An Unusual Step During Endochondral Bone Formation: Mesenchymal Stem-Dependent Cartilage Growth at the Onset of Ossification

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Abstract
Endochondral ossification is proceeded by initial development of cartilage model. Cartilage grows to gain the size and the shape before ossification process. Two prevalent types of cells contribute to cartilage growth; chondrocytes and perichondrial cells. Recently, mesenchymal cells have been further mentioned during cartilage growth. The current study investigated contribution of mesenchymal cells in growth of cartilage model at the onset of ossification. Samples of quail embryos of 8, 9 and 10 post-incubation days were processed for the light microscopic examination. Cartilage templates of the developing femur and tibia were formed and were entirely covered by a perichondrium. Differential organization of the growth cartilage was distinguished in both cartilage templates. At the onset of ossification (9 day), aggregations of subperiosteal cells was observed having typical profile of undifferentiated mesenchymal cells. The subperiosteal Mesenchymal cells niche occupied a long distance in the central hypertrophic zone and some mesenchymal cell penetrated the interior of the cartilage template. Differentiating chondrocytes secrete proteoglycan-rich cartilage matrix which appeared basophilic by H and E, and was stainable positive with both PAS-positive, and safranin O. Differentiating chondrocytes were observed adjacent to the vacated lacunae. At day 10, subperiosteal mesenchymal cells niche was reduced to solitary mesenchymal-like cells which retained the chondrogenic potential. In conclusion, the subperiosteal mesenchymal cells niche provided the cartilage templates by chondrogenic cells for cartilage growth and regeneration. Understanding mechanisms of mesenchymal cells invasion and chondrogenic differentiation is a promise in regenerative medicine.

Keywords
Subperiosteal Mesenchymal Cells; Embryos; Bone Development

Introduction
The skeletal system is derived from the mesoderm-embryonic germ layer. Skeletal mesenchymal cells considered as heterogeneous cell populations that have the ability to differentiate into estrogenic, chondrogenic, and myogenic cells [1]. Mesenchymal cells participate in bone growth and development via two modes; the intramembranous and Endochondral ossification. The intramembranous ossification is proceeded by ossification...
of the highly vascularized membranous mesenchymal sheath where the mesenchymal cells are directly acquired estrogenic cell lineage. Endochondral ossification depends on development of preliminary cartilage model. During its development, the cartilage model grows to an appropriate bone size and shape before the onset of endochondral bone formation. Two forms of cartilage growth have been commonly known, the appositional growth and the interstitial growth. Chondrocytes enter the cell cycle to generate cell progenies which yield new interstitial matrix. Perichondrial cells serve as reservoir stem cells which retain chondrogenic potential. The activation of the perichondrial cells driven appositional growth in which they secrete new circumferential matrix [2]. The role of mesenchymal cells in cartilage formation is confined to the initial steps of cellular condensation. However, the contribution of mesenchymal cells in cartilage growth has been recently described in avian, aquatic and mammalian species. Mesenchymal cells express CD117 are identified in the different skeletal cartilaginous elements of the quail and camel embryos. Mesenchymal cells secrete proteolytic enzymes such as MMP-9 to helpmin the invading of the cartilage matrix. The invading cells express cartilage-specific proteins particularly type II collagen and proteoglycans. The authors speculated involvement of the mesenchymal cells in interstitial cartilage growth [3-5]. The mesenchymal-dependent mode of cartilage growth is also described during development of the cartilaginous support of the air-breathing organ of catfish. Penetration of mesenchymal cells to the cartilage is accompanied by vascular invasion. In catfish, mesenchymal cells play a role in cartilage growth with vascular invasion. The current study explored the pattern of mesenchymal cells-dependent cartilage growth of the prospective femur and tibia in quail embryos in relation to ossification events.

**Material and Methods**

The study used fertile quail (Coturnix japonica) eggs obtained from the Research Quail Farm founded by the Department of Histology, Faculty of Veterinary Medicine, South Valley University in, Qena, Egypt. The fertilized eggs were incubated in a c10 “POULTRY TECHNICAL OFFICE, Alexandria, Egypt” at 37.5°C with a relative humidity of 65%. The eggs were rotated automatically every 6 hours after the 3rd day of incubation. We collected 21 quail embryos at day 8, 9 and 10 days of incubation (Figure 1). Embryonic stages were determined upon the onset of incubation. Eggs were kept at -20°C for 4 hours prior to collecting. Eggshells were opened at the broad end, and apparently healthy embryos were carefully excised from their shells. All embryos were immediately fixed in 10% buffered formalin for 3 days or in Bouin’s solutions for 8 hours.

