Correlations Between Uptake of Technetium, Calcium, Phosphate, and Mineralization in Rat Tibial Bone Repair

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Technetium-99m- (^{99m}Tc) phosphates are extensively used for detection of bone formation and resorption. The present is a study of ^{99m}Tc incorporation during bone remodeling. Uptake of ^{99m}Tc-labeled phosphate was studied in an animal model of primary osteogenesis following tibial marrow injury and incorporation was correlated to that of calcium-47 (^{47}Ca), phosphorus-32 (^{32}P), and with matrix vesicle calcification. Isotope uptake on Day 6 in the whole bone was increased compared to controls. On this day, an increase in vesicular diameter and distance from the calcified front was previously observed. Technetium-99m-labeled phosphates were detected only in the organic phase. Phosphorus-32 and ^{47}Ca were detected in both organic and inorganic phases. It is suggested that ^{99m}Tc serves as a specific marker to the anabolic phase of remodeling. Increased incorporation of ^{99m}Tc during bone healing indicates enhanced organic matrix formation and not calcification.

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Technetium-99m-labeled (^{99m}Tc) phosphates have served for the diagnostic imaging of a wide range of pathologic bone conditions because of their special predilection for foci of bone remodeling (*1–4*). Three different mechanisms have been suggested to explain the increased radionuclide uptake in active bone sites viz local hypervascularity, interaction with the organic matrix, and incorporation into the mineral phase during the process of calcification (*5*), however, the precise mechanism for ^{99m}Tc uptake has not yet been clarified (*6*). The possibility that local hypervascularity is responsible for the high uptake of the isotope is supported by its abundance in the cartilage bars associated with the vascular loop in the growth plate (*7*). In addition, autoradiographic studies demonstrated that the distribution of pyrophosphate in normal bone reflects the arrangement of the blood supply (*1,8,9*). Other investigators claim that the pyrophosphate (PYP) does not accumulate in cartilage and that both $^{99m}TcO^-$ and ^{99m}Tc-PYP bind to immature collagen (*1,10*).

Incorporation of phosphate into the hydroxyapatite crystals during osteogenesis has been established previously. It was suggested that the binding of ^{99m}Tc-phosphate occurs at the calcifying front and not in the organic matrix (*8,11,12*). Microautoradiographic studies of ^{99m}Tc localization in rabbit bone revealed isotope concentration along mineralization fronts and not in bone cells of a forming or resorbing nature (*13*). It has been demonstrated by a recent study that osteogenesis after injury to the rat tibial bone could serve as a model for the investigation of the uptake mechanism of labeled ^{99m}Tc-phosphate compounds (*14*). This is a highly reproducible model of bone healing, which is triggered by removal of the tibial bone marrow. Tissue regeneration in this model consists of the following stages: on the first week after injury, the bone cavity is occupied by a large quantity of partially calcified young bone trabecules. Further calcification of this primary bone is accompanied by signs of resorption during the second week. The third week is characterized by completion of calcification with a concomitant bone resorption process and restitution of the marrow to its complete maturation on the fourth week (*15–18*).

It has been clearly demonstrated that primary bone formation following rat tibial bone injury is mediated by extracellular matrix vesicle mineralization. These trilaminar membrane-bound organelles were found to participate in calcification of different tissues in normal and pathologic conditions (*19–24*). Matrix vesicles have biochemical properties that serve the process of formation of the initial hydroxyapatite crystal (*25*). According to this hypothesis, the osteoblastic cells secrete empty vesicles. Once in the matrix the vesicles accumulate calcium and phosphate to form hydroxyapatite crystals that rupture the vesicular membrane. Released crystals attach to the forming calcified fronts. Our recent quantitative electron microscopic studies on vesicular behavior render support to this hypothesis.

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The purpose of the present study was to examine the uptake of different labeled technetium phosphate compounds during bone formation and calcification via extracellular matrix vesicles. The uptake of calcium-47 (^{47}Ca) and phosphorus-32 (^{32}P) in the organic and inorganic phases, as well as vesicular behavior served as comparative parameters for the uptake of ^{99m}Tc in this model of newly formed bone (*18–24*).

