

## Increased Osteocyte Apoptosis during the Development of Femoral Head Osteonecrosis in Spontaneously Hypertensive Rats

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We investigated the presence of osteocyte apoptosis in the necrotic trabeculae of the femoral head of spontaneously hypertensive rat (SHR) using the *in situ* nick end labeling (TUNEL) method and transmission electron microscopy. The occurrence of osteonecrosis and ossification disturbance was significantly higher in SHR compared with Wistar Kyoto (WKY) rats, and Wistar (WT) rats used as control animals ( $P < 0.01$ ). A high population of TUNEL positive osteocytes was detected mainly in 10- and 15-week-old SHRs. Sectioned examination of the femoral head of SHRs and WKY rats by electron microscopy revealed apoptotic cell appearances such as aggregation of chromatin particles and lipid formation. In contrast, a positive reaction was significantly lower in osteocytes in the femoral heads of WT rats ( $P < 0.01$ ). Our results indicate that apoptosis forms an important component of the global pathologic process affecting the femoral head of SHR, which leads to osteonecrosis in this region.

**Key words:** apoptosis, spontaneously hypertensive rat, osteonecrosis of the femoral head

A number of studies have examined the pathogenic mechanisms of osteonecrosis in the femoral head. The vascular theory presents the most widely accepted etiologic mechanism of this condition. Jacobs (1) suggested that the underlying mechanism of osteonecrosis of the femoral head might be damage or abnormality of the reticular vessels. Clinically, osteonecrosis of the femoral head is closely associated with several factors, such as chronic alcoholism, smoking (2), sickle cell disease (3),

decompression sickness, and corticosteroid therapy for either collagen disease or renal and cardiac transplantation (4, 5). These conditions are thought to be associated with increased intraosseous pressure (6), intravascular coagulation, or fat embolism in the bone (7). Although it has not been elucidated, a similar mechanism might be applied for osteonecrosis secondary to alcoholism.

The spontaneously hypertensive rat (SHR) (8) is produced by selective inbreeding of the Wistar (WT) rat, which has spontaneous hypertension. The Wistar Kyoto (WKY) rat is produced by selective inbreeding of SHR which does not have hypertension. In addition, SHR is reported to be a model of Perthes' disease (9) and frequently shows ossification disturbance in the femoral head (10). Iwasaki *et al.* (9) proposed 2 causes of osteonecrosis and ossification disturbance in SHR, the first relates to dysfunction of the endocrine system, while the second involves blockade of the vascular bed, which is indispensable for the ossification of epiphysis. We hypothesized that these conditions might cause osteocyte apoptosis leading to bone necrosis.

Apoptosis is an important process in various pathological conditions, such as post-irradiation cell death (11), stimulation by glucocorticoid (12), withdrawal of glucocorticoid (13), and changes induced by growth hormones (14). In the present study, we hypothesized that the pathological process in the femoral head of SHR also includes apoptosis of osteocytes followed by osteonecrosis (15). For identification of apoptotic osteocytes in the necrotic area of the femoral head in growing SHR and control rats, we used the *in situ* nick end labeling (TUNEL) method and transmission electron microscopy. Our results should throw new light on the elucidation of

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the pathomechanism of osteonecrosis.

## Materials and Methods

**Tissue preparation.** Five-week-old male SHR ( $n = 5$ ), WKY ( $n = 5$ ), and WT ( $n = 5$ ), and 10-week-old rats of same types ( $n = 15$ ) were purchased from Charles Riv. Inc. (Yokohama). Five rats of the 10-week-old SHR, WKY, and WT types were maintained until they were 15-week-old, and another 5 rats of each type were maintained until they were 20-week-old. All animals were fed an oriental stock chow diet (MF, Oriental Yeast Co., Tokyo) at the Laboratory Animal Center for Biochemical Research, Okayama University Medical School under good conditions. Ultimately we obtained 5, 10, 15 and 20-week-old SHR, WKY and WT. After their body weight was measured, they were sacrificed by inhalation of diethyl ether. The femoral heads were fixed with 2% paraformaldehyde and 2% glutaraldehyde mixture in 0.1 M phosphate-buffered saline (PBS). Adipose tissue was removed using a mixture of chloroform and methanol (1:1) and decalcified in 0.24 M EDTA-2Na and 0.22 M EDTA-4Na for 1 week at 37 °C before paraffin embedding. The paraffin sections were mounted on poly-L-lysine-coated slides. They were stained with hematoxylin and eosin, or TUNEL method.

