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Hypertrophic Chondrocytes in Avian Growth Cartilage Do Not Die by Apoptosis

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Abstract: Chondrocytes in avian growth cartilage undergo proliferation, hypertrophy and then die by a mechanism that has widely been described as apoptosis. The aim of the current study was to investigate the morphology of dying hypertrophic chondrocytes in duck and quail growth cartilage. Growth cartilage from post-hatching growing ducks and quail was fixed in 10% formalin or 5% glutaraldehyde and processed for light and electron microscopic examination. Growth cartilage from ducks and quail was similar to other avian species; the chondrocytes were organized into three zones; resting, proliferative and hypertrophic. Ultrastructural studies of the tissue specimens from ducks and quail suggested that there were two morphologically different types of hypertrophic chondrocytes; hypertrophic dark chondrocytes with electron-dense cytoplasm and hypertrophic light chondrocytes with electron-lucent cytoplasm. Dark and light hypertrophic chondrocytes were dying by mechanisms morphologically different from apoptosis. Dying hypertrophic dark chondrocytes appeared to undergo extrusion of their contents into the extracellular matrix. Whereas, dying hypertrophic light chondrocytes appeared to disintegrate within the cell membrane. No a typical example of apoptotic death of hypertrophic chondrocytes was observed at any stage of development. From the observations presented in this study, it was concluded that dark and light hypertrophic chondrocytes appeared to be different cell populations with different morphology. These cells showed non-apoptotic physiological modes of death. For improving production, further understanding growth and mechanisms of bone and cartilage diseases in avian, the results reported here should be considered.

Key words: Avian, bone, histology, endochondral ossification, physiological cell death

INTRODUCTION

Avian dyschondroplasia is an important orthopedic disease affecting growing meat type birds causing a high economic loss to the poultry industry (Burton *et al*., 1981). Dyschondroplasia is usually associated with retention of a non-calcified avascular cartilage plug below the growth plate which extends into the metaphsyseal long bone tissue (Orth and Cook, 1994). The exact cause of avian dyschondroplasia is not known, however it has widely been mentioned that it results from a failure in the normal process of endochondral ossification (Poulos, 1978).

Endochondral ossification is the normal process of replacement of the embryonic cartilaginous skeleton with bone tissue and subsequent development of long bones. During embryonic limb development, mesenchymal cells differentiate into chondrocytes which secrete specific collagen type II and proteoglycan-rich cartilage matrix which is organized into a model with a similar shape to that of the future bone. A Primary Ossification Centre (POC) appears in the middle of the cartilage model followed by a Secondary Ossification Centre (SOC) in both extremities. The ossification process expands and the cartilage is gradually replaced by bone except at two sites: the Physeal Growth Cartilage (PGC) or growth plate which is present between the POC and SOC and the Articular Epiphyseal Growth Cartilage (AEGC) between the articular surface and the SOC. By the time skeletal maturity is reached, these centres of ossification completely replace the cartilage except at the articular surfaces (Mackie *et al*., 2008). Although, the growth cartilage is normally avascular, the embryonic cartilage is invaded by many vascular channels known as cartilage canals which gradually chondrify and later disappear in post-hatching growing birds (Lutfi, 1970; Hunt *et al*., 1979). Chondrocytes in growth cartilage are arranged into morphologically distinct zones: resting, proliferative and hypertrophic which reflect dynamic changes in morphology and the function of these cells (Orth and Cook, 1994). The resting zone has small round chondrocytes randomly distributed throughout their extracellular matrix. The proliferative zone contains rapidly dividing flattened chondrocytes arranged in columns

parallel to each other and to the longitudinal axis of the bone diaphysis. In the hypertrophic zone, the chondrocytes are rounded and their volume dramatically increases in association with calcification of the extracellular matrix.

One important step in the pathway of endochondral ossification is the physiological death of hypertrophic chondrocytes that leave empty spaces within the calcified cartilage matrix allowing entry of the invading cells of the ossification front (osteoclasts, osteoblasts and bone marrow cells) along with blood vessels; the end result is the replacement of cartilage remnants by bone tissue (Mackie *et al*., 2008). The mode of death of hypertrophic chondrocytes is a matter of disagreement between researchers. Many researchers have described hypertrophic chondrocytes as dying by apoptosis under physiological circumstances and some have attributed dyschondroplasia in birds to an increase in the number of apoptotic cells in the hypertrophic zone (Rath *et al*., 1998) and others to a decrease in the number of apoptotic cells (Ohyama *et al*., 1997). These studies have based their conclusions on the detection of DNA breaks and other molecular methods however the definitive method of identifying cells undergoing apoptosis is the examination of ultrastructural morphology. Apoptosis has unmistakable features including solid nuclear condensation, cell fragmentation into membrane-bound apoptotic bodies and phagocytosis of these fragments by other cells (Kerr *et al*., 1972; Ahmed *et al*., 2007a).

