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The mechanical consequences of mineralization in fetal bone

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Results

A Kaplan Meier survival analysis showed that the probability of surviving one year after the hip fracture was 0.82. During the year of follow-up, the average survival time per patient was 10.6 months. Utilization of all services increased dramatically after discharge from the initial hospitalization and declined gradually during the year. By the end of the year, direct medical costs were almost as low as before the hip fracture; the direct non-medical and informal care costs were lower. Average monthly expenditures during the 6 months prior to the hip fracture equalled $1,857. The average cost per hip fracture hospital admission was $5,790 using Medicare reimbursement rates and $16,214 using the average per diem charge reported by the AHA. Average monthly expenditures during the first and second months post-discharge equalled $4,259. During the 3rd-6th months post-discharge, the average monthly expenditure was $2,267. During the 7th-12th months post-discharge, the average monthly expenditure was $1,466. A detailed breakdown of cost estimates is presented in Table 2.

Table 2 - Costs prior to and in the year following hip fracture adjusted for mortality (excludes cost of index hospitalization) (Dollars per month)

	Direct Medical	Direct Non-medical	Informal Care
6 mos prior	431	182	1244
1-2 mos	2562	372	1325
3-6 mos	813	262	1192
7-12 mos	539	144	783

Discussion

Hip fractures pose a heavy economic burden on society. In addition to direct medical care costs, the formal non-medical and informal care costs are substantial. Although analyses account for differential survival in the population, they do not account for differences in comorbidity and functioning following the fracture. This may explain why informal care costs between 7-12 months post-fracture are lower than costs for these services pre-fracture. These cost estimates are especially relevant and should be taken into consideration for health care policy and planning.

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THREE DIMENSIONAL POROUS POLYMER-CERAMIC SYSTEMS FOR BONE REGENERATION: THE FORMATION OF MINERAL CONTAINING MATRICES

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Present methods used for the replacement of bone generally center on the use of autografts and allografts. Each has its specific drawbacks. Our interest has been in the design of synthetic systems for the replacement of bone. We report on the fabrication of three dimensional systems of degradable polymers and ceramics able to form mineralized matrices of bone in vitro.

Materials and Methods

3-Dimensional Matrix Preparation

Macroporous 3-dimensional polymer matrices were fabricated using techniques recently described. Briefly, a 40% w/v solution of 50:50 poly(lactide-co-glycolide) (PLAGA) in chloroform was used to suspend particulate hydroxyapatite (HA) and NaCl crystals (150-250 μm in diameter). The w/w ratio of polymer to salt was 1:1 and the ratio of polymer to ceramic was also 1:1. To this suspension, a 1% aqueous solution of poly(vinyl alcohol) was added. The resulting emulsion was cast to a cylindrical mold, air dried for 48h, and vacuum dried for an additional 48h. At this point, the polymer was demolded and the salt leached by immersion in deionized water at 37°C.

Cell culture

Primary osteoblast cultures were established using the method described by Schwartz. Cells were isolated by enzymatic digestion of neonatal rat calvaria and plated onto 75 cm^2 polystyrene tissue culture flasks. Cells were grown to confluence in Ham's F-12 medium (GIBCO), supplemented with 12% fetal bovine serum (Sigma) and seeded onto 3-dimensional matrices at a plating density of $1x10^4$ cells per cm^2, and then cultured for periods ranging from 24h to 21 days.

Formation of mineralized matrix

Cell were grown on the matrix up to 11 days and then incubated for 10 more days in medium supplemented with 10 μM β-glycerophosphate, ascorbic acid, and 5mM glucose. The cells were fixed and stained by the method of von Kossa to visualize mineralized areas.

Electron microscopy

Polymer-cell samples were also prepared and examined with an AMR Model 1000 scanning electron microscope (SEM).

Results

Osteoblast cell attachment and growth were studied on 3-dimensional degradable polymer-ceramic matrices over a 21 day period. The rate of proliferation of cells on the 3- dimensional PLAGA/HA matrices was similar to that of tissue culture polystyrene controls. At day 21, cells were found to retain their typical polygonal morphology and grew in layers. The retention of osteoblast-like phenotype was confirmed in these cells through measurements of alkaline phosphatase and osteocalcin production. The rate of proliferation of cells on the 3 dimensional matrices polymer was similar to that of tissue culture polystyrene controls.

Cells were found to form a mineralized matrix when cultured in the presence of a phosphate donor. Mineral deposits could be visualized in the form of opaque areas throughout the polymer-ceramic system. In control experiments, no mineralization occurred in three dimensional matrices when β-glycerophosphate or cells were absent. This implied that the presence of HA was not essential for mineralization to occur in this system.