MATERIALS AND METHODS

Animal Model

A total of 240 albino rats of the Hebrew University "Sabra" strain were used in three separate experiments. The rats weighed between 350-400 grams and were maintained on a free supply of food and water. The bone marrow injury was carried out under ketamine- (Ketalar, Parke Davis, Detroit, MI) induced anesthesia (35 mg/kg body weight i.p.). The proximo-medial aspect of the right tibial bone was exposed 3 mm distal to the knee joint, and a dental burr (size 5/0) rotating at 5000 rpm was used to penetrate through the cortical bone into the marrow cavity. Bone marrow was evacuated by repeated washings with saline introduced into the intrabony space by a cannule. The skin wounds were then sutured. In the sham-operated control rats, tibial exposure was performed without bone penetration and marrow evacuation.

Experimental Design

In each of the three experiments, 80 rats were used. Four groups of 20 rats were injured 21 days, 14 days, 6 days, and 3 days before radionuclide injection. Half of the rats (10 animals) from each time interval, (a total of 40 animals) were then injected with one of the radioactive compounds, while the other half received a second radioactive compound. In each time interval subgroup of 10 rats, four were sham-operated controls.

Radionuclidic Studies

The following compounds were administered subcutaneously in 0.3 ml aliquots: ^{47}Ca 37,000 Bq (1 μCi); $H_3{}^{32}PO_4$ 0.37 MBq (10 μCi), or ^{99m}Tc-phosphate 1.85 MBq (50 μCi). Rats were killed 18 hr after administration of the radiochemicals. Tibial bones were removed, cleaned, weighed, and counted for ^{47}Ca and ^{99m}Tc.

All bones were then demineralized in 10% trichloroacetic acid (TCA) for 6 hr on a reciprocating table rotating at 60 rpm. The TCA extracts (inorganic phase) were sampled for counting, as well as the demineralized bones (organic phase). To confirm that demineralization with TCA removed all of the calcium and phosphorus that was not part of the organic matrix, bones were ashed before and after undergoing demineralization. No significant differences were found between the two methods. The ^{32}P containing demineralized bones were dissolved (50% of the bones before and 50% after extraction) in 2 ml tissue solubilizer (Soluene 350, Packard), bleached with 0.2 ml 30% H_2O_2 and diluted 1 + 49 with Lumax:Toluene 1:3 (Frutarom, Israel) before counting. All samples were counted for 1 min. Decay calculations were not necessary as results were expressed as cpm/mg of injured over noninjured limb. The radionuclides used were: (a) ^{47}Ca in saline (Amersham, England), 200 μCi/mg; (b) ^{32}P-orthophosphate (carrier-free) in 0.1 N HCl (Nuclear Research Center-Negev, Beer Sheva, Israel); (c) Technetium (^{99m}Tc) as pertechnetate, MDP, and PYP kits (Soreq Nuclear Center, Yavne, Israel); and (d) HDP kit (Malinckrodt Diagnostica, Petten, Holland). Counting was performed using a autogamma scintillation spectrometer (Packard) and a liquid scintillation counter (Betamatic, Kontron). In each of the three experiments, a different technetium compound was administered together with either ^{47}Ca or $H_3{}^{32}PO_4$.

Statistical Evaluation

Radionuclide uptake data were approximately assessed by calculating the ratio of the treated-to-control leg uptake for each animal. The mean treatment/control ratio and standard error of the mean (s.e.m.) were calculated for each timed treatment group. The differences between the treated legs and the control leg were analyzed using the Wilcoxon matched-paired test. P levels of <0.05 were considered significant.

RESULTS

Analysis of the uptake of radiolabeled isotopes in the whole-bone specimens revealed a significantly enhanced uptake ratio of all radionuclides (^{99m}Tc, ^{47}Ca, and ^{32}P) on the 6th day reaching 146%, 162%, and 163% of the control side for ^{99m}Tc, ^{47}Ca and ^{32}P, respectively (Fig. 1). Examination of the radionuclide uptake in the organic phase revealed a similar pattern with increases of 165%, 179%, and 177% for ^{99m}Tc-MDP, ^{47}Ca and ^{32}P, respectively, on the 6th day after injury (Fig. 2). However, radionuclide uptake in the inorganic phase revealed increases in the calcium phosphorus uptakes (43% and 61%, respectively) on Day 6, while no increase in ^{99m}Tc-MDP uptake could be detected (Fig. 3). The treatment/control ratios of radiolabel uptake in the sham-operated rats ranged between 1.00-1.13 throughout the study but was not significantly different from 1.