**Classification of histological findings in hematoxylin and eosin stained sections.** The pathological feature of epiphysis of the femoral head, in which histological ossification occupies less than 70% of the head, was determined to be "ossification disturbance". The pathological findings in the femoral head were classified into the following 5 categories (Fig. 1): A) no ossification, B) ossification disturbances without osteonecrosis, C) ossification disturbances with osteonecrosis, D) abnormal ossification with osteonecrosis, and E) normal ossification without osteonecrosis.

**Detection of apoptosis by TUNEL method.** TUNEL staining was carried out using the method described previously (16). Briefly, the paraffin embedded sections were incubated twice in a xylene bath for 10 min, hydrated with serial dilutions of ethanol (70% to 90%), and immersed in double-distilled water. The samples were treated with 0.1% trypsin diluted with 0.01 M PBS for 30 min at room temperature to facilitate the following enzymatic reactions. They were incubated with 0.3% H<sub>2</sub>O<sub>2</sub> that had been diluted with methanol for 30 min at room temperature to block endogenous peroxidase activity.

Each section was rinsed in 0.01 M PBS and immersed in terminal deoxynucleotide transferase (TDT) buffer (140 mM sodium cacodylate, and 1 mM cobalt chloride in 30 mM Trizma base, pH 7.2), TDT (0.3 unit/ $\mu$ l) (Boehringer Mannheim, Germany), and biotinylated dUTP (Boehringer Mannheim) or digoxigenin-rodamine at 3'-OH ends of double-stranded DNA breaks (Boehringer Mannheim) at 37 °C for 40 min. After washing PBS, ABC (Vectastain *elite*, Vector Laboratories Inc, Burlingame, CA, USA) was allowed to react for 30 min at room temperature. After washing, the sections were immersed in 3,3'-diaminobenzidine (Nichirei, Tokyo, Japan) H<sub>2</sub>O<sub>2</sub> (0.01%) for 5 min to develop reaction products and counter stained with methyl green.

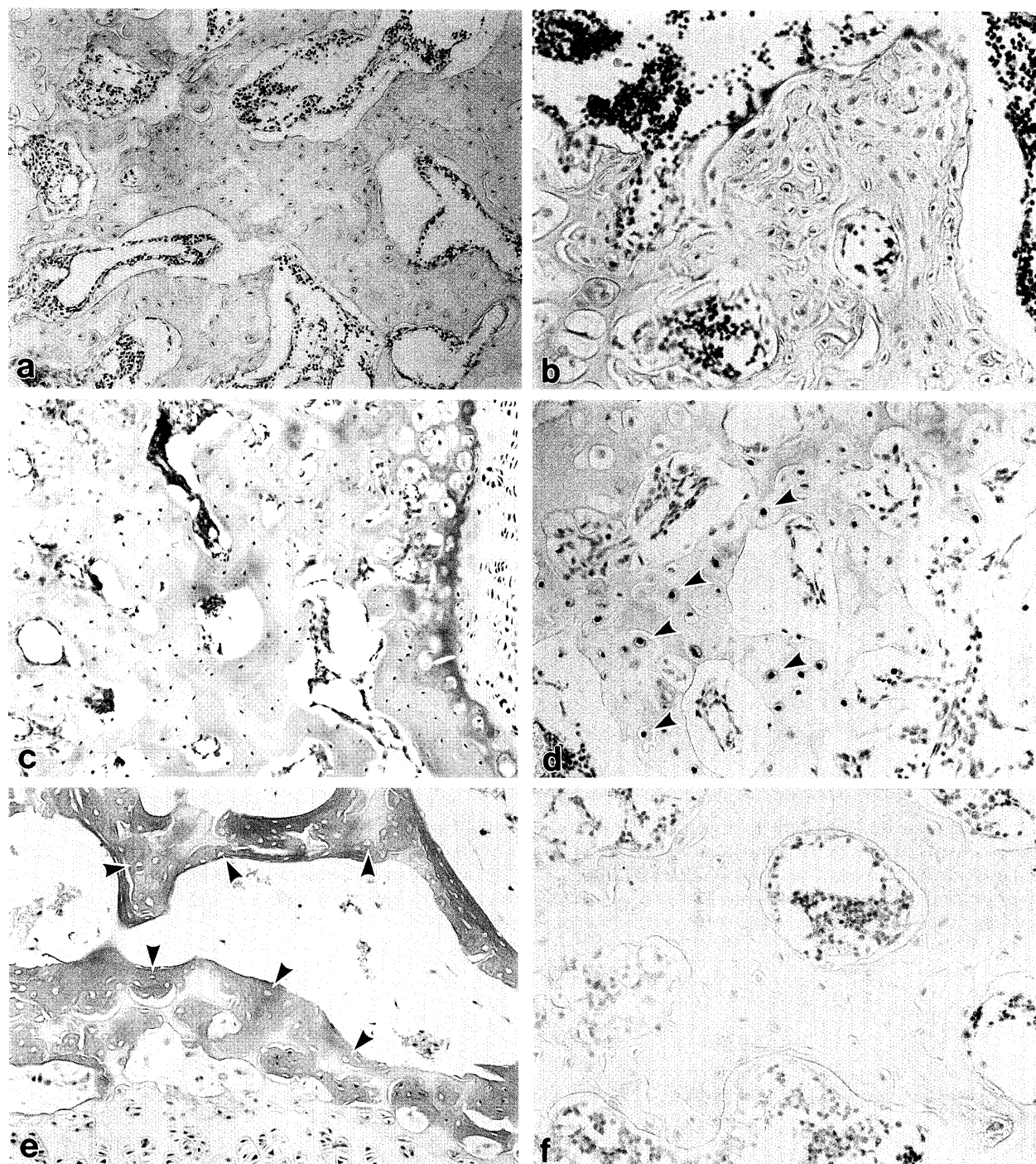
**Transmission electron microscopy.** Small pieces (1 × 1 × 1 mm) of the femoral head containing the necrotic area were immersion-fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS. After being rinsed in 0.1 M PBS containing 5% sucrose, the tissue pieces were stained with 1% OsO<sub>4</sub> aqueous solution, embedded in epoxy resin, and cut into ultrathin sections. The sections were stained with uranyl acetate and lead citrate and examined by a transmission electron microscope (H-7100S, Hitachi, Japan).

