the present study, in which bone haematopoietic cells sustained oxidative injury soon after steroid administration. Kabata et al. [6] have already reported on the role of apoptosis in steroid-induced osteonecrosis and characterized bone haematopoietic cell apoptosis as the stage preceding osteonecrosis.

When steroids are administered, breakdown of in vivo antioxidant mechanisms leads to tissue peroxidation and protein modifications, promoting the development of oxidative stress and oxidative injury in bone tissue soon thereafter. This in conjunction with the influence of multiple factors that have been reported hitherto, including fat embolism [14–16], and adipocyte hypertrophy [17] is considered to induce apoptosis in bone, thereby promoting the development of bone and bone marrow necrosis.

Recently, the efficacy of lipid-lowering agents (lovastatin [18] and probucol [19]) and reduced glutathione [5] in the prevention of
FIG. 2. Development rate of osteonecrosis and 8-OhdG %PA in each group. In this model osteonecrosis occurred by 14 days after steroid administration in 70% of animals (group C). From the changes in 8-OhdG %PA, it can be seen that significant oxidative injury had already occurred before histopathological confirmation of the development of osteonecrosis (by 5 days after steroid administration; group B) ($P<0.001$).
steroid-induced osteonecrosis has been reported in animal models. The fact that these agents exert an antioxidant effect further implicates bone oxidative injury in the development of steroid-induced osteonecrosis. The administration of prophylactic agents timed so as to coincide with the period of greatest susceptibility to steroid-induced injury is thought to be important, with clinical application of this concept anticipated in the near future.

The authors have declared no conflicts of interest.

References