The changes in radionuclide uptakes were noticed

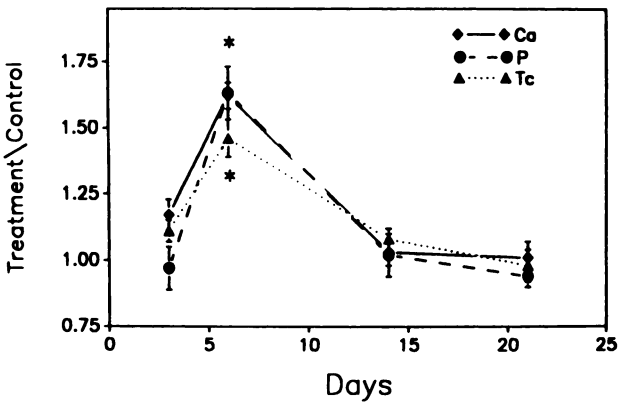

FIGURE 1
The whole-bone uptake of ^{99m}Tc-MDP (Tc), ^{47}Ca (Ca), and ^{32}P (P) uptake at 3, 6, 14, and 21 days of tibial bone healing. The data are expressed as the mean s.e.m. of the treatment/control ratios. *Significant difference between the treated and control legs using the Wilcoxon matched-paired test ($p < 0.05$).

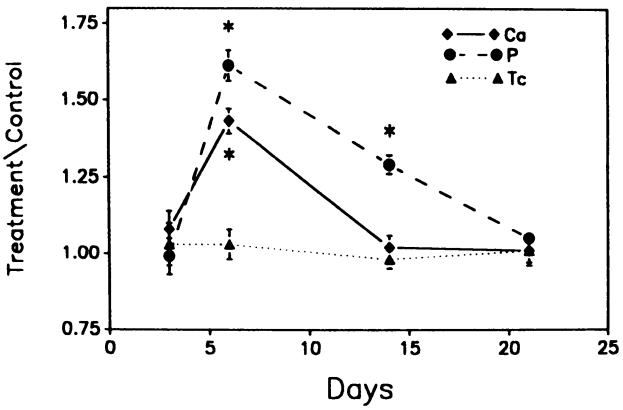

FIGURE 2
Uptake by the inorganic phase of ^{99m}Tc-MDP (Tc), ^{47}Ca (Ca), and ^{32}P (P) uptake at 3, 6, 14, and 21 days of tibial bone healing. The data are expressed as the mean s.e.m. of the treatment control ratios. *Significant difference between the treated and control legs using the Wilcoxon matched-paired test ($p < 0.05$).

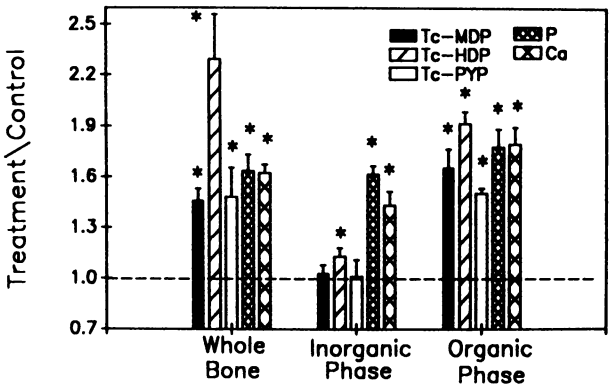

FIGURE 4
A summary comparing the uptake of ^{99m}Tc-MDP, ^{99m}Tc-HDP, ^{99m}Tc-PYP, ^{32}P, and ^{47}Ca (from Figs. 1–3) and data not presented in the text) by the whole bone, the inorganic, and organic phases at the 6th day of healing. The data are expressed as the mean s.e.m. of the treatment/control ratios. *Significant difference between the treated and control legs using the Wilcoxon matched-paired test ($p < 0.05$).

only on the sixth day. Figure 4 summarizes the differences in uptake of the three ^{99m}Tc-labeled phosphates, ^{32}P, and ^{47}Ca in the whole bone, the approximate inorganic, and the organic phases on Day 6. All three ^{99m}Tc-labeled phosphates showed similar patterns of incorporation with significant increases in uptake in the whole bones and the organic phase while no increase in their uptake in the inorganic phase was noted with two of the ^{99m}Tc compounds (Tc-MDP and Tc-PYP). Technetium-HDP, however, showed a small but significant increase in the inorganic phase. In contrast, the ^{32}P and ^{47}Ca uptake increased in all three phases.

