Complement Proteins Are Present in Developing Endochondral Bone and May Mediate Cartilage Cell Death and Vascularization

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Normal endochondral bone formation follows a temporal sequence: immature or resting chondrocytes move away from the resting zone, proliferate, flatten, become arranged into columns, and finally become hypertrophic, disintegrate, and are replaced by bone. The mechanisms that guide this process are incompletely understood, but they include programmed cell death, a stage important in development and some disease processes. Using immunofluorescence we have studied the distribution of various complement proteins to examine the hypothesis that this sequence of events, particularly cell disintegration and matrix dissolution, are complement mediated. The results of these studies show that complement proteins C3 and Factor B are distributed uniformly in the resting and proliferating zones. Properdin is localized in the resting and hypertrophic zone but not in the proliferating zone. Complement proteins C5 and C9 are localized exclusively in the hypertrophic zones. This anatomically segregated pattern of distribution suggests that complement proteins may be important in cartilage-bone transformation and that the alternate pathway is involved. © 1996 Academic Press, Inc.

INTRODUCTION

The form for an endochondral bone is specified by the cartilaginous model that precedes it during fetal development. Even though chondrocyte hypertrophy and cartilage resorption precede vascularization [1], a critical event leading to bone formation is the invasion of the midregion of the cartilaginous model by blood vessels. During this process chondrocytes undergo a series of sequential events that lead to their differentia-

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and synthesized in articular cartilage of hamsters [3], where it may play a role in collagen degradation [4], we know of no reports of the presence of other complement proteins in cartilage. In both osteoarthritis and rheumatoid arthritis, complement is thought to play a role in cartilage destruction. In these conditions, however, complement is thought to be derived from noncartilaginous sources [5–7].

To examine our hypothesis that cartilage–bone transformation is complement mediated we have mapped the distribution of various complement proteins in the tibias or femurs of 19-day-old fetal rats using immunofluorescent techniques. In addition, we used a marker for apoptotic cells to compare their distribution with that of complement.

**MATERIALS AND METHODS**

Tissues and antibodies. Tibias or femurs from 19-day-old fetal Fischer rats were cleaned of adhering soft tissue, fixed for 2 h in 4% phosphate-buffered paraformaldehyde, and stored at −70°C. Frozen sections, 8 μm in thickness, were cut at −20°C using a Miles Tissue-Tek cryostat. Alternatively, the tibias or femurs were fixed in Bouin’s solution and embedded in paraffin. Sections 5 μm in thickness were used for immunofluorescence and histological staining.

Goat antibodies to rat C3 (IgG fraction) and goat antiserum to human complement component C5 and C9 were purchased from Cappel (West Chester, PA). Goat antiserum to human properdin and FITC-labeled rabbit anti-goat IgG were purchased from Calbiochem (La Jolla, CA). Human antibodies were tested for cross-reactivity to rat complement proteins by the suppliers.

Fluorescence. Paraffin sections were deparaffinized by two 10-min changes of xylene and then hydrated in a series of alcohol:water mixtures and finally water. The sections were allowed to air dry and incubated with 10% normal goat serum diluted with phosphate-buffered saline (PBS). After 30 min the goat serum was removed and the appropriate primary antibody added. After 1 h the slides were washed three times, 5 min each with PBS followed by the addition of FITC-conjugated secondary antibody. Appropriate controls were also used. After an additional hour of incubation the slides were washed with PBS, coverslipped, examined by fluorescence microscopy, and photographed.

After the sections were photographed for fluorescence, the coverslip was removed, and the sections were stained with the hematoxylin–eosin or toluidine blue stains. Since cartilage matrix is known to bind proteins, including nonspecifically antibodies, particular care was employed to block and wash the tissue sections carefully. The fluorescence signals are specific; first, because tissues incubated with control antibody did not fluoresce; second, each antibody had a different distribution of binding sites in the tissue, which would not occur if the binding were nonspecific. An antibody directed against an envelope protein of herpes simplex virus, type I, was used as a negative control.

Apoptosis. Rat fetal bone was fixed in 10% neutral Formalin for 2 h, dehydrated in ascending concentrations of ethanol and eliminate xylene, and embedded in paraffin. Sections from rat fetal bone, and some from human fetuses obtained from our surgical pathology files, 8 μm thick, were deparaffinized, rehydrated with phosphate-buffered saline (PBS), and digested with proteinase K (20 mg/ml) for 15 min at room temperature. After washing in distilled water, the sections were treated with 2% H2O2 in PBS for 5 min to quench endogenous peroxidase activity and then with reagents from a kit (ApopTag; Oncor, Inc., Gathersburg, MD). In this procedure 3′ OH ends of DNA generated by DNA fragmentation during apoptosis are bound to dioxigenin complexed nucleotides using terminal deoxynucleotidylase as the catalyst. A peroxidase-coupled anti-dioxygenin antibody is then added. After washing, the sections were incubated with 0.02% H2O2 and 0.05% diaminobenzidine to yield a brown reaction product. Controls (thymus glands from dexamethasone-treated mice) were treated identically and gave the expected results.

