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Methotrexate effects on heterotopic bone in rats

We studied the effects of high-dose methotrexate on heterotopic bone formation induced by implants of demineralized bone matrix in the abdominal wall of growing rats. Methotrexate induced an arrest in normal weight gain of the animals, more pronounced the younger the animals were. The youngest animals had reduced ash weight and decreased isotope uptake in the tibiae and teeth. However, implants from these animals, given methotrexate 10 days before implantation of bone matrix, had a 33 per cent increase in ash content. When methotrexate was given at, or 10 days after, implantation, heterotopic bone formation was reduced by 40 and 22 per cent, respectively, whereas orthotopic bone was considerably less affected in these older animals. In a second experiment, no difference in elimination rates of ^{45}Ca between methotrexate-treated and control rats in implants, teeth, or tibiae were found.

It appears that a less detrimental effect of methotrexate on new bone formation can be expected if the drug is given before, or a substantial period after, surgery requiring bone formation for healing.

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Chemotherapy used in the treatment of skeletal sarcomas (Jaffe & Pald 1972, Jaffe et al. 1974, Rosen et al. 1974, Eilber et al. 1984) may disturb the healing after skeletal reconstruction. The commonly used chemotherapeutic agents methotrexate and adriamycin have been shown to reduce bone formation, both in normal bone (Ragab et al. 1970, Nesbit et al. 1976, Friedlander et al. 1984) and after fracture (Hajj et al. 1981, Friedlander et al. 1983, Pelker et al. 1985). They also delay the incorporation of autografts in segmental cortical defects in dogs (Burchardt et al. 1981).

We have recently reported on the adverse effects of high-dose methotrexate with leucovorin rescue on heterotopic bone, induced by demineralized bone matrix implanted in the abdominal wall of rats (Nilsson et al. 1984). The methotrexate was given simultaneously with the implantation of bone matrix. We now report on high-dose methotrexate given 10 days before, or after, the implantation of the bone matrix, with the aim of reducing the inhibiting effect of cytotoxins on bone induction.

Materials and methods

Demineralized cortical bone matrix implants were prepared from growing male Sprague-Dawley rats as previously described (Bauer et al. 1984, Nilsson et al. 1984). The implants had a mean dry weight of 11 (9–13) mg. The pieces of bone matrix were implanted in muscle pouches in the abdominal wall of male Sprague-Dawley rats. There were six implants per animal.

In *Experiment A*, 32 rats were divided into four equal groups. One group (4-week-old animals) received a single dose of methotrexate 10 days before the implantation of demineralized bone matrix; another group at the time of implantation (5–6-week-old animals); and a third group was treated 10 days after implantation (8-week-old). The fourth group (implanted when 5–6 weeks old) served as controls. The animals were killed 20 days after the implantation of demineralized bone matrix. A single i.v. injection of carrier-free ^{45}Ca (6 μCi/kg body weight) was given 24 hours before death.

In *Experiment B*, 48 4-week-old rats were divided into six equal groups, three receiving methotrexate and three NaCl solution 10 days before implantation of the bone matrix. All animals were given a single i.v. injection of ^{45}Ca 19 days after implantation.

Groups of 8 control and 8 methotrexate-treated animals were killed 20, 26, and 33 days after implantation.

Methotrexate treatment. In ether anesthesia, the inferior epigastric vein was dissected free, and animals in treatment groups were given an i.v. injection of methotrexate 250 mg/kg body weight (Lederle, Cyanamid Nordiska AB, Sweden) and control animals NaCl (0.9 g/l). The wounds were closed with one suture. Exactly 2 hours after the first injection, all the animals were given an injection of leucovorin, 3 mg/kg body weight, in the same vein, also under ether anesthesia. Additional leucovorin was given in the same amount subcutaneously after 6, 24, and 48 hours.

