The immunohistochemical localization of superoxide dismutase activity in the avian epithelial growth plate

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Summary

Superoxide dismutase (SOD) is a 'scavenger' enzyme which catalyses the dismutation (reduction–oxidation) of the superoxide anion (O2⁻), a toxic free radical generated during normal cellular respiration. Light microscopy employing immunohistochemistry was utilized for localizing SOD activity in the chick epiphyseal cartilage. Antibodies to mammalian liver CuZn-SOD were prepared and the avidin-biotin-peroxidase technique (ABC complex) was utilized to localize activity for this enzyme in the growth plate cartilage. The localization of enzyme activity varied in accordance with the characteristic zonation pattern of the growth plate (zone of proliferation, zone of maturation, zone of cell hypertrophy and zone of matrix calcification). In the upper regions of the epiphyseal cartilage (the zones of proliferation and maturation), where the vascularity is poor and the oxygen tension low, SOD activity was localized within the chondrocytes. No extracellular activity was observed. However, in the lower regions of the growth plate (the zones of cell hypertrophy and matrix calcification), where both the vascularity and the oxygen tensions are increased, SOD activity was intense in both the chondrocytes and the surrounding extracellular matrix. Thus, the distribution of SOD enzyme activity in this tissue seems to vary in accordance with the level of oxygen present. The significance of the extracellular SOD activity, seen in the lower aspects of the growth plate cartilage, may indicate the sensitivity of matrix components, especially collagen, to toxic free radicals such as the superoxide anion.

Introduction

Superoxide dismutase (SOD) or superoxide oxidoreductase (EC 1.15.1.1) is a major oxygen 'scavenging' enzyme. Thus it is a component of the biological defence mechanism against toxic oxygen species generated during normal cellular respiration (Fridovich, 1975, 1978). For example, SOD catalyses the dismutation of the superoxide anion (O2⁻). The latter is a toxic free radical produced during the monovalent reduction of oxygen. SOD activity appears to be present in most, if not all, differentiated cells (Fridovich, 1978; Freeman & Crapo, 1982). Immunohistochemical studies have reported the presence of this enzyme in a wide variety of mammalian cells and tissues (Thaete et al., 1983, 1985). However, these light microscope studies do not mention the presence of SOD activity in either cartilage or bone. In a preliminary study, we noted the presence of SOD in the chondrocytes and extracellular matrix of the rat epiphyseal growth plate (Davis et al., 1988a). We have been employing electron microscope and light microscope immunohistochemical procedures to localize SOD activity in other tissues as well (Davis et al., 1988b,c,d).

It is well known that extremes in oxygen tension can affect various biochemical processes in both cartilage and bone (Brighton et al., 1969). Because of these data, we were prompted to investigate SOD activity further in vertebrate epiphyseal cartilage, a tissue in which an inherent gradient in oxygen tension is known to exist.

Materials and methods

White Leghorn chicks were obtained from a local hatchery. The animals were raised for 6 weeks from 1 day post-hatching. Chicks were housed in temperature-regulated (72°C) brooders and were exposed to a day–night cycle of
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12 h-12 h. The birds were allowed food and water freely. Food consisted of a commercially available diet (Startena) containing a normal regimen of calcium, phosphorus and vitamin D (cholecalciferol). A total of 12 chicks were used for this study.

Chicks were sacrificed by decapitation. Blood was collected for serum analysis. Serum calcium levels were determined by either atomic absorption spectrophotometry or by serum analysis using the SMAC-28. Both femora were removed from each chick and subsequently cleaned of adhering soft tissue. The proximal femoral heads were removed from each femur. These were immediately sectioned in half and the tissues placed in fixative (see below). While under fixative, each femoral head was further diced into multiple 1.0-3.0 mm-thick sections. The latter were placed in fresh fixative. This tissue preparation generally required less than 2.0 min and was repeated for each chick.