Discussion

This is the first study to demonstrate the ability of synthetic bioerodible polymer-ceramic matrices to support bone cell proliferation and mineralization. With its features of biological compatibility and mechanical stability, this 3-dimensional system may ultimately find usefulness as a synthetic bone graft material.

THE MECHANICAL CONSEQUENCES OF MINERALIZATION IN FETAL BONE

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Introduction: Proper understanding of cell processes regulating development of fetal bones is important for the prevention and treatment of musculoskeletal congenital deformatics. The mineralization in long bones commences in the mid-diaphysis, in the center of the primary area of hypertrophic cartilage. From there it progresses, towards the periphery and then longitudinally towards the epiphyses. Mineralization always occurs in hypertrophic cartilage, which separates the gradually expanding mineralized central part from the unmineralized hyaline cartilage. Using *in vitro* organ cultures of fetal mouse metatarsal bones, the mineralization rate *in vivo* was stimulated by intermittent hydrostatic pressures of 13 KPa (1).

The goal of the present study was to evaluate the mechanical consequences of mineralization for the bone, in terms of stiffness, deformations and stresses. The mechanical properties of the unmineralized and mineralized tissues were determined. This data was used for a FE analysis to simulate the *in vitro* organ culture experiment and evaluate stress and strain signals which may potentially stimulate the mineralization process.

Methods: Six to ten 16 to 17 day old embryos were harvested from each of three pregnant mice. Under microscopic control, ribs were removed from the embryos, cleaned of adhering soft tissue and transversely cut in pieces consisting entirely of either unmineralized tissue (cartilage plus an osteoid sleeve) or mineralized tissue (calcified cartilage and bone). Pieces of at least 1.5 mm long with reasonably small longitudinal curvature were mechanically tested in four-point bending, using a servo-controlled micro-mechanical system (2). Each bone was tested at least twice at an actuator-displacement rate of 100 μm/s. Average diameter, force and displacement were registered and the Young's (bending) modulus was calculated, assumming linear-elastic beam theory for a solid circular cross section. Later, using FE analysis of the test, correction factors were calculated for the aspect ratio of the specimen (length/diameter), unroundness of the cross section (ellipticity), longitudinal curvature, and effects of 'sticking' boundary conditions. 3-D FEA models.

Finally, a 3-D FEA model of a whole metatarsal bone, in several stages of the mineralization process, was constructed, using the moduli determined. Assuming 13 KPa hydrostatic external pressure (1), the stress and strain

distributions in both tissues were estimated. The strain-energy density (SED), osteogenic index (3), maximal principal stress and strain, and the maximal shear stresses and strains were evaluated as to their potential roles in providing a mechanical stimulus for mineralization to the cells.

Results: The bending experiments yielded 8 successfull tests with suitable unmineralized rib specimens (average diameter 313 (±61) μm). An average modulus of 1.76 (±1.08) MPa (variation 0.23-3.40) was determined with beam theory. After correcion by the FE model, an average of 1.11 (±0.62) MPa for the unmineralized tissue was estimated.

For the mineralized rib specimens, successfull tests with 9 specimens were performed (average diameter 229 (±45) μm). An average modulus of 250.0 (±145.0) MPa (variation 76.9-557.8) was found with beam theory. After corrections based on the FE-analysis, an average of 117.0 (±62.2) MPa was established.

FE-analysis of the mineralizing metatarsal bone rudiments showed that of the mechanical variables studied the distribution of the maximal shear *strain* (deviatoric strain) was most consistent with the hypothesis that the mineralization process is stimulated by local mechanical signals, owing to its high values and gradients precisely near the hypertrophic cartilage (4,500-9,000 μstrain). SED, osteogenic index (3), principal stress and principal strain distributions did not adequately discriminate the mineralization region. The maximal shear *stresses* were particularly prominent in the already mineralized tissue, and not in the unmineralized cartilage.

Discussion: To our knowledge, this is the first time that mechanical properties of fetal mineralized and unmineralized bone tissue were measured. Due to the small dimensions of the bones and their rapidly expanding mineralization, these measurements are extremely tedious. Admittedly, bending is not the ideal testing mode for the tissues, but by using FEA simulation of the tests at least some of the inaccuracies could be corrected. More serious are the bi-phasic characteristics of the unmineralized cartilage and its associated time-dependent behavior, neglected in these tests. Hence the Young's moduli found should be considered as approximate indications of tissue stiffness.