**RESULTS AND DISCUSSION**

When a section of a 19-day-old embryonic tibia is reacted with an antibody to complement protein C3, the cells of the resting area fluoresce intensely without localization to any specific area of the cell (Fig. 1a). In the proliferative zone, the fluorescence persists and, in those cells adjacent to the hypertrophic area, appears to be localized preferentially at the cell periphery (Fig. 1b). In the hypertrophic area the fluorescence is markedly diminished and it appears at the cell periphery (Fig. 1c).

The distribution of Factor B of the alternate complement pathway shows a distribution similar to that of C3; however, in the resting area cells do not appear uniformly fluorescent. Some cells are intensely positive, whereas others show only faint fluorescence (Fig. 2a). In the proliferative and hypertrophic zones fluorescence is markedly diminished and, in the area closest to bone, largely limited to the cell periphery (Fig. 2b).

The distribution of properdin, another protein of the alternate pathway of complement, shows a pattern of distribution different from that of C3 or Factor B. Properdin staining appears in the resting, but not the proliferative, area (Fig. 3a). In the resting area properdin is not present in all cells. It is also present in the hypertrophic area, where it localizes at the cell periphery (Fig. 3c). Figure 3b shows all the morphological characteristics of a typical growth plate in a section of 19-day-old embryonic tibia; adjacent to the joint is the resting zone (R) with small, uniformly distributed chondrocytes. Adjacent to the joint is the proliferative area (P) of flattened cells arranged in columns and between this zone and the bone (B) is the zone of hypertrophy (H).

Complement proteins C5 and C9 are demonstrable only in the hypertrophic area, where they are localized at the cell periphery (Fig. 4). They are not uniformly distributed and cells vary in staining intensity.

Apoptotic nuclei, demonstrated by the visualization of 3′ OH DNA ends were unusual, with rare scattered stained cells in the distal hypertrophic zone at the cartilage–bone junction.

Complement proteins are synthesized mainly by macrophages or the liver but other cells also have this capacity. Fibroblasts, for example, can synthesize C1q, C1r, and C1s [8]. Hyaline cartilage has also been shown...
FIG. 1. Distribution of complement protein C3 in cartilage. In the resting zone (a) C3 appears evenly distributed in all chondrocytes. In the proliferative zone (b) C3 appears to be localized to the cell periphery, especially in the areas closer to bone. In the hypertrophic zone (c) not all cells stain for C3; in positive cells C3 appears as patches on or near the cell surface. ×165 (a), ×250 (b), ×350 (c). *Resting zone of the adjacent femur.

FIG. 2. Distribution of complement protein Factor B in cartilage. In the resting zone (a) Factor B is not present in all cells and more abundant in some than in others. In the proliferative and hypertrophic zones (b) not all cells are positive and the fluorescence tends to be at the cell periphery. ×200 (a, b).

to synthesize C1s [3], and synovial fibroblast-like cells can synthesize C1r, C1s, C1 inhibitor, C2, C3, Factor B, and Factor H [9].

It has been shown that cartilage dissolution occurs when complement is added to cultured cartilage under conditions in which it is activated [2]. Complement is thought to be involved in cartilage destruction in osteoarthritis and rheumatoid arthritis [5, 10, 11], in which
FIG. 3. The distribution of complement protein properdin in cartilage. Properdin appears evenly distributed in many cells of the resting zone (a, upper portion). In the proliferative zone (a, lower portion; c, upper portion) properdin staining is not seen. In the hypertrophic zone (c, lower portion) properdin is also present. Autofluorescence is visible in bone and perichondrium. (b) Hematoxylin-eosin-stained section for orientation: R, resting zone; P, proliferative zone; H, hypertrophic zone; B, bone. ×200 (a, c), ×100 (b).

