Bone Formation Studied by Proton NMR Microscopy

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Introduction: Bone yields very little signal in proton NMR images, but trabecular architecture and hence strength and fracture risk of cancellous bone can be assessed because of the high intensity signal from the intervening bone marrow (1). A few investigators have applied solid state imaging techniques to detect phosphorus-31 nuclei immobilized in the inorganic matrix of bone with some encouraging results (2). In this work, proton NMR imaging has been used to investigate bone formation *de novo* in a mineralizing cell culture system. Small mineral deposits in the cultures do not result in a complete loss of signal and thereby provide a unique opportunity to relate water proton NMR properties to mineral quantity and quality.

Methods: To study the earliest stages of the endochondral ossification process, a three-dimensional mineralizing cell culture system, based on a hollow fiber bioreactor, was developed (3). All NMR microscopy experiments were performed on a Varian Inova spectrometer (Varian, Palo Alto, CA) coupled to a 4.7 T magnet (200.6 MHz for 1H). The radio-frequency probe consisted of a three-turn solenoid coil wrapped around individual bioreactors. The probe was inserted into a radio-frequency resonant circuit. A field-of-view of 8 mm, a slice thickness of 2 mm, and a matrix size of 128 x 128, yielding a nominal in-plane resolution of 62 µm, were used. Throughout data acquisition, bioreactors were maintained under incubator-like conditions (37°C, 5% CO2). Measurements were made of the water proton longitudinal (T1) and transverse (T2) relaxation times, the magnetization transfer rate (km) constant, and the water diffusion coefficient (D), at 4, 5, and 6 weeks after inoculation of bioreactors with hypertrophic chondrocytes isolated from the cephalic half of 17-day-old chick embryo sterna.

The spatial distribution of developing mineral deposits in the cultures was detected with von Kossa and Alizarin Red stains, which gave the locations of phosphate and calcium, respectively. To verify that the mineral deposits in the bioreactor-derived cartilage were hydroxyapatite (HA), tissue sections were prepared using anhydrous techniques and the mineral crystals, detected by transmission electron microscopy, were subjected to selected area electron diffraction and x-ray microanalysis (4).

Results and Discussion: The earliest evidence of mineral formation was apparent in water proton diffusion maps of cartilage 4-weeks post-inoculation. The cartilage tissue was relatively homogeneous ($2.04 \pm 0.22 \times 10^{-5}$ cm^2/s) except for a dark pre-mineralized zone ($1.72 \pm 0.30 \times 10^{-5}$ cm^2/s), which appeared prior to a detectable change in both the water proton T1 and T2 values for the same zone. This result confirms that the water in this cartilage zone is rapidly tumbling but has reduced diffusivity. At week 4 the km values of the pre-mineralized zone (0.73 ± 0.09 s^{-1}) were slightly higher than for cartilage zones that did not mineralize (0.63 ± 0.15 s^{-1}), data suggesting collagen enrichment prior to mineralization. By week 5 mineral deposits had formed in the collagen-rich zones, reducing water proton T1, T2, and D values and increasing km values. These results support the hypothesis that mineralization proceeds by means of a collagen template. In non-calcifying zones, after 6 weeks of growth, water proton T2 values decreased by 13% and water diffusion values increased by 7% compared to the same tissue one week earlier. These changes could be attributed to the formation of small mineral inclusions possibly associated with matrix vesicles which are thought to play a role in the endochondral ossification process.

In summary, NMR images acquired before and after the onset of mineralization of cartilage tissue grown in a bioreactor can provide novel information concerning compositional changes of a cartilage organic matrix as it undergoes mineralization as well as mineralization events themselves.

Acknowledgements: We thank Dr. Iren Horkay (NIH) and Ms. Jennifer Hillyer (NEOUCOM) for the preparation of tissue samples, Dr. William Swaim (NIH) for the preparation of histologic sections, and Mr. Scott Chesnick (NIH) for the radio-frequency probe used in these experiments.

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