**Figure 1:** Cartilage Models of the Prospective Femur and Tibia in The 8day Embryonic Stage

Paraffin sections of the quail embryo showed femur (A, B) stained by Crossman’ trichrome and tibia (C, D) stained with H&E. A: cartilage template of the femur was distinguished into particular cartilage, proliferating zone of the physeal growth cartilage while the centre of the femur shaft mostly contained Non-hypertrophic chondrocytes. Note some chondrocytes undergo hypertrophy (h), perichondrium (pr). C, D: cartilage template of the tibia had the typical zone organization of the growth cartilage. Note Proliferating zone (P), hypertrophic zone (h), perichondrium (pr).
Fixed samples were dehydrated in ascending grades of ethanol (70%, 80%, 90% and 100%) for 90 minutes in each concentration. The samples were cleared using methyl benzoate, embedded in Paraplast (Sigma Aldrich) and serial sections of 3-5µm thick were cut using a Richert Leica RM 2125 Microtome, Germany and mounted on glass slides. Slides were kept in an incubator at 40°C for dryness.

Sections were stained with H&E stained as a general histological stain and cartilage matrix specific stain including safranin O [7], Crossmon trichrome [8] and periodic acid-Schiff reaction (PAS) according to McManus [9]. Stained sections were examined using a DMLS light microscope (Leica, Germany) outfitted with MC120 HD camera (Leica, Germany).

**Results**

Cartilage templates of the prospective femur and tibia were formed at the 8 days of incubation. Zonal organization of the growth cartilage was obviously detected in the tibia (Figure 1C, D). While non-hypertrophic chondrocytes dominated the center of the femur shaft (Figure 1B). Both cartilage templates of femur and tibia were covered by a perichondrium with no signs of ossification were found (Figure 1A-D).

Cartilage model of the femur undergoes ossification in the day 9. The bone collar was established around the hypertrophic zone. Aggregations of subperiosteal cells were observed with typical profile of undifferentiated mesenchymal cells. They were small cells and had a flattened shape (Figure 2 A-C). The subperiosteal mesenchymal cells niche occupied a long distance in the central hypertrophic zone (Figure 2D). Some mesenchymal cell penetrated the interior of the cartilage matrix. Differentiating chondrocytes secrete proteoglycan-rich cartilage matrix which appeared basophilic by H&E (Figure 3 E, F), and satined positive with PAS-positive (Figure 2E, Figure 3B) and safranin O positive matrix (Figure 3 A, C). Different chondrocytes were observed adjacent to the vacated lacunae (Figure 3 D).

By the day 10, growing femur and tibia had known cartilage model features. They had a distinct zonal organization of the growth cartilage; proliferating, hypertrophic zones and the particular cartilage. No obvious interstitial mesenchymal cell differential was identified in the cartilage templates of both femur and tibia (Figure 4 A, B, D, F). Subperiosteal mesenchymal cells niche was reduced to solitary mesenchymal-like cells, which were closely opposed to bone collar. Chondrogenic differentiation of mesenchymal-like follows much the same pattern as in the appositional growth (Figure 4 F).

**Figure 2: Identification of Mesenchymal-Like And Chondrogenic Cells in Cartilage Model of the Femur in the 9-Day Embryonic Stage**

Paraffin sections of the quail embryo showed femur stained by Crossman’ trichrome (A-C) and PAS (D, E). A-B: cartilage templates of the femur undergo ossification. Note bone collar (B) around the hypertrophic zone (H). Sub periosteal cells (S) which have typical profile of mesenchymal cells. Mesenchymal cells penetrated to the interior of the hypertrophic zone (arrow). Some cells acquired rounded-shape which seems to be different chondrocytes (d). C: higher magnification of the Subperiosteal Mesenchymal cells niche (S), the arrows refer to mesenchymal cells; some of which penetrate the cartilage matrix. Note Bone (B). D: cartilage templates of the femur having the central hypertrophic zone (h). note Subperiosteal Mesenchymal cells niche (S). E: Hypertrophic zone of the growth cartilage (h). The arrow refers to a thin rim of bone collar. Subperiosteal Mesenchymal cells niche (S). Differentiating chondrocytes (d) secrete PAS positive matrix.
Figure 3: Identification of Mesenchymal-Like And Chondrogenic Cells in Cartilage Model of the Tibia in the 9-Day Embryonic Stage

Paraffin sections of the quail embryo showed tibia stained by Safranin O (A, C), PAS (B) and H&E (D-F). A: cartilage template of the tibia. Note hypertrophic zone (h). Subperiosteal Mesenchymal cells niche (S), different chondrocytes secrete Safranin O positive matrix. B: cartilage template of the tibia. Note hypertrophic zone (h). Subperiosteal Mesenchymal cells niche (S), different chondrocytes secrete PAS positive matrix. C: hypertrophic chondrocytes (h) surrounded by different chondrocytes (d) which secrete Safranin O positive matrix. Note Subperiosteal Mesenchymal cells niche (S). Bone (B). D: Hypertrophic zone of the growth cartilage (h). Note Vacated lacunae (V) nearby the differentiating chondrocytes (d). Subperiosteal Mesenchymal cells niche (S). Bone (B). E: Subperiosteal Mesenchymal cells niche (S). Note mesenchymal cells penetrate to the interior of the cartilage template. hypertrophic chondrocytes (h) Bone (B). F: Subperiosteal Mesenchymal cells niche (S), different chondrocytes (d). Subperiosteal Mesenchymal cells niche (S). Hypertrophic chondrocytes (h) bone (B).

