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Changes in the Gene Expression of Collagens, Fibronectin, Integrin and Proteoglycans During Matrix-Induced Bone Morphogenesis

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Subcutaneous implantation of demineralized bone matrix in rat results in the local cartilage and bone development. This in vivo model of bone formation was used to examine the expression patterns of cartilage and bone specific extracellular matrix genes. The steady state levels of mRNA in implants for cartilage specific type II collagen, type IX collagen, proteoglycan link protein and cartilage proteoglycan core protein (aggrecan) were increased during chondrogenesis and cartilage hypertrophy. Fibronectin mRNA levels were high during mesenchymal cell migration, attachment and chondrogenesis. Integrin (β1 chain) mRNA was expressed throughout the endochondral bone development. Type I collagen mRNA levels in implants increased as early as day 3, reached its peak during osteogenesis. These gene markers will be useful in the study of the mechanism of action of bone morphogenetic proteins present in the demineralized bone matrix.

Endochondral bone formation and fracture repair are complex biological processes. These processes can be mimicked by subcutaneous implantation of demineralized bone matrix in rat (1,2). The multistep developmental cascade of matrix-induced bone formation consists of 1) A transient inflammatory response on day 1; 2) Appearance and proliferation of mesenchymal cells in close proximity to the implanted matrix on day 3; 3)Chondrogenesis on days 5 through 7; 4) Vascular invasion, appearance of osteoblasts and new bone formation on days 9-14; and 5) Bone remodeling and formation of bone marrow on days 11 through 21 (3,4). Recent work has identified osteogenin and related bone morphogenetic proteins in the demineralized bone matrix (5,6). Bone morphogenetic proteins are members of the transforming growth factor-β super-family which plays

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an important role in tissue development and repair(5). It is of interest to determine the signalling mechanisms for the temporal expression of genes during the developmental sequence of bone formation. As a first step this study investigated the chages in the steady state levels of mRNA for cartilage and bone matrix proteins.

Materials and Methods

RNA Preparation

Bone formation was induced in 28-day Long-Evans rats by implantation of 25 mg of rat demineralized bone matrix subcutaneously on each side of the thoracic region as described (1). At selected days after implantation, the implants were dissected out. Six to ten implants were pooled and homogenized in 4 M guanidium thiocyanate buffer, total RNA was isolated (7) and poly (A^+) RNA was isolated by oligo(dT) cellulose affinity column (8). The quantity and quality of RNA were routinely examined by determination of absorbance ratio at 260 and 280 nm and ethidium bromide staining of RNA after electrophoresis in agarose gels.

Northern Blot Hybridization

Equal aliquots of total RNA (25µg) were denatured in formamide at 65°C for 5 min and separated in a1% agarose gel containing 3.6% formaldehyde. The RNA was transferred to a Nytran membrane (Schleicher & Schuell) in 20 x SSC by capillary method. The RNA was immobilized on membrane by baking in a 80°C vacuum oven for 2 hours. All prehybridizations and hybridizations were performed at 45 °C with 5 x SSC, 50% formamide, 1 x Denhardts' solution, 200 µg/ml salmon sperm DNA, 1% glycine and 0.05M phosphate, pH 6.4. Following hydridization, the membranes were washed once in 2x SSC and 0.5% SDS for 10 min, then once at 60 °C in 0.4 x SSC and 0.1% SDS for 10 min. for nonhomologous probes and 60 min. for homologous probes. Most blots were stripped in 0.1 x SSC, 0.1% SDS at 100 °C for 10 min for removal of the probes. The blots were then subjected to a second hybridization with a different probe. The resulting autoradiographs were quantitated by scanning laser densitometry and all values were normalized against the relative fluorescence of EtBr-stained ribosomal RNA in a given lane. DNA probes used for hybridization were the following: rat α1 type I collagen cDNA probe (9); rat (α1) type II collagen cDNA probe (10); rat α1 type IX collagen cDNA probe (11); rat link protein cDNA probe (12); rat large chartilage proteoglycan core (aggrecan) cDNA (13); rat fibronectin cDNA probe (14); human intergrin receptor ($ß_1$chain) cDNA probe (15) was obtained commercially from Telios Pharmaceuticals Inc.. All the probes were labeled with ^{32}P-dCTP by random primer kit following manufacturer's protocol (BRL). The size of mRNA was estimated by comparison with RNA ladder size marker (BRL).