DISCUSSION

The results of this study indicate that there is a significant increase in the uptake by the whole bone of all the radionuclides tested on the sixth day after injury. This increase, however, is not similarly distributed between the organic and inorganic constituents of the bone. The ^{47}Ca and ^{32}P increase occurs in both the organic and inorganic phase of the injured bones while the ^{99m}Tc is only increased in the organic phase. The morphologic changes that occur in this model and those that we have reported previously (*19–24*) can be summarized as follows. Three days after evacuation of the marrow, a blood clot is present with no apparent woven bone formation. By the sixth day, there is organization of the clot with extensive cellular proliferation and the formation of small, osteoid-rich trabecules of primary bone with presence of a minimal number of foci of mineralization. At this time point, the matrix vesicles are at their largest diameter and are at the furthest distance from the calcifying front and the number of mature matrix vesicles is in a (tendency of increase). On Day 14, the clot is completely replaced by primary bone. The majority of matrix vesicles are of the mature, hydroxyapatite crystal-containing type and mineralization of this bone is now prominent. By Day 21, the primary bone trabecules are undergoing resorption and new bone marrow is being formed.

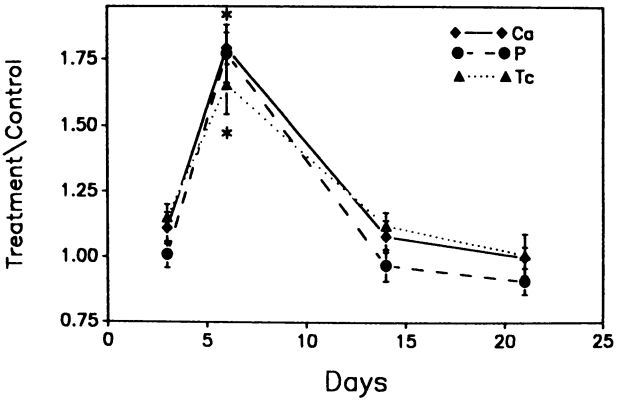

FIGURE 3
Uptake by the organic phase of ^{99m}Tc-MDP (Tc), ^{47}Ca (Ca), and ^{32}P (P) uptake at 3, 6, 14, and 21 days of tibial bone healing. The data are expressed as the mean s.e.m. of the treatment control ratios. *Significant difference between the treated and control legs using the Wilcoxon matched-paired test ($p < 0.05$).

The interpretation of these data suggest that the significant increases in radionuclide uptake occur at the time point when cellular activity and organic matrix production are at a maximal values, just prior to the time point when primary matrix vesicle mineralization becomes predominant. At Day 14, when most of the mineralization has already approximately occurred, and at Day 21 when remodeling is approximately occurring, the uptake of the radionuclides is not different from that observed in the noninjured legs.

The significant increases in the ^{47}Ca and ^{32}P in both the organic phase and the mineral phase of the bone,

probably reflects the incorporation of these two elements into the structural components of the cell and the mineral crystals associated with the matrix vesicles, respectively. The incorporation of the ^{99m}Tc into the organic phase only, and not into the inorganic phase, probably results from the separation of the ^{99m}Tc from its phosphate molecule to which it was bound prior to the phosphates' incorporation into the mineral phase. Alternatively, but less likely, is the possibility that ^{99m}Tc bound to the phosphate prevents its incorporation into the mineral phase. The small but significant uptake of ^{99m}Tc-HDP in the inorganic phase suggests that there might be slight differences in the uptake of different ^{99m}Tc-labeled compounds. These differences could have potential clinical implications.

The observation that the increased uptake of ^{99m}Tc occurs at a time when bone formation is predominant, and before any bone resorption, might indicate that it is a marker of bone formation and that ongoing resorption does not necessarily have to be present for the increased uptake of ^{99m}Tc to occur. The lack of uptake of ^{99m}Tc by the inorganic phase of bone could suggest that although it is a reliable marker for bone matrix formation, as suggested previously (*1,10*), it is not an indicator of bone mineralization activity (*8,11,12*).

In conclusion, these studies tend to support the hypothesis that increased ^{99m}Tc uptake in bone remodeling is associated with bone matrix formation. However, the suggestion that it is associated with local hypervascularity (*7*) can not be excluded.

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