Figure 4: Cartilage Model of theProspective Femur and Tibia in the 10-Day Embryonic Stage

Paraffin sections of the quail embryo showed femur (A-C) and tibia (C-F) stained by Safranin O (A, C, D), Crossman’ trichrome (B) and H&E (F). Cartilage template had a distinct zone organization of the growth cartilage; proliferating (p), hypertrophic zones (h) and the particular cartilage (a). Differentiating chondrocytes (d). Note mesenchymal-like cells (arrow) were observed in adjacent to bone (B).
Discussion

The current study described pattern of mesenchymal cells-dependent cartilage growth at the onset of ossification in the prospective femur and tibia in quail embryos. In the current study, cartilage templates of the prospective femur and tibia were well-established by the day 8 of incubation. They more or less exhibited the general organization of the growth cartilage and were entirely covered by a perichondrium. The onset of ossifications occurred at day 9 when the cartilage templates acquired masses of undifferentiated cells that had a mesenchymal cell profile. These cells were located adjacent to the periosteal bone. Mesenchymal cells underwent differentiation and secreted proteoglycan-rich matrix as confirmed by positive staining with PAS and safranin O. Thus, we speculated that the subperio steal mesenchymal cells niche was a population of undifferentiated cells that had a chondrogenic tendency. The results also provide evidence for chondrogenic differentiation of mesenchymal cells may build up the subperiosteal region of the cartilage templates. Moreover, some mesenchymal cells penetrated the interior of cartilage to commit secretion of the interstitial matrix (interstitial growth). Different chondrocytes were also located close to the vacated lacunae where chondrocytes expected to die. These results suggest that chondrogenic differentiation of mesenchymal cells may replace the dying chondrocytes and help in cartilage regeneration.

Mesenchymal cells have been studied in the elastic cartilage supporting the air-breathing organ of catfish. The authors suggest a contribution of mesenchymal cells in cartilage growth, regeneration, and replacement. Mesenchymal cells secrete photolytic enzymes particularly MMP-9 to be enabled to penetrate the cartilage matrix [3, 4, 6]. MMP-9 break down collagen types IV, V, XI, XIX, and elastin, aggrecan, link protein, decorin, laminin, entactin, SPARC, myelin basic protein, Mn, Pli, IL-1β, proTNF k[10]. Mesenchymal cells acquire chondrogenic cell lineage and expresses type II collagen. They commit an interstitial cartilage growth. Mesenchymal cells also occupy the empty lacunae which are left by dead chondrocytes. They could replace the hyaline cartilage, located at the base of the air-breathing organ, the elastic type. Thus, mesenchymal cells participate in the structural and functional maintenance of various types of tissues [3, 4, 6]. In the current study, mesenchymal cartilage was randomly distributed and limited to the subperiosteal region. However, CD117-positive mesenchymal cells were identified in cartilage templates of camel and quail embryos. Mesenchymal cells have a chondrogenic potential and express type II collagen. They also produce MMP-9. A different mesenchymal cell organization was described in various skeletal elements during different developmental stages. Mesenchymal cells invasion is not related to ossification processes. Mesenchymal cells originated from either the perichondrium or the surrounding mesenchymal. Cellular invasion is not confined to the subperiosteal region but could be occurred in the focal distribution of mesenchymal cells and may be at multiple sites in the cartilage templates of the developing flat bone or in the resing, proliferating and hypertrophic zone in the cartilage template of the prospective long bone. Mesenchymal cells in quail and camel embryos have different organization either single cell, cellular streaks of high and low cellular densities. The authors concluded that mesenchymal cell participated in production of new interstitial matrix, and consider this type as an interstitial type of cartilage growth [4, 5]. It known that cartilage grows by two modes. The first mode achieves an increase in the cartilage diameter and depends on the undifferentiated perichondrium cells to secrete new matrix adding to the outer circumference of the cartilage. Hence, this type is known as appositional growth. The second mode is a chondrocytes-dependent, in which chondrocytes proliferate and the daughter cells produce interstitial matrix (an interstitial type of cartilage growth [11, 12].

Penetration of mesenchymal cells in the cartilage templates of prospective long bone has been described as an initial step in Endochondral ossification. Mesenchymal cells acquire an estrogenic cell lineage to serve in bone formation [13, 14]. Mesenchymal cells may be also as a constituent of the cellular elements of the cartilage canals. These canals are derived from perichondrial papillae to provide a nutritional support to the particular cartilage. Development of cartilage canals is limited to the epiphysis and associated with vascular invasion [15]. Two Different cell lineage are described in the cartilage canals; type II collagen expressing cells [16, 17] and type I collagen and, peristatin expressing cells [18-20].

Conclusion

The existence of subperiosteal mesenchymal cell niche was a provisional event in both cartilage model of both femur and tibia. The subperiosteal mesenchymal cell niches provide the cartilage model by chondrogenic cells. The aim of the Chondrogenic differentiation of mesenchymal cells was to construct the cartilage model.
and may be considered as a preparatory stage before proceeding of ossification.

References