**Counting of TUNEL positive osteocytes.** Without knowing the type of epiphysis, we accurately counted the number of TUNEL positive osteocytes using a light microscope which was fitted with a special eyepiece containing a 1 square cm grid. The averages of cell numbers in 3 randomly selected areas (250 × 250  $\mu$ m) located within the trabeculae of the epiphysis were used for statistical analysis. They were evaluated by means of one way functional ANOVA (analysis of variance) and Fisher's analysis;  $P < 0.05$  was considered significant.

## Results

**Time course of osteonecrosis and ossification disturbance in SHR and control rats (Table 1).** The femoral heads from SHR, WKY and WT rats were classified into 5 types according to histological appearances. In 5-week-old SHR and WKY rats, no ossification was detected in the femoral head (type A) (Fig. 1A). In 5-week-old WT rats, 3 heads among 10 heads showed ossification disturbance without osteonecrosis (type B) (Figs. 1B, 2a and 2b). In 10-week-old WKY rats, no ossification disturbance (type D and E) (Fig. 1D, 1E) was detected in 8 of 10 femoral





**Fig. 2** Photomicrographs of the epiphysis of the femoral head in a representative 10-week-old (a-d) and 20-week-old (e, f) spontaneously hypertensive rat. **a**, HE stain of the ossified area of type B head; **b**, *in situ* nick end labeling (TUNEL) stain of the same area with (a) in a serial adjacent section counterstained with methyl green. Histological features were not different from those of Wistar Kyoto (WKY) and Wistar rats; **c**, HE stain of the ossified area of type C head; **d**, TUNEL stain of the same area with (c) in a serial adjacent section counter stained with methyl green. Note that > 20% of osteocytes are TUNEL-positive (arrowhead); **e**, HE stain of type D head. Abnormal ossification was seen with empty lacunae (arrowhead) in trabeculae of epiphysis; **f**, TUNEL stain of type E head. Note the TUNEL positive osteocytes in the trabeculae rarely seen. Histological features were not different from those of WKY rats. Original magnification, a, c, e,  $\times 160$ ; b, d, f,  $\times 320$ . HE stain, see legend to Fig. 1.

