Matrix Metalloproteinase Activity During Endochondral Ossification

Introduction: Endochondral ossification requires the coordination of programs regulating chondrocyte differentiation, extracellular matrix (ECM) remodeling, and the formation of new bone vessels. ECM remodeling is intricately linked to the vascular invasion of the cartilage element, an event that precedes ossification. Matrix metalloproteinases (MMPs) are calcium- and zinc-dependent endopeptidases that can degrade the ECM by cleaving matrix molecules, or by modulating the activities of signaling factors or other enzymes (1). At least two MMPs (MMP-9 and MT1-MMP) have been implicated in the process of ECM remodeling during endochondral ossification (1,2). Because of their ability to act on a wide variety of ECM molecules in cartilage and bone, MMPs and the tissue inhibitors of MMPs (TIMPs) are likely regulators of bone formation during both fetal development and adult repair. However, the role of MMPs during endochondral ossification remains incompletely understood. The purpose of this study is to investigate the spatio-temporal expression of genes encoding for a subset of ECM-degrading enzymes (MMPs and TIMPs) during endochondral ossification in skeletal development and adult fracture repair.

Materials and Methods: To study their role in endochondral ossification, mmp and timp expression were analyzed during the stages of initial vascular invasion of the cartilage matrix both during fetal development and adult fracture repair. The expression domains of mmps and their inhibitors were compared with those of molecular markers of cartilage (collagen type II (coll2), collagen type X (col10), and indian hedgehog (ihh)) and bone (osteocalcin (oc)). For this purpose, mouse upper and lower limbs were harvested at embryonic days 14 (e14) and 15 (e15) (n=6), and during various stages of tibial fracture repair, including the intermediate (soft callus) and late (hard callus) phases of healing (n=20). The closed fractures were created in 10-12 week old 129B6 mice. Following the creation of the fractures, radiographs were taken to confirm the extent of injury. Mice were capable of bearing weight within 24 hours. All procedures were approved by the UCSF Committee on Animal Research.

Tissues were decalcified, dehydrated, and embedded. Tissue sectioning, probe preparation, and in situ hybridization were performed. cdNAs of the mouse genes mmp2 (gelatinase A), mmp9 (gelatinase B), mmp13 (collagenase-3), mt1-mmp, timp2, timp3, col10, ihh, and oc were used to generate antisense (35S)UTP-labeled riboprobes. Slides were dewaxed and hybridized at 53–58º C. Post-hybridization washes were performed at a high stringency (53-59º C, 1-2X SSC / 50% formamide). Tissue sections (4-5 micron thickness) were examined using simultaneous darkfield and fluorescence microscopy. Near adjacent and adjacent serial images were captured on a digital camera as double exposures (darkfield image represented the silver grain density of the hybridization signal and the fluorescent image represented the Hoescht nuclear dye). Near-adjacent serial sections were processed by the Hoescht nuclear dye. Near-adjacent serial sections were processed by the Hoescht nuclear dye. Near-adjacent and adjacent serial images were captured on a digital camera as double exposures (darkfield image represented the silver grain density of the hybridization signal and the fluorescent image represented the Hoescht nuclear dye).

Results: At e14, the skeletal tissues in the mouse femur were composed entirely of cartilage with resting, mature, and hypertrophic chondrocytes in the central region of the skeletal elements, as seen by the expression of coll2, col10, and ihh. Endochondral cells, as outlined by PECAM staining, were visualized adjacent to the cartilage. A few scattered perichondrial cells next to the hypertrophic cartilage expressed mmp9, and cells in the resting zone of the cartilage, in the perichondrium, and the surrounding mesenchymal tissue expressed low levels of mmp2. Mmp13 was not detected at this stage. Perichondrial cells also expressed mt1-mmp, timp2, and timp3. At e15, the bone consisted entirely of cartilage. In some embryos, vascularization of the peristeal edge of hypertrophic cartilage was evident by PECAM staining, while in others vascularization was not yet apparent, indicating that this process is initiated rapidly. Chondrocytes in the resting, proliferative, and mature zones expressed mmp2. In the areas of terminally differentiating chondrocytes, mmp13 was upregulated. Late hypertrophic chondrocytes weakly expressed mmp9 and mt1-mmp, a domain that extended more centrally in the hypertrophic zone, suggesting that chondroclast/osteoclast activity precedes endothelial cell invasion. The perichondrium and surrounding mesenchymal cells expressed timp2 and timp3, although cells between the early and late hypertrophic zones only expressed timp3.

In the adult, in the intermediate stage of fracture healing, the soft callus contained areas of hypertrophic cartilage and early woven bone as indicated by coll2, col10, ihh, and oc expression. New blood vessels appeared at the periphery of the cartilaginous regions, as indicated by PECAM staining. The peripheral hypertrophic chondrocytes in the cartilaginous regions of the callus expressed mmp2 and mmp9, including those areas with blood vessel penetration. Mmp13 and mt1-mmp were expressed throughout the callus. The cells at the periphery of the cartilaginous portions of the callus also expressed timp2 and timp3. As the callus matured with an increase in vascularization and ossification, mmp expression also increased, particularly at the interface of the hypertrophic cartilage and the newly formed blood vessels. Late in the repair process, both mmps and timps were downregulated.

Discussion/Conclusion: These data indicate that a variety of ECM-degrading molecules are involved in the process of endochondral ossification during both fetal skeletogenesis and adult fracture repair. Although the function of these molecules may be redundant in some cases, distinct patterns of mmp and timp expression during vascular invasion of the hypertrophic cartilage indicate that these molecules also have different functions in endochondral ossification. Therefore, analyzing the individual roles of each of the ECM degrading enzymes and their in vivo substrates will be critical to understanding the complex processes of ECM remodeling, angiogenesis, and osteogenesis. We are currently determining the function of these enzymes by using mice with null mutations in mmps. This work demonstrates similarities between fetal and adult bone formation, further supporting the hypothesis that fractures heal by recapitulating the molecular program for fetal skeletal development.

References:

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