Results and Discussion

The temporal expression of cartilage and bone matrix genes associated with developmental sequence of endochondral bone formation was

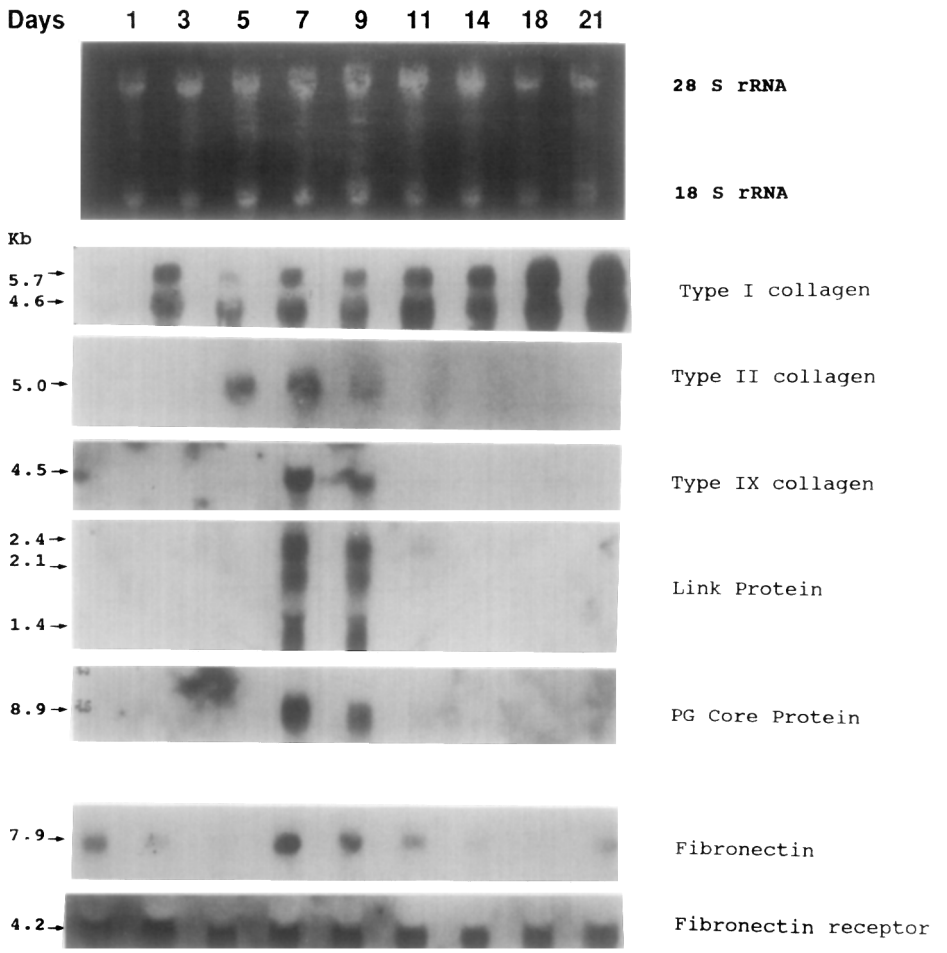

Figure 1. Northern blot analysis of mRNA of extracellular matrix during endochondral bone formation. The ethidium bromide-stained blot represents equal amount of total RNA (25 μg) isolated from implants at the indicated times (days 1, 3, 5, 7, 9, 11, 14, 18 and 21). RNA was separated in a 1% agarose gel containing formaldehyde, and immobilized on Nytran membrane. The autoradiographs depict results of a series of hybridizations of mRNA on the blots to radio-labeled cDNA probes for type I collagen, type II collagen, type IX collagen, link protein, aggrecan, fibronectin and integrin. The size of respective mRNAs is noted in the figure.

examined by determination of steady state levels of mRNA throughout the 21-day period during matrix-induced endochondral bone formation. Fig. 1 shows the autoradiographs of different mRNAs hybridized with several probes in a Northern blot analysis. The relative abundance of the mRNAs at different time points determined from autoradiography scans is shown in Figure 2. The steady-state level of type II collagen mRNA increased on day 5, and reached its peak on day 7, then decreased rapidly to a undetectable level after day 9. Type II collagen is a cartilage specific marker (16). In demineralized matrix-induced bone formation model, chondroblasts first appear on day 5, and is correlated with the appearance