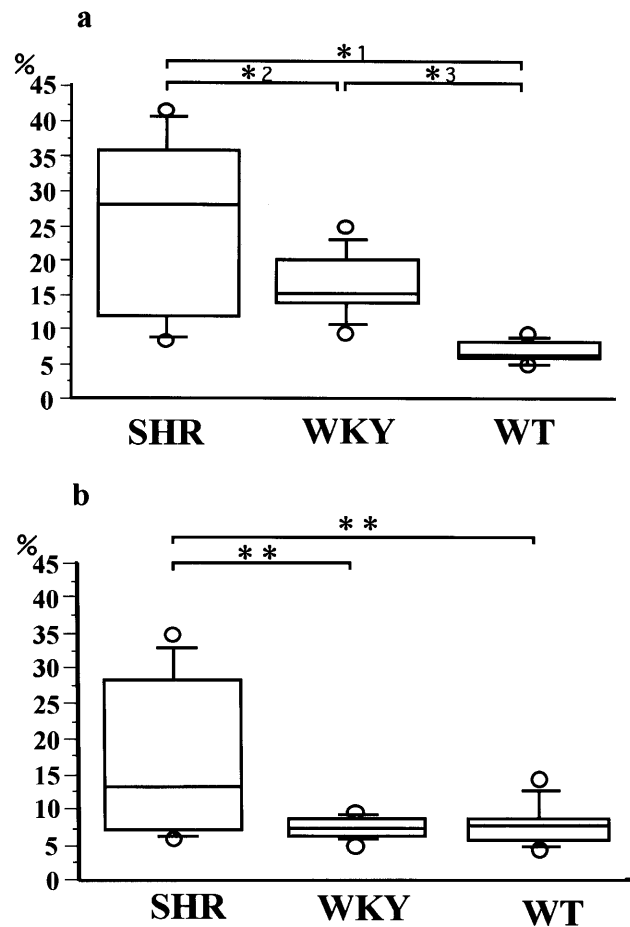
heads; the remaining 2 heads showed type B. In contrast, among 10-week-old SHR normal ossification (type E) (Fig. 2f) was noted in only 2 femoral heads; of the other heads, type A appeared in 4; type B in 2, and ossification disturbance with osteonecrosis (type C) (Figs. 1C, 2c and 2d) in 2 heads. In 10-week-old WT rats, no osteonecrosis was seen; among them 6 femoral heads showed type E. In 15-week-old SHR, osteonecrosis was noted in 7 of 10 femoral heads (types C and D); one of type C showed complete osteonecrosis with empty lacunae. In 15-week-old WKY, type B was noted in 1 head, while type D (Fig. 2e) was present in 2 heads. The remaining 7 heads showed type E. In 15-week-old WT rats, type C was noted only 1 head. The remaining 9 heads showed type E. In 20-week-old SHR, 2 heads showed type C; and types D and E were found in 8 of 10 heads. In WKY and WT rats of the same age, ossification disturbances were not observed in any of the 10 femoral heads. Only 1 head of WKY in type D included a necrotic area.

**Detection of apoptosis by TUNEL.** In the ossified area (types C, D and E), at 10-weeks, the number of TUNEL positive osteocytes was not significantly different among the 3 kinds of rats. At 15 weeks, the number of TUNEL positive osteocytes in SHR was significantly higher than that in both WKY and WT rats (Fig. 3a;  $^{*1}P < 0.01$ ,  $^{*2}P = 0.019$ ). The number of TUNEL positive osteocytes in WKY was also significantly higher than that of WT ( $^{*3}P = 0.011$ ). At 20 weeks, the number of TUNEL positive osteocytes in SHR was significantly higher than that in both WKY and WT rats (Fig. 3b;  $^{**}P < 0.05$ ).

**Transmission electron microscopy.** Transmission electron microscopy revealed various stages of osteocyte apoptosis with necrotic trabeculae, chromatin condensation, and nuclear fragmentation (Fig. 4). Apoptosis of osteocytes was not detected in areas with new bone formation. There were no apoptotic cells in sections of the epiphysis that did not contain necrotic areas (types A, B and E).