The fixative used was freshly prepared 4.0% formalin buffered with 2.0% calcium acetate (pH 7.5). The total fixation time was 2.0 h; this included several changes to fresh fixatives during this interval. This was followed by a 2.0 h rinse in water to remove excess formalin. Tissues were then dehydrated in a series of graded ethanols and subsequently placed in fresh fixative. This tissue preparation generally required less than 2.0 min and was repeated for each chick.

Since SOD activity is highest in liver tissue (Freeman & Crapo, 1982), the copper-zinc superoxide dismutase (CuZn-SOD) was isolated and purified from the chick liver using the technique of Crapo & McCord (1976). The purity of the enzyme was checked by both gel electrophoresis and enzymic assay (Redmond et al., 1984). Antiserum to chick liver SOD were produced in New Zealand White rabbits and subsequently purified (Crouch et al., 1981; Thaete et al., 1983). Tissue localization was accomplished by a modified immunoenzyme bridge procedure utilizing the avidin-biotin-peroxidase complex (ABC-complex) of Hsu et al. (1981).

Tissue sections were cut at 6.0 μm on a standard microtome. They were mounted on glass slides and subsequently deparaffinized and rehydrated. They were then treated with 3.0% H2O2 for 10 min. This step allows for the inactivation of any endogenous tissue peroxidase activity. Following this step, sections were washed in phosphate-buffered saline (PBS, pH 7.2). Next, they were treated with normal goat serum (diluted 1:5) to block non-specific binding. The sequence involved in the formation of the ABC complex was as follows: (1) treatment of sections with rabbit anti-(chick SOD) diluted 1:800 with PBS, overnight at 4°C; (2) treatment of sections with biotinylated goat anti-(rabbit IgG), diluted 1:50, for 1.0 h at room temperature; (3) this was followed by treatment of the sections with rabbit peroxidase anti-peroxidase complex (Vector Labs, Burlingame, CA, USA) for 1.0 h at room temperature. Several washes in PBS were performed between each step. After this, tissue sections were incubated in a modified Graham–Karnovsky (1966) medium for 15 min at room temperature. The latter consisted of 0.03% 3,3'-diaminobenzidine and 0.006% H2O2 prepared in PBS. Following this, sections were dehydrated, stained with OsO4, and mounted with Permount. Adjacent tissue sections were stained with Haematoxylin and Eosin for routine histological examination. For immunostaining sections, the above procedure was used with the exception that the rabbit anti-(chick SOD) serum was replaced with rabbit preimmune serum. These sections were also stained with OsO4. All specimens were examined and photographed on a Zeiss Photomicroscope III.

Results

Routinely prepared sections, stained with Haematoxylin and Eosin, of the normal chick proximal femoral epiphyseal growth plate are shown in Figs 1 and 2. Four zones, based primarily on the morphology of the chondrocytes, are clearly seen in these sections. These zones are as follows (from epiphysis to metaphysis). (1) The zone of cell proliferation, characterized by the presence of numerous poorly differentiated ‘flattened’ chondrocytes (Fig. 1). (2) The zone of chondrocyte maturation (Fig. 2). Here, the cells have lost their flattened appearance and show some differentiation. (3) The zone of cell hypertrophy (Figs 1 and 2). The chondrocytes in this zone are large, swollen and characterized by their cytoplasmic vacuolation and concomitant degeneration. (4) In the fourth zone (the zone of provisional or matrix calcification), the staining characteristics of the extracellular matrix have changed (Fig. 2). The basophilia seen is indicative of matrix calcification. From these sections it is clearly evident that the avian epiphyseal cartilage is not nearly as well organized as that seen in the various mammals routinely studied.

Figures 3, 4 and 5 show chick growth plates immunostained for the presence of SOD. Figure 3 is a low magnification of the growth plate. Note that the chondrocytes in the upper regions of the cartilage are intensely stained for this enzyme while the extracellular matrix in these areas is devoid of SOD activity. Figure 4 is a higher magnification of the lower portion of the growth plate. As in Fig. 3, the cells in the zone of cell proliferation as well as the chondrocytes in the zone of cell maturation show an intense cytoplasmic immunoreaction for SOD. The adjacent matrix again appears free of immunoreactivity. In the lower two