Shapiro *et al*. (2005) introduced the hypothesis that hypertrophic chondrocytes die by autophagic cell death which is characterized by digestion of the cytoplasmic contents with increasing numbers of lysosomes. More recently, researchers have provided evidence that hypertrophic chondrocytes die by mechanisms involving neither apoptosis nor autophagy (Ahmed *et al*., 2007b; Chen *et al*., 2010a). Researchers have proposed that the two populations of hypertrophic chondrocytes, light (with electron-lucent cytoplasm) and dark (with electron-dense cytoplasm (Wilsman and Van Sickle, 1971; Hwang, 1978; Wilsman *et al*., 1981; Carlson *et al*., 1985) which also differ in their gene expression die by morphologically distinct, cell type-specific modes of physiological cell death (Ahmed *et al*., 2007a). The dark chondrocytes die by a process involving extrusion of their cytoplasmic contents into the extracellular matrix while the light chondrocytes undergo progressive digestion of cytoplasmic contents within the plasma membrane as they die.

The aim of the current study was to investigate the morphology of hypertrophic chondrocytes undergoing physiological death in non-mammalian species. Researchers have chosen to study the growth cartilage of two avian species, ducks and quail to understand the biology of growth in such species and subsequently assist in the understanding of the mechanisms of developmental orthopedic diseases of birds, especially dyschondroplasia. Researchers hypothesized that in birds as in mammals, hypertrophic light and dark chondrocytes exist in the growth cartilage and die by non-apoptotic modes of physiological cell death.

MATERIALS AND METHODS

Sample collection: Ten apparently healthy post-hatching Muscovy ducks (1, 7, 15 and 30 days) collected from a commercial farm in Assiut city, Egypt and 20 apparently healthy post-hatching (1, 7, 15, 30 and 45 days) Japanese quail raised in the quail research centre belonging to the Histology Department, Faculty of Veterinary Medicine, South Valley University were used for the current experiments. The birds were euthanized using ether inhalation and then the humerus and femur were carefully dissected without touching the articular surfaces with the scalpel and washed thoroughly in phosphate-buffered saline.

Specimen processing for light and electron microscopy: Cartilage with some adjacent bone was excised and fixed in either 10% formalin (pH; 7.4) or 5% glutaraldehyde/4% paraformaldehyde in buffered phosphate saline at 4°C for 3 days. The specimens were decalcified in 0.33 M Ethylene-Diamine-Tetra-Acetic Acid (EDTA). For light microscopy, formalin-fixed specimens were embedded in paraffin using standard methods. Paraffin sections (5-7 μm) were taken and stained with hematoxylin and eosin for general examination of the growthcartilage. For electron microscopy, glutaraldehyde/ paraformaldehyde-fixed specimens were postfixed in 1% osmium tetroxide and embedded in Spurr's resin. Semi-thin sections (0.5-1 μm) were stained with methylene blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and Reynold's stain and examined under a transmission electron microscope (JIOL, 1010).

RESULTS AND DISCUSSION

Histology of growth cartilage of the post-hatching ducks and quail: The chondrocytes of the duck and quail growth cartilage in all samples examined were organized into three zones; resting, proliferative and hypertrophic (Fig. 1A). The resting cells were rounded and randomly distributed through abundant extracellular matrix (Fig. 1B). The proliferative chondrocytes were flattened and organized

into columns parallel to the long axis of the bone (Fig. 1C). The hypertrophic chondrocytes were rounded and either completely or partially filling the lacunae (Fig. 1C). The hypertrophic chondrocytes appeared to degenerate with increasing proximity to the cartilage-bone interface (Fig. 1D) where lacunae were opened and fused together, allowing invasion by vascular channels containing osteoblasts and osteoclasts (Fig. 1F). Two morphologically different types of hypertrophic chondrocytes could be identified when examining semi-thin sections; dark (dense-stained cytoplasm) and light (pale-stained cytoplasm) chondrocytes (Fig. 1E). During the cartilage development in 1 day post-hatching birds, the cartilage matrix contained cartilage canals (Fig. 1A) which gradually disappeared after 30 days with decreased cartilage thickness.