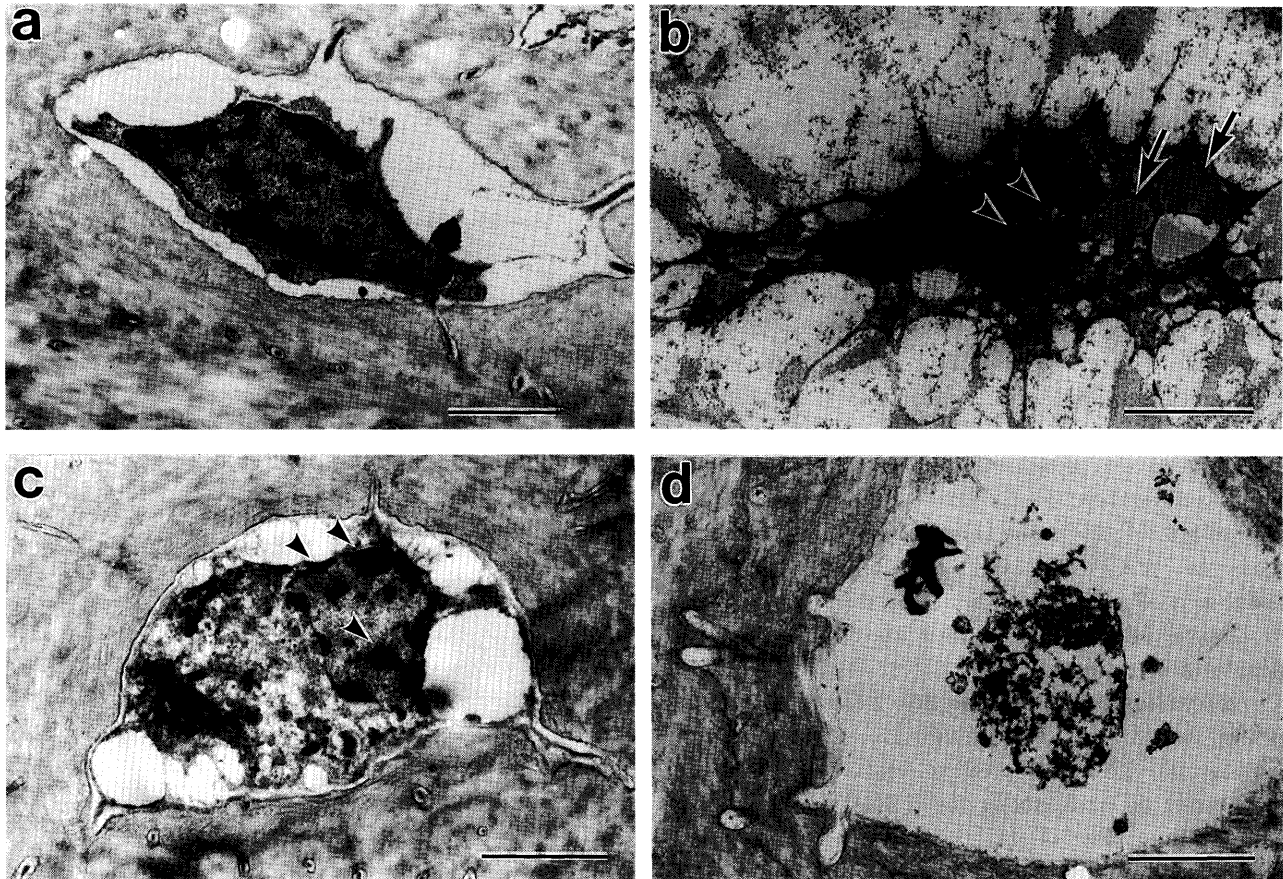
## Discussion

In the present study, we examined the apoptosis of osteocytes of the femoral head in SHR, a useful model of Perthes' disease with osteonecrosis and ossification disturbance in the femoral head (9). We used the TUNEL method to detect apoptosis, noting earlier uses of the



**Fig. 3** The population of *in situ* nick end labeling (TUNEL)-positive osteocytes. **a**, 15 week-old rats. The number of TUNEL positive osteocytes in spontaneously hypertensive rat (SHR) was significantly higher than that in both Wistar Kyoto (WKY) and Wistar (WT) rats ( $^{*1}P < 0.01$ ,  $^{*2}P = 0.019$ ). At 15 weeks, significantly higher population of TUNEL positive osteocytes was seen in all 10 femoral heads of WKY compared with WT ( $^{*3}P = 0.011$ ); **b**, 20 week-old rats. The number of TUNEL positive osteocytes in SHR was significantly higher than that in both WKY and WT rats ( $^{**}P < 0.05$ ).

