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Immunohistochemical localization of Nox1, Nox4 and Mn-SOD in mouse femur during endochondral ossification

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ABSTRACT

Enzymes synthesizing reactive oxygen (Nox family) have recently been identified. Elucidation of the production mechanism has been initiated, and the involvement of reactive oxygen in metabolism, intracellular transport, signal transmission and apoptosis has been reported. We immunohistochemically investigated expression and localization of the Nox family in endochondral ossification using a normal mouse femur. Weakly positive reactions with Nox1, Noxa1, and Noxo1 were observed in the zones of proliferative and prehypertrophic chondrocytes at 3 weeks of age. Nox4 was widely positive from the resting over the hypertrophic cell zone. At 18 weeks of age, none of the Nox types was expressed in chondrocytes as the zones disappeared. On the other hand, positive reactions with Nox1, Noxa1, Noxo1, and Nox4 were observed in osteoblasts in the zone of ossification at 3 weeks of age, and each Nox was also positive in osteoblasts arranged on the bone marrow side in the epiphyseal cartilage at 18 weeks of age. In addition, a reactive oxygen-eliminating enzyme, Mn-SOD, was observed only in prehypertrophic chondrocytes at 3 weeks of age, and not detected in osteoblasts. It was suggested that the Nox family is closely associated with endochondral ossification of the mouse femur, and Nox1 and Nox4 are closely involved in the chondrocyte maturation process and bone matrix formation.

1. Introduction

Seven homologs have been identified in the Nox family, reactive oxygen-synthesizing enzymes (Nox1, Nox 2, Nox 3, Nox 4, Nox 5, Duox 1 and Duox 2) (Geiszt, 2006; Bedard and Krause, 2007). Noxa1 (Nox activator 1) and Noxo1 (Nox organizer 1) are essential for reactive oxygen production through Nox1 (Bedard and Krause, 2007). Different homologs are expressed depending on the organs, tissues, and cells, and investigation of the types and functions of enzymes expressed in various tissues is underway (Krause, 2004; Geiszt, 2006). Regarding chondrocytes, reactive oxygen production and the induction of apoptosis have been shown in vitro (Kim et al., 2010). However, there has been no report on the Nox family involved in reactive oxygen synthesis in cartilage tissue.

The longitudinal growth of long bone is regulated by the endochondral ossification of epiphyseal cartilage. In this endochondral ossification process, chondrocytes proliferate and differentiate, osteoclasts enter the region and are activated, and osteoblasts add bone matrix (Kronenberg, 2003; Goldring et al., 2006). Although the involvement of reactive oxygen synthesis in osteoclasts in differentiation and functional expression has been suggested (Sasaki et al., 2009), reactive oxygen synthesis in chondrocytes in endochondral ossification or its function has not been analyzed. It has been reported that Nox was expressed in cultured chondrocytes (ATDC5) and associated with the differentiation of cultured cells, and the apoptosis of cultured chondrocytes was induced by a decrease in Nox (Kim et al., 2010). Regarding in vivo studies, Nox family expression in the embryonic period (Dong et al., 2010) and Nox expression in ataxia telangiectasia (Morita et al., 2007) have been investigated, but no study has been performed on Nox family expression in the in vivo endochondral ossification process or reactive oxygen and its functional significance and intercellular regulatory system in various cells associated with the process, such as chondrocytes, osteoblasts, and osteoclasts. Furthermore, there has been no report on the expression of a reactive oxygen-eliminating enzyme (superoxide dismutase (SOD)) with the Nox family in the endochondral ossification.

To identify reactive oxygen-producing cells and their function in the endochondral ossification process, we immunohistochemically

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investigated the Nox family and Mn-SOD expression localization with growth of the mouse femur.

2. Materials and methods

The femurs of C57BL/6J mice each at 3 and 18 weeks of age (Charles River Japan) were used. Mice were maintained following the Ohu University Animal Experiment Regulation (approval 2009–18).

Under anesthesia by intraperitoneal pentobarbital injection, the thorax was opened and saline was perfused through the left ventricle. The femur was excised after perfusion fixation with 4% paraformaldehyde solution (pH 6.2). After 24-h fixation in the same fixative, the bone was decalcified in 10% EDTA solution (pH 7.0, 4 °C, 3 months) and paraffin-embedded, and 5 μm-thick serial sections were prepared using a microtome.

The sections were deparaffinized and treated with 0.1% glycine, 0.1% sodium azide solution for 1 h to inactive endogenous peroxidase. The sections were then blocked with 10% goat, rabbit, horse serum (VECTASTAIN® Elite ABC Kit, VECTOR Lab., USA) for 20 min, followed by reaction incubation with the primary antibody. The primary antibodies used were rabbit anti-human NOX1 polyclonal antibody (Antagene, Inc., USA), rabbit anti-mouse NOXA polyclonal antibody (Santa Cruz Biotechnology Inc., CA, USA), rabbit anti-human NOXO polyclonal antibody (Rockland Inc., USA), goat anti-human Nox4 (Santa Cruz Biotechnology Inc., CA, USA), and mouse anti-Mn-SOD (CHEMICON International Inc., CA, USA). These were reacted for 10 h. In the secondary antibody reaction, biotinylated anti-rabbit, goat, mouse immunoglobulin (VECTASTAIN® Elite ABC Kit, VECTOR Lab., USA) was reacted at room temperature for 30 min, followed by reaction with peroxidase-labeled streptavidin (VECTASTAIN® Elite ABC Kit, VECTOR Lab., USA) for 30 min. Immunoreactivity was visualized with 0.3% H_2O_2-containing 0.05% 3.3-diaminobenzidinetetra-hydrochloride (DAB) solution (0.05 M Tris–HCl buffer, pH 7.6), the nuclei were stained with 5% methyl green (Muto pure chemicals Co., Ltd., Tokyo, Japan), and the sections were observed under a light microscope. For washing and dilution of antisera, 0.05 M Tris–HCl buffer (pH 7.6) was used.