FIG. 4. Distribution of complement proteins C5 (a) and C9 (b). These antigens are demonstrated at the cell periphery in the hypertrophic, but not the proliferative, zone. ×400 (a), ×200 (b). *Cartilage-bone junction.

cases it is believed to be of extracartilaginous origin. Our results show that transforming cartilage contains proteins of the alternate complement system. The presence of the complement proteins C3, Factor B, and properdin, but not C5 or C9, in the resting zone of cartilage suggest that these components may be synthesized by cartilage cells. This notion is based on their distribution: in the resting zone there is diffuse staining of the
FIG. 5. Distribution of the alternative complement pathway proteins and hypothetical reactions in the various zones of the cartilage growth plate. In the resting zone (R) C3 is activated to C3i, which binds to B to form C3iB. C3iB binds to the cell surface where Factor D hydrolyzes B to give rise to C3 convertase, and C3iBb is inactivated by Factors H and I, leaving C3i attached to the cell and available for further degradation. In the resting zone the presence of properdin (P) suggests that C3 convertase could be stabilized; however, even a stabilized C3 convertase can be inactivated by Factors H and I, when their concentration is appropriate. In the proliferative zone the absence or decreased concentration of Factors H and I allows C3 convertase to hydrolyze C3 to form the other form of C3 convertase, C3bBb, or C5 convertase C3bBbC3b. The C5 convertase is stabilized in the late proliferative zone by P (C3BbC3bP). Upon meeting C5, diffusing from the blood, C5 convertase hydrolyzes C5 into C5b and C5α, thus starting the attack phase of the complement cascade resulting in cell lysis. \( \times 50. \)

Cells, in the proliferative and hypertrophic zones these components are more localized at the cell periphery, and perhaps importantly, the extracellular matrix does not stain. An alternate source of complement proteins would be via diffusion from the blood vessels at the osseous end of the growth plate. If that were the case, the concentration and hence presumably the staining intensity of these proteins would be expected to be higher closer to the bone, but such is not the case. On the other hand, the presence of properdin in the resting and hypertrophic zones and our failure to demonstrate generation of properdin in resting cartilage suggest that the alternate complement pathway may play a role in the normal development of this tissue.

Endochondral bone formation and growth involve a series of tightly regulated events that lead to chondrocyte hypertrophy, disintegration, and replacement of the cartilage matrix by bone. Even though endochondral bone formation has been extensively studied, the agents that mediate the process are incompletely understood. Two events are thought to be critical for the generation of endochondral bone, the disintegration of chondrocytes and invasion of the cartilaginous model by blood vessels. The nature of the chemoattractants for the invading vessels is not known. It is possible that a fragment of one of the complement proteins (C3a or Ba) may act as such as agent. C3a is known to be chemotactic for a number of cells and to enhance vascular
permeability [12–14]. The presence of C5 and C9, members of the membrane attack assembly of the complement cascade, only in the hypertrophic area supports the concept that complement localization accompanies and perhaps mediates chondrocyte cell death. A possible scenario is that as the proliferating cells migrate toward the bone, spontaneous conversion of C3 into C3i occurs. C3i complexes with B to form the inactive C3 convertase, C3iB, which is then activated by Factor D (C3Bb). Once activated the C3 convertase hydrolyzes C3 into C3b and C3a, expanding the process and generating the C5 convertase, R-C3BbC3b. As the cell carrying C3bBbC3b migrates further toward the midshaft, properdin diffusion from the vessels present in the bone binds and stabilizes the C5 convertase. The complex hydrolyzes C5 into C5a and C5b. C5b then binds to cell surface which might initiate the cytolytic attack phase. Figure 5 summarizes the major steps in this conjecture and how the various events may be related to the distribution of the various complement proteins in prenatal cartilage. We have tried to perturb this system by injecting complement into mice and zymosan into rats. These experiments yielded inconclusive results with regard to growth plate morphology.

A recent study [15], using organ cultures of chick embryonic femurs demonstrates apoptosis in the distal hypertrophic zone. Apoptotic cells, including endothelial cells, can bind and activate components of the alternative pathway of the complement system [16, 17]. The accumulation of these proteins near the cartilage–bone junction may thus be a consequence or accompaniment of cell death rather than its cause. Even though cell death in this area is programmed, this appears to be unlikely for two reasons. First, except for the dying chondrocytes at the very distal end of the growth plate, chondrocyte nuclei are not shrunken, as they are in apoptotic cells. Second, DNA OH− ends were demonstrable only rarely in the very restricted area of the shrunken, dead chondrocytes, while complement proteins were present throughout the cartilage. In addition, the striking, apparently regulated pattern of distribution of the various complement proteins in developing cartilage argues strongly that they play a significant role, perhaps, as we suspect, by promoting matrix dissolution. The possibility of an interaction between apoptotic cells and complement, however, remains, since nuclear changes are not the first step in the apoptotic pathway.

Finally, the integration of local events in preosseous cartilage is critical to cartilage transformation. Inhibition of mineralization in rickets results not only in retardation of cartilage mineralization, but also widening of the growth plate and its failure to vascularize. Whether complement distribution is changed in this condition remains to be seen. It is, however, intriguing that mice given toxic doses of vitamin D develop more extensive vascular calcification if injected with complement and that hypervitaminotic rats show less calcification when injected with zymosan [18, 19].

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