The values indicate that the stiffness of the unmineralized tissue is similar to articular cartilage and that of the mineralized tissue somewhat lower than cancellous bone. Within a very short period of time, the tissue increases in stiffness by about two orders of magnitude; mechanically speaking a process of 'catastrophic' dimensions. As a result, given continuity in external loads, the deformations in the post-mineralization matrix suddenly reduce roughly by the same order of magnitude, implying drastically reduced mechanical signals to the cells. As noted earlier (3), the interface between the mineralized and unmineralized tissues provides a 'stress riser', due to their distinct differences in stiffness. The facts that this 'catastrophe' coincides with the onset of muscular activity in the embryo (4), that the rate of the mineralization process is susceptible to external mechanical loads in organ cultures (1), and that the chondrocytes in the post-mineralization matrix subsequently die, all indicate a mechanical component in the regulation process.

If this is the case, the FEA simulation of the organ-culture experiments suggest that the maximal shear (deviatoric) strain is the most likely candidate for the mechanical signal, with values well above those proposed to stimulate cells in the hypertrophic zone. The inconsistency of principal stress, strain and SED patterns with the conditions for a mineralization signal suggest a different kind of stimulation than for bone remodeling, to which these variables do correlate. The potential efficacies of shear stresses or the osteogenic index as signals for mineralization, suggested in earlier FEA simulations of the same organ-culture experiments (3), could not be confirmed in this study.

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IMMUNOHISTOCHEMICAL DETECTION OF NONCOLLAGENOUS PROTEINS OF BONE IN HUMAN BONE TUMORS

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INTRODUCTION

Noncollagenous proteins (NCP), which comprise about 10% of the organic component of bone matrix, have recently been the focus of intense investigation because of their potential influence on bone tissue morphogenesis and differentiation[1]. Moreover, since different levels of NCP synthesis are retained by neoplastic bone cells[2], they have been suggested for the differential diagnosis of bone tumors. To ascertain the value of NCP as diagnostic markers of bone tumors, the expression of osteonectin (ON), osteopontin (OP), bone sialoprotein (BSP), and osteocalcin (OC) has been evaluated by immunohistochemistry in 34 cases of bone neoplasms.

MATERIALS AND METHODS

Cases. We have evaluated 2 cases of osteoblastoma, 14 cases of osteosarcoma (2 low-grade central, 4 osteoblastic, 3 chondroblastic, 1 fibroblastic, 2 small cell, and 2 lung metastasis), 8 cases of chondrosarcoma (2 grade I, 3 grade II, and 3 grade III), 3 cases of malignant fibrous histiocytoma, 4 cases of giant cell tumor, and 3 cases of Ewing's sarcoma. Tissue sections were obtained from nondecalcified specimens fixed with 10% buffered formalin and embedded in paraffin.

Antibodies. Polyclonal antibodies against ON (LF-bONII), OP (LF-19), BSP (LF-6), and OC (LF-32) were raised in rabbits and purified as described[3-5].

Immunohistochemistry. The ABC method was used for immunostaining. Samples were pretreated with pepsin (10 min at 37°C). Primary antibodies were incubated overnight at 4°C at the following dilutions: 1:150 for LF-bONII, 1:100 for LF-19, 1:100 for LF-6, and 1:200 for LF-32. Development was obtained with 3-amino-9-ethyl-carbazole and nuclei were counterstained with Gill's hematoxilin.

RESULTS

Among the NCP considered, only BSP immunostaining was present in all the cases, with no apparent variation of the labeling intensity. Osteogenic tumors showed a positive stain for ON, OP, and OC both in cells and in the matrix. Of note is that small cell osteosarcoma showed a lower positivity to these NCP than the other osteosarcoma subtypes. Metastatic osteosarcoma did not show positivity for OC but featured staining similar to that of primary lesions for the other NCP.

In chondrosarcoma, cells but not chondroid matrix were positive for NCP. However, at the perifery of the tumor, where reactive osteoid is formed, positivity for NCP could be detected. Moreover, in this tumor a remarkable difference was observed between grade I and grade II-III lesions, the latter showing higher levels of expression of NCP. Among the other nonosteogenic lesions, only giant cell tumor showed a scattered positivity for ON in mononuclear cells. Of particular interest is that Ewing's sarcoma was entirely negative to ON, OP, and OC, whereas the two cases of small cell osteosarcoma were positive to all three NCP.

DISCUSSION

Immunohistochemical evaluation of ON, OP and OC can be useful for the differential diagnosis between small cell osteosarcoma from Ewing's sarcoma.

In chondrosarcoma, NCP immunostaining may also be helpful for a better determination of the histologic grade, low-grade lesions showing a lower positivity compared to high-grade tumors. The absence of immunostaining in the chondroid matrix may be explained by the role of these NCP in the mineralization via hydroxyapatite binding of bone but not of cartilage[6].

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BONE METASTASIS VIA ARTERIAL INJECTION OF HUMAN BREAST CANCER IN NUDE MICE.

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Purpose: The local and/or systemic mechanisms that preferentially direct metastatic human breast cancer cells to bone are not well