In both experiments, implants, tibiae, and lower incisor teeth were collected, dissected free from adhering tissue and used for determination of ash weight and isotope activity. The tibiae were divided into metaphyseal bone (from the physis to the tibial tuberosity) and diaphyseal bone (from the tuberosity to the tibiofibular synostosis). The implants, teeth, and the two parts of tibiae were ashed in a muffle furnace at 600°C for 24 hours, and weighed. The ash was then dissolved in 3 ml of 1 M HCl and counted in a liquid scintillation counter (Intertechnique L 2000) after addition of 10 ml Aquasol®. All isotope activity was expressed as the percentage of activity of the given dose (μCi/kg body weight). Specific ^{45}Ca activity designates percentage of the given dose per gram ash. The ash weight of the implants was also calculated as a percentage of the dry weight of the implanted bone matrix (w/w).

Three implants from different animals in each group were prepared for histologic examination. Sections were stained with hematoxylin, eosin, and azure.

The mean value of the implants from each animal was calculated. For statistical evaluation, the groups were compared by the Wilcoxon rank sum test.

Results

Experiment A. An arrest in the weight gain of the rats was noted after treatment with methotrexate (Figure 1). The younger the animal, the more pronounced was the effect of the treatment. In the 2 youngest groups, an actual weight loss was seen 4 days after treatment. A rapid gain in body weight then ensued, so that only the group that received methotrexate 10 days before the implantation had significantly lower (10 per cent) body weight than the controls at the end of the experiment. The animals in this group had a mean body weight of about 100 grams at the time of methotrexate treatment. This 10 per cent lower body weight at the end of the experiment was paralleled by a similarly reduced ash weight of tibial metaphyses and teeth (Table 1). The ^{45}Ca uptake was also reduced, even though the methotrexate had been given 30 days before isotope injection. In contrast, the implants from these rats given methotrexate 10 days before implantation of bone matrix had a 33 per cent higher ash content than controls. The ^{45}Ca uptake of these implants was equal to the controls, and as a consequence the ^{45}Ca-specific activity was reduced by 25 per cent (Table 1).

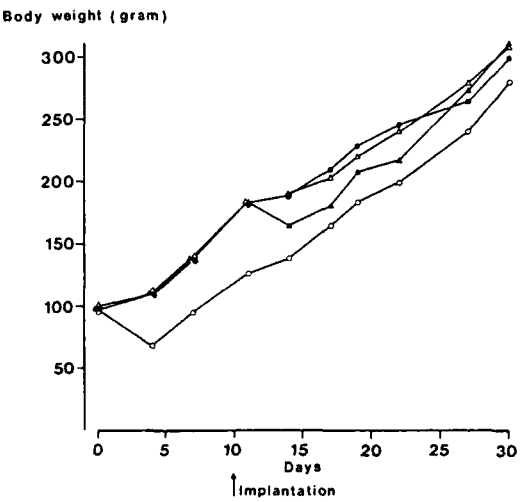

Figure 1. Mean body weights of animals in Experiment A. The symbols refer to groups given: o, methotrexate injection 10 days before implantation of bone matrix (Day 0); ▲, methotrexate injection at implantation (Day 10); ●, methotrexate injection 10 days after implantation (Day 20); and, △ controls.

Treatment with methotrexate at the time of implantation or at 10 days after implantation resulted in decreased ash content of the implants, indicative of a decreased net bone formation (Table 1). This was most pronounced in the former group where an almost 40 per cent lower ash content was noted. The uptake of the isotope at the end of the experiment was equally decreased, giving an unchanged specific activity of the implants.

Experiment B. Methotrexate treatment 10 days before implantation of demineralized

Table 1. Experiment A. Effect of methotrexate treatment at different intervals from implantation of demineralized bone matrix on ash and ^{45}Ca activity of tibiae, teeth, and bone matrix implants recovered 20 days after implantation.