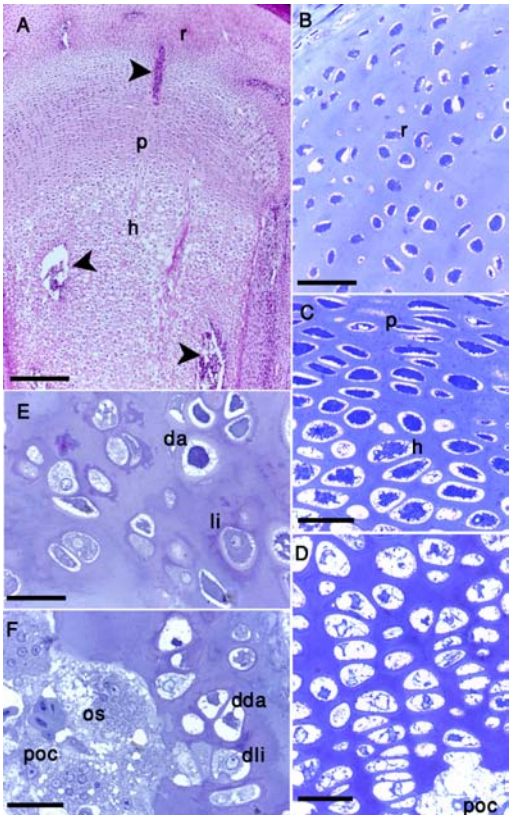

Fig. 1: Morphology of avian growth cartilage; A) Light micrographs of paraffin sections from the growth cartilage from the proximal end of the humerus of a 1 day post-hatching quail stained with hematoxylin and eosin (B-F) and semi-thin-sections from the growth cartilage from the proximal end of the humerus of a 1 day post-hatching ducks stained with methylene blue; A) Zones of growth cartilage; resting (r), proliferative (p) and hypertrophic (h), and cartilage canals (arrowheads); B) Resting chondrocytes (r); C) Proliferative (p) and hypertrophic chondrocytes (h); D) Dying hypertrophic chondrocytes close to Primary Ossification Centre (POC); E) Light (li) and dark (da) hypertrophic chondrocytes; F) Dying hypertrophic light (dli) and hypertrophic dark (dda) chondrocytes close to primary ossification centre (poc); note osteoclasts (os). Bars = 50 μm in A. Parts B-F have the same magnification; bars = 5 μm

Morphological investigation of physiological cell death of hypertrophic chondrocytes in avian growth cartilage: Ultrastructural studies were conducted on specimens of growth cartilage from the cartilage-bone border area of the humerus and femur of post-hatching ducks and quail from 1-45 days and no morphological differences were observed in specimens from humerus or femur. Researchers have observed two morphologically different types of hypertrophic chondrocytes; light and dark cells (Fig. 2A-E and 3A-D) in each species during different stages of development. The majority of the cells were of the light type with a low percentage of dark chondrocytes. The light hypertrophic chondrocytes had electron-lucent cytoplasm (Fig. 2A, B and 3B) while the dark hypertrophic chondrocytes had electron-dense cytoplasm (Fig. 2D, E and 3A). Hypertrophic light and dark chondrocytes had well developed rough endoplasmic reticulum (Fig. 2B, D and 3A, D) and dark chondrocytes have an obvious Golgi apparatus (Fig. 2D and 3A). In all specimens examined, researchers failed to find a typical example of a cell undergoing apoptosis as earlier described (Kerr *et al*., 1972). Instead, researchers observed that light and dark hypertrophic chondrocytes appeared to be undergoing a non-apoptotic mode of physiological death (Fig. 2B-F and 3C, D) as earlier described in the horse (Ahmed, 2007). With increased proximity to the cartilage-bone interface, light cells showed increasing cytoplasmic disintegration within their cell membrane while the nucleus remained intact and apparently functional until the cytoplasm was completely disintegrated (Fig. 2B, C and 3D). The cytoplasm of dark cells showed extrusion into the extracellular matrix with increasing proximity to the cartilage-bone interface, these cells showed decreasing cytoplasmic volume and increasing nuclear condensation (Fig. 2E, F and 3A, C).

Neither dying light nor dark chondrocytes showed the characteristic intense solid accumulations of chromatin or the formation of apoptotic bodies typical of apoptosis.