In the negative control reaction, normal goat serum (DAKO Corp., CA, USA) was used instead of the primary antibody.

3. Results

3.1. Epiphyseal cartilage in 3-week-old mice

In the femoral epiphyseal cartilage, the resting cell zone, proliferative cell zone in which many flat cells were present, and hypertrophic cell zone in which the chondrocyte morphology changed to a cube were observed, showing the endochondral ossification stages (Figs. 1–3). Reaction with anti-Nox1 antibody was negative in chondrocytes in the resting cell zone, but Nox1-positive cells were diffusely present in the proliferative over the prehypertrophic cell zone in which the chondrocyte morphology changed from flat to cubic (Figs. 1a and 2a). In contrast, no chondrocyte was positive for Nox1 in the hypertrophic cell zone, but strong Nox1 positivity was observed in osteoclasts in the zone of ossification forming bone matrix, and some osteoblasts were weakly positive for Nox1 (Figs. 1a and 3a). Some chondrocytes in the resting cell zone were positive for Noxa1, and many cells were Noxa1-positive in the proliferative and prehypertrophic cell zones, whereas no cell was Noxa1-positive in the hypertrophic cell zone (Figs. 1b and 2b). In the zone of ossification, positive reaction with Noxa1 was noted in many osteoblasts (Figs. 1b and 3b). Noxo1 reactivity was similar to those of Nox1: Noxo1-positive chondrocytes were present in the proliferative over the prehypertrophic cell zone, whereas no positive cell was noted in the resting or hypertrophic cell zone (Figs. 1c and 2c). In the zone of ossification, Noxo1 positivity was noted in osteoblasts and osteoclasts (Figs. 1c and 3c). On immunostaining of Nox4, many chondrocytes were positive in endochondral ossification, unlike Nox1, Noxa1 and Noxo1: chondrocytes diffusely present in the resting cell zone and chondrocytes with cubic or large cytoplasm in the prehypertrophic and hypertrophic cell

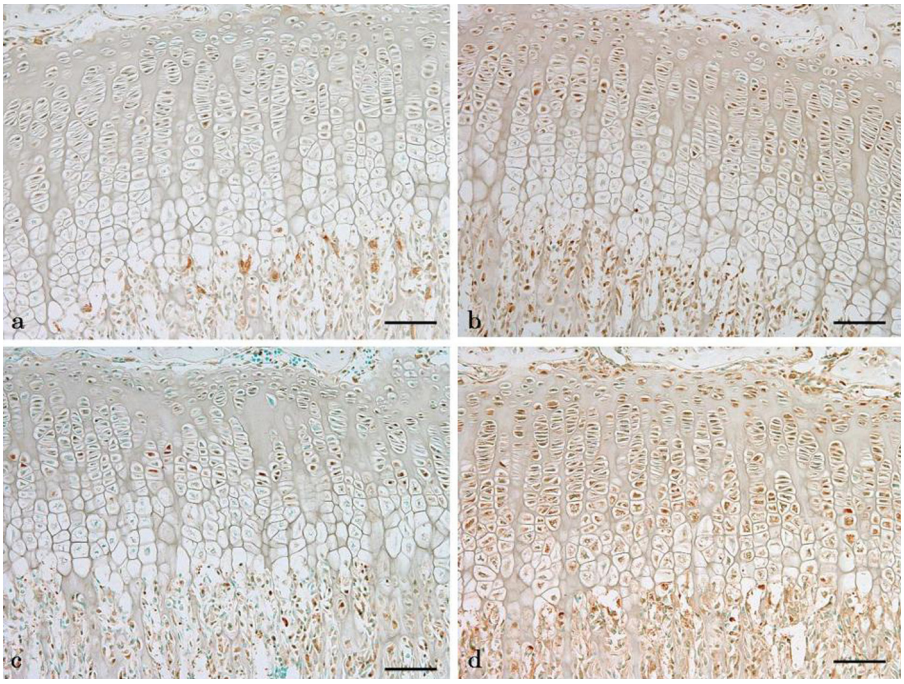

Fig. 1. Immunostaining of the mouse femur at 3 weeks of age with anti-Nox antibodies. (a) Nox1, (b) Noxa1, (c) Noxo1: positive reaction was noted in some chondrocytes, and positive cells were also present in the zone of ossification. (d) Nox4: positive reaction was noted in many cells in the resting cell zone through zone of ossification. Bar = 100 μm.