		Control	Methotrexate treatment		
			10 days before implantation	At implantation	10 days after implantation
Tibial metaphyses	Ash	59.1(5.0)	47.3(5.2)***	59.9(3.8)	56.3(5.6)
	^{45}Ca abs.	0.458(0.062)	0.328(0.072)**	0.411(0.067)	0.434(0.108)
	^{45}Ca spec.	7.79(1.15)	6.92(0.89)	6.87(1.10)	7.57(1.37)
Tibial diaphyses	Ash	70.0(5.3)	62.3(8.8)	65.4(2.9)	68.4(4.1)
	^{45}Ca abs.	0.111(0.028)	0.102(0.016)	0.105(0.016)	0.111(0.024)
	^{45}Ca spec.	1.59(0.21)	1.66(0.18)	1.62(0.26)	1.61(0.30)
Teeth	Ash	41.4(1.5)	38.6(1.3)***	38.5(1.9)**	40.5(2.0)
	^{45}Ca abs.	0.106(0.012)	0.079(0.010)***	0.085(0.014)**	0.105(0.020)
	^{45}Ca spec.	2.57(0.34)	2.05(0.28)**	2.22(0.40)	2.62(0.47)
Implants	Ash	4.2(0.9)	5.6(0.6)**	2.7(0.9)**	3.3(1.2)
	^{45}Ca abs.	0.043(0.012)	0.042(0.006)	0.027(0.010)*	0.033(0.015)
	^{45}Ca spec.	10.45(1.39)	7.60(0.99)***	9.23(2.03)	10.15(2.28)
	Ash/matrix	43(9.7)	56(6.6)**	27(8.9)**	34(13.8)

Values are means (S.D.). Eight animals in each group. Ash is given as mg. ^{45}Ca abs. means per cent of given dose (μCi/kg body weight). ^{45}Ca spec. means per cent of given dose/g ash. Ash/matrix denotes (w/w) of ash recovered in the implants per weight unit of implanted demineralized bone matrix. * denotes $P < 0.05$, ** $P < 0.01$ and $P < 0.01$ using Wilcoxon two-tailed rank sum test.

Table 2. Experiment B. Ash weight of diaphyses and metaphyses of tibiae, and of teeth and implants from methotrexate- and control-treated rats at different time intervals after implantation of demineralized bone matrix. Methotrexate was given 10 days before implantation.

	20 days after implantation		26 days after implantation		33 days after implantation	
	Controls	Methotrexate	Controls	Methotrexate	Controls	Methotrexate
Tibial metaphyses	61.0(7.5)	63.1(7.1)	74.2(9.5)	68.0(8.0)	83.9(7.0)	74.2(7.8)*
Tibial diaphyses	52.9(4.1)	48.4(2.5)*	55.1(6.4)	52.7(6.2)	64.4(4.2)	58.2(4.3)*
Teeth	40.8(1.9)	35.5(3.3)**	41.6(2.2)	38.4(2.5)*	44.8(1.9)	41.1(6.1)
Implants	5.6(0.5)	6.7(1.2)*	8.7(0.9)	7.9(0.7)	9.2(0.7)	10.4(1.0)*

Values are means (S.D.) (mg). Eight animals in each group. Statistics were performed using Wilcoxon rank sum test.

bone matrix caused an increase in ash content of the implants when assayed 20 days after implantation, which was similar to what was found in Experiment A (Table 2). This higher ash content became equalized 26 and 33 days after the implantation procedure. There was a continuous increase in ash content of implants from both methotrexate and control groups during the whole observation period. The ash content of tibiae and teeth was only slightly reduced in methotrexate-treated groups.

The resorptive activity in implants, tibiae, and teeth was studied by analysis of the elimination rates of ^{45}Ca. The decrease in specific activity of implants and metaphyses was rapid, indicating high turnover, whereas the specific activity of diaphyseal bone remained almost constant at a low level (Figure 2). Teeth had a slight increase in specific activity of ^{45}Ca throughout the whole experimental period. Methotrexate treatment did not induce any difference in the elimination rates of ^{45}Ca in implants or tibiae.