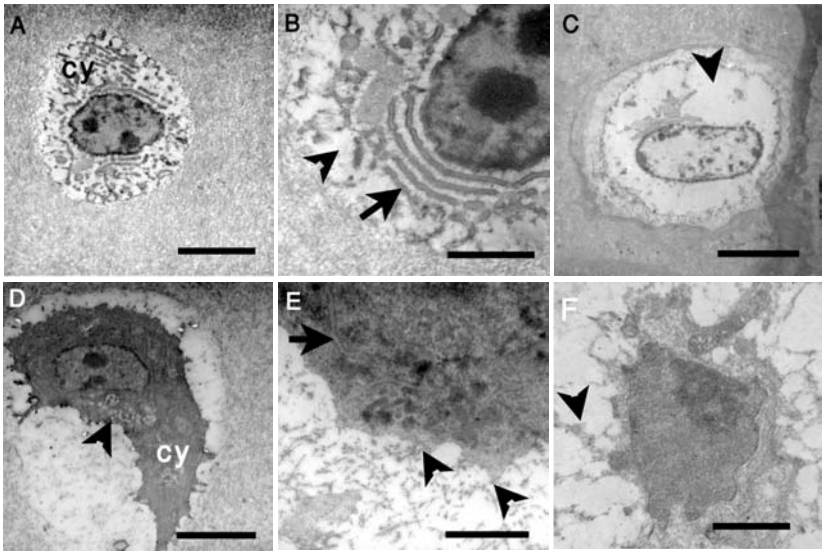

Fig. 2: Modes of physiological death of hypertrophic chondrocytes of 1 day post hatching ducks and quail. Electron micrographs of duck hypertrophic light (A-C) and quail hypertrophic dark (D-F) chondrocytes; A) Hypertrophic light chondrocyte with electron-lucent cytoplasm (cy); B) Higher magnification of a hypertrophic light chondrocyte; note well-developed rough endoplasmic reticulum (arrow) and cytoplasmic disintegration (arrowheads); C) Dying hypertrophic light chondrocyte; note cytoplasmic disintegration within the plasma membrane (arrowhead); D) Hypertrophic dark chondrocytes with electron-dense cytoplasm (cy); E) Higher magnification of hypertrophic dark chondrocyte; note well-developed rough endoplasmic reticulum (arrow) and cytoplasmic extrusion (arrowheads); F) Dying hypertrophic dark chondrocytes; note cytoplasmic extrusion into the extracellular matrix (arrowhead). Parts A and D have the same magnification; bars = 2 μm. Parts B, E, F have the same magnification; bars = 0.25 μm. Bar = 0.5 μm in C

The aim of the current study was to clarify how the hypertrophic chondrocytes of avian growth cartilage die. Light microscopy of samples from different stages of development of post-hatching ducks and quail showed that growth cartilage is similar to that in other avian species (Simsa and Ornan, 2007). The chondrocytes were organized into 3 zones; resting, proliferative and hypertrophic. The resting zone may contain stem-like cells that give rise to proliferative chondrocytes (Abad *et al*., 2002) and may secrete factors that inhibit their hypertrophy. Furthermore resting chondrocytes are thought to produce morphogens responsible for the arrangement of the proliferative zone into columns parallel to the long axis of the bone (Abad *et al*., 2002). Chondrocytes in the proliferative zone undergo mitotic division which decreases with increasing age (Aizawa *et al*., 1997). Chondrocyte hypertrophy makes an important contribution to bone growth. It has been suggested that chondrocyte hypertrophy is responsible for about 50% of long bone growth and the remaining 50% is due to cellular proliferation and ECM accumulation (Ballock and O'Keefe, 2003).

Light and dark hypertrophic chondrocytes have earlier been described in growth cartilage from the domestic fowl (Erenpreisa and Roach, 1998) but these cells have not previously been described in quail or duck. The electron microscopic studies demonstrated that the majority of the duck and quail hypertrophic chondrocytes were identifiable as light cells and only few chondrocytes were of the dark type. It was previously demonstrated that hypertrophic chondrocytes of a variety of species (Hwang, 1978; Wilsman *et al*., 1981; Carlson *et al*., 1985; Ahmed *et al*., 2007a; Chen *et al*., 2010b) exist as light and dark chondrocytes with well developed rough endoplasmic reticulum indicating that these cells are likely to have an important role in extracellular matrix formation. In no specimen examined was an example of apoptosis seen among the dying hypertrophic chondrocytes. However, the light and dark chondrocytes were dying by very characteristic non-apoptotic modes of physiological cell death. In the dying light chondrocytes, cytoplasmic organelles were undergoing gradual disintegration within the plasma membrane. However, the dark chondrocytes showed cytoplasmic extrusion into the extracellular matrix.