same method (16–18). TUNEL has been criticized as being non-specific for apoptosis since several other forms of DNA damage could also be detected by TUNEL (19). Because of this criticism, we also used electron microscopy to confirm the presence of apoptosis. Electron microscopy is an acceptable method for identification of apoptotic cells and has also been used in several similar studies (20, 21). The major finding of the present study was the presence of a large number of apoptotic



**Fig. 4** Electron micrographs of apoptotic osteocytes in the epiphysis of the femoral head of a representative 10-week-old spontaneously hypertensive rat (Figs. 1C, 1D) **a**, a normal osteocyte showing scattered chromatin within nucleus; **b**, nuclear segmentation (arrowhead) and lipid formation (arrow) in the cytoplasm; **c**, apoptotic osteocyte showing chromatin condensation (arrowhead) and paucity cytoplasm; **d**, cell debris within the pericellular matrix. a-d, bar = 3  $\mu$ m.

osteocytes around necrotic areas, mainly in the femoral head of 15- and 20-week-old SHR. The same pathology was also found, though in smaller amounts, in 15- and 20-week WKY and WT. Our results suggest that the apoptosis of osteocytes may be an important process in the femoral head of SHR, leading to the osteonecrosis and destruction of bone structure.

There is very little available information on the mechanism of apoptosis of osteocytes *in vivo* and *in vitro*. Glucocorticoid administration not only decreases the bone formation rate and bone mineral density (BMD) but also increases apoptosis of mature osteoblasts and osteocytes (22). Noble *et al.* (23) indicated that in normal and pathological conditions, apoptosis of osteocytes in both adults and children might reflect the modeling and remod-

eling activity of the osseous tissue. Interestingly, the distribution of potentially apoptotic cells in infants and pathological bone was not uniform, suggesting a functional relationship between bone turnover and controlled cell death of osteocytes. In the same study, apoptotic changes were detected in lacuna osteocytes in rapidly modeling or remodeling human bone, but were almost absent in the adult bone. In our study, TUNEL positive osteocytes were present in the necrotic area of the epiphyseal nucleus. These results suggested that apoptosis of osteocytes might be closely associated with the process of modeling or remodeling of the bone.

Several studies have emphasized the role of vascular pathology in the femoral head in SHR. In particular, microangiographic and histologic examinations have

shown that interruption of blood flow outside the epiphyseal nucleus causes osteonecrosis of the femoral head in SHR (10). The lateral epiphyseal vessels (LEVs) in SHRs mainly supply the epiphyseal nucleus of the femoral head. The anastomosing branches of LEVs between the epiphysis and femoral neck are scarce even in the normal femoral head. Furthermore, the branches of LEVs in growing SHRs frequently show marked segmental stenosis and obstruction (10). Nishimura (24) reported that osteonecrosis in SHR is caused to a large extent by obstruction of LEVs probably following abnormalities of the femoral head cartilage induced by mechanical stress. Because osteocytes are versatile cells, they undergo changes in response to environmental changes, such as ischemia or increased blood flow. Usui *et al.* (25) have shown that ischemic hypoxia may result in a temporary injury of osteocytes. They reported that the principal morphological changes of ischemia in osteocytes are pyknosis of nuclei followed by the appearance of empty lacunae; there may be at least 2 types of necrotic processes in osteocytes that eventually lead to cell death. Based on our results, it is possible that ischemic changes caused by vascular abnormalities in SHR might induce apoptosis of osteocytes either directly or indirectly.

SHR is known to exhibit a variety of hormonal imbalances. Amador *et al.* (26) reported the presence of high plasma levels of prolactin and follicle-stimulating hormone and low levels of plasma testosterone in SHR. Other reports described low serum concentrations of ionized calcium (Ca) (27), diminished intestinal Ca absorption (28), increased levels of serum immunoreactive parathyroid hormone (PTH) (29), and abnormal metabolism of vitamin D (28) in SHR as compared with WKY. These abnormalities could have an effect on the process of bone modeling; they could also result in osteocyte apoptosis. Tomkinson *et al.* (30) suggested that estrogen might play a role in the control of osteocyte apoptosis. The disturbance of ossification observed in SHR may also correlate with these metabolic abnormalities.

In conclusion, we provided histological and electron microscopic evidences for apoptosis of osteocytes in the femoral head of SHR. The population of apoptotic osteocytes was significantly higher than that found in control rats at an age of 15 and 20 weeks. Our results could contribute to the development of new therapeutic strategies for treating idiopathic osteonecrotic disease.

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