Histologic examination revealed newly formed bone and small areas of cartilage in implants from both control and methotrexate-treated animals 3 weeks after implantation (Figure 3). Remnants of the implanted bone matrix could be recognized, and the induced bone formation was seen closely adjacent to or in the implanted bone matrix.

Discussion

The bone induction model, employed in the present investigation, provides a useful tool in

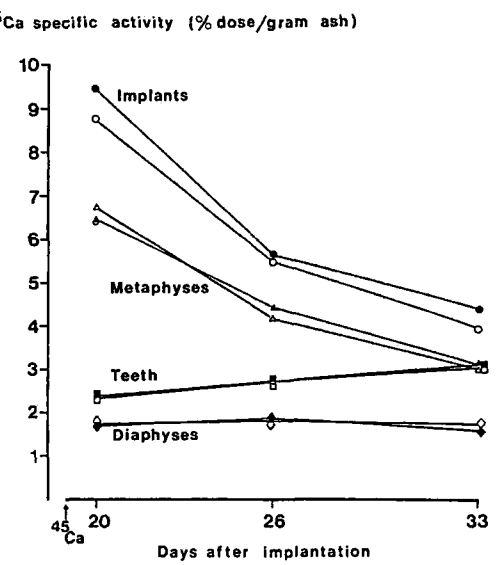

Figure 2. Mean ^{45}Ca-specific activity in implants, metaphyses, and diaphyses of tibiae and teeth, recovered 1, 7, and 14 days after isotope injection in Experiment B. Filled symbols refer to group given methotrexate injection 10 days before implantation, and empty symbols are controls.

bone research. Bone is predictably formed in practically every implant, allowing reliable evaluation, especially since several implants can be placed in each animal. The net bone formed during the experimental period can be estimated with accuracy at any given time since the heterotopic site contained no bone at implantation. Initiation of the inductive process is determined by the implantation procedure and can thus be modified in relation to other procedures, in these experiments treatment with methotrexate. The morphologic and biochemical events of bone induction have been analyzed in detail and are well defined (Urist et al. 1983). The sequence of events preceding the formation of mature bone mimics that of organogenesis of the fetus; undifferentiated mesenchymal cells are induced by a protein called Bone Morphogenetic Protein (BMP), released from the implanted matrix, to proliferate and differentiate into first cartilage and then bone (Urist et al. 1983). An ossicle is formed containing cortical and trabecular bone, and blood-forming cells.

We wished to study to what extent the inhibitory effect of methotrexate on bone formation can be reduced by altering the timing of high-dose methotrexate treatment in relation to initiation of bone induction. We chose to implant the animals at the same age since the age of the recipient affects the amount of bone formed (Irving et al. 1981, Syftestad & Urist 1982). It became apparent from the growth curves that the smaller the animals, the more the treatment affected the growth rate. In rats treated at a body weight of 100 grams, methotrexate initially caused a significant reduction in body weight. Thirty days after a single dose of methotrexate, the body weight and ash content of tibiae and teeth were still not equal to controls. Rates of bone formation at this time were also not normalized, as reflected by the considerable reduction of ^{45}Ca incorporation. Methotrexate given to older rats, weighing 160 grams and 230 grams, caused much milder effects on body weights, teeth, and orthotopic bone, both immediately and after recovery from the acute toxic effects. The age of the animal is thus an important parameter when analyzing the effects of methotrexate on bone.

In agreement with our previous results, high-dose methotrexate treatment at the time of implantation of bone matrix caused reduced amount of ash formed (Nilsson et al. 1984). The inhibitory effect persisted 20 days after the methotrexate treatment, since the ^{45}Ca uptake was similarly reduced. Methotrexate thus caused an irreversible reduction of heterotopic bone formation when the drug was given during the early phases of bone induction. This is similar to the inhibitory effects of irradiation (Craven & Urist 1971) and of indomethacin (Törnkvist et al. 1985). Ten days after implantation of bone matrix, enchondral ossification is normally well under way. Methotrexate inhibited bone formation to a lesser degree when given during this phase of the bone induction process, that is, when proliferation and differentiation to bone-forming cells already have occurred.