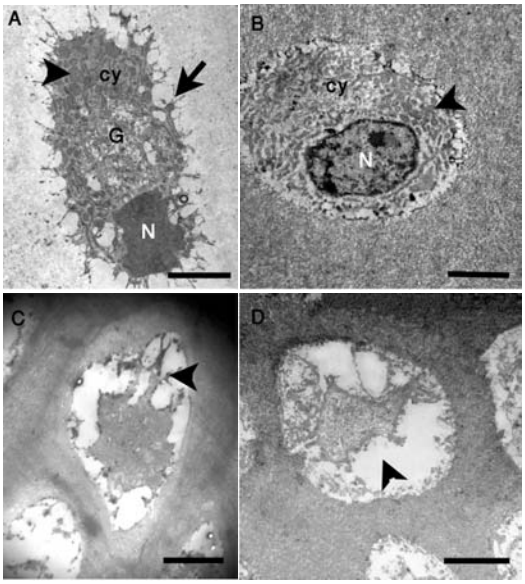

Fig. 3: Modes of physiological death of hypertrophic chondrocytes of 30 days post hatching quail and ducks. Electron micrographs of quail hypertrophic dark (A, C) and duck hypertrophic light (B, D) chondrocytes; A) Hypertrophic dark chondrocyte with electron-dark cytoplasm (cy), dark Nucleus (N), well-developed rough endoplasmic reticulum (arrowhead) and Golgi apparatus (G); B) Hypertrophic light chondrocyte with electron-lucent cytoplasm (cy), light Nucleus (N) and well-developed rough endoplasmic reticulum (arrowhead); C) Dying hypertrophic dark chondrocyte; cytoplasmic extrusion into the extracellular matrix (arrowhead); D) Hypertrophic light chondrocytes; cytoplasmic disintegration within the plasma membrane (arrowhead). Bars = 2 μm (A and B) and 1 μm (C and D)

Many earlier studies have reported that hypertrophic chondrocytes die by apoptosis (Roach *et al*., 1995; Aizawa *et al*., 1997; Adams and Horton, 1998; Cheung *et al*., 2003). An important step in apoptosis of most cells is phagocytosis of dying cells by macrophages or adjacent cells (Kerr *et al*., 1972). In cartilage, phagocytosis is not possible due to the absence of such specialized phagocytic cells and the presence of chondrocytes within lacunae that are separated from each other by abundant extracellular matrix making it difficult for these cells to be phagocytosed by the adjacent cells. Furthermore, researchers did not find such characteristic features of apoptosis. Studies reporting apoptosis in growth cartilage relied on methods such as TUNEL staining, caspase activity detection, flow cytometry and gel electrophoresis, all of which detect features that are associated with apoptosis but may have problems with specificity (Matsuo *et al*., 2001; Lee *et al*., 2007; Teixeira *et al*., 2007). A review study, Shapiro *et al*. (2005) has suggested that hypertrophic chondrocytes may undergo autophagic cell death. If that were true, it would be possible to observe the increased number of double-membraned autophagosomes characteristic of this form of death but that was not the case in either light or dark chondrocytes in the current study.

The result supports the conclusions made on the basis of the earlier study in equine growth cartilage (Ahmed *et al*., 2007b) and that of Chen *et al*. (2010a) in mouse growth cartilage; light and dark chondrocytes appear to be two different populations that die by distinct non-apoptotic modes of physiological cell death. Chen *et al*. (2010b) reported that the majority of chondrocytes in mouse cartilage are of the light type while we reported that in equine growth cartilage the ratio of light: dark hypertrophic chondrocytes varies from a location to another (Ahmed, 2007). The proportion of each cell type is likely to be related to species, age and/or anatomical location.

CONCLUSION

The results presented in the current study suggest that hypertrophic chondrocytes of avian growth cartilage are mostly of the light type with few dark hypertrophic chondrocytes. Both cell types appeared to be dying by non-apoptotic modes of physiological cell death, similar to those observed in mammalian species. The current study was conducted on growth cartilage from normal post-hatching ducks and quail. It will be very important to explore the mode of physiological death in pathological condition such as avian dyschondroplasia to obtain an improved understanding of the pathophysiology of these conditions.

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