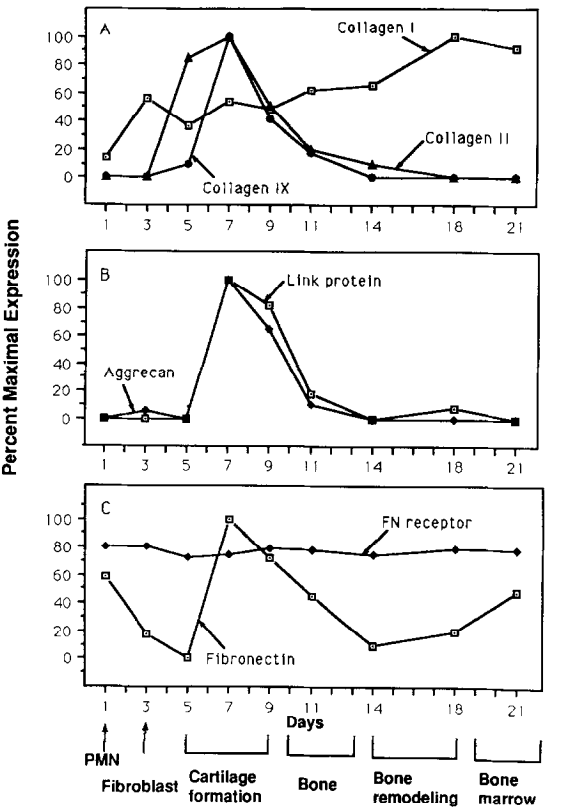

Figure 2. Temporal expression of extracellular matrix genes during endochondral bone formation. Northern blots were prepared and hybridized to the cDNA probes as noted in Figure 1. The resulting blots were quantitated by scanning densitometry and the results plotted relative to the maximal expression of each transcript. Each value represents the mean values of three Northern blot hybridizations to the same probe after normalization against rRNA staining. Panel A, ▫ represents the mRNA levels of type I collagen; ▲ represents the mRNA levels of type II collagen; • represents type IX collagen. Panel B, ▫ represents mRNA levels of proteoglycan link protein; ◆ represents the mRNA levels of aggrecan. Panel C, ▫ represents the mRNA levels of fibronectin; ◆ represents the mRNA levels of fibronectin receptor. The developmental events of bone formation during the 21-day period are described at the bottom.

of type II collagen mRNA. By the time cartilage is replaced by bone on day 11, the expression of type II collagen gene diminished to a undetectable level. In a similar fashion, type IX collagen mRNA was transcribed at high levels from day 7, then gradually decreased to a undetectable level after day 9 (Fig. 2). Although type IX collagen expression is also cartilage specific, there is a two-day lag as compared to type II collagen expression indicating that this is a late marker. We also observed exactly the same pattern of expression as type IX collagen for other cartilage specific genes

encoding proteoglycan core protein (aggrecan) and link protein. These patterns of expression reflect important roles of their respective gene products in cartilage formation. As shown in Figure 2, fibronectin mRNA was detectable throughout the 21-day period. However, a higher level of the mRNA appeared first on day 1, during mesenchymal cell proliferation and attachment to implanted matrix (17). The highest level was detected on day 7, concomitant with early chondrogenesis. The mRNA declined when the cartilage matrix calcified on day 9. The mRNA level increased again on day 21, during haematopoiesis(18). In contrast to fibronectin, integrin mRNA was expressed continuously throughout 21-day period (Fig.1). The level of type I collagen mRNA was detectable as early as on day 3 and reached its peak on day 18 (Fig. 2). Since there are different cell types within an implant, a major proportion of osteoblasts in late stage implants may account for the peak level of type I collagen mRNA observed in day 18 implants. Alkaline phosphatase, another osteoblast differentiation marker gene, was also expressed at higher levels during the osteogenic phase (days 11-14 implants) (Paralkar, unpublished data).

In conclusion, the expression patterns of several cartilage and bone matrix genes were studied during demineralized bone matrix induced endochondral bone formation. These patterns of gene expression provide us initial insights into the molecular mechanisms associated with bone formation and repair. This will form the basis for further study of the relationship between cellular proliferation and differentiation in response to the actions of hormones, growth factors and other novel cartilage and bone morphogenetic proteins.

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