The most surprising and interesting result was the 30 per cent increase in ash content of implants from rats given methotrexate before implantation of bone matrix, although these animals had lower body weights and lighter tibiae. This finding was confirmed in Experiment B, in which the implants had 20 per cent higher ash content than controls. The animals

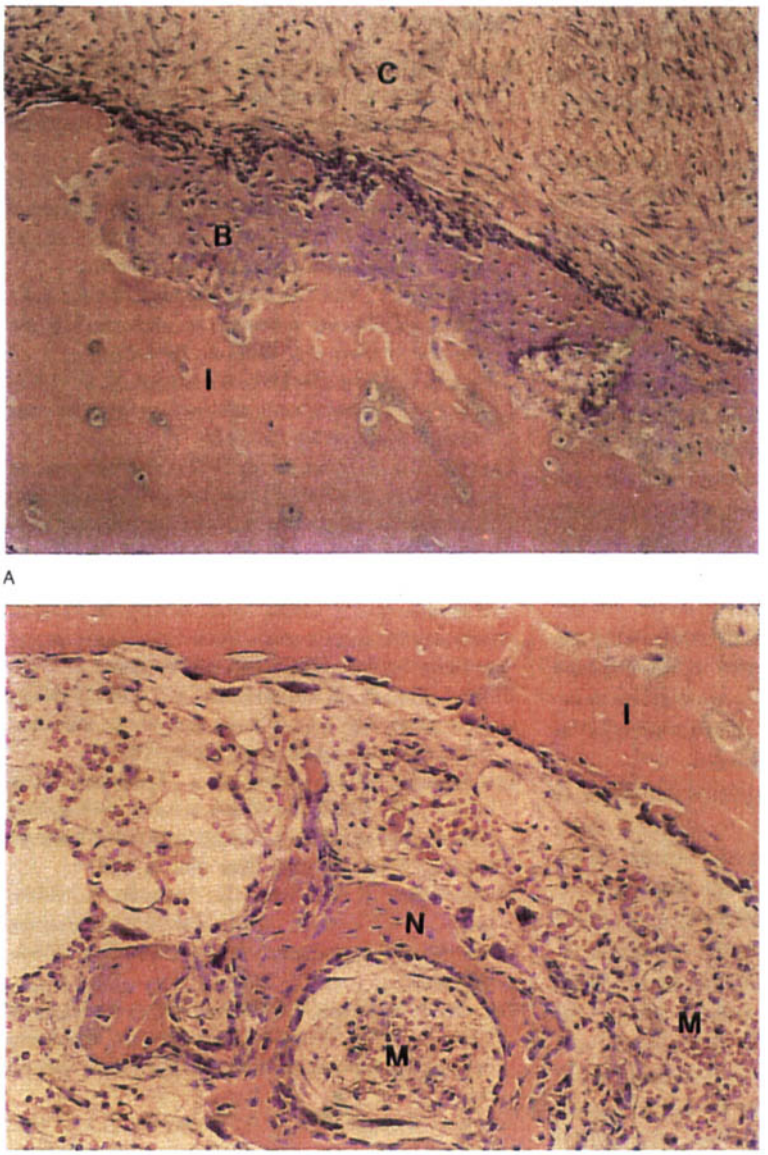

Figure 3. Histologic appearance of bone matrix implant recovered 20 days after implantation. A. Note new bone formation (B), remnant of implanted bone matrix (I) with a few induced chondrocytes, and surrounding connective tissue (C). Demineralized sections stained with hematoxylin-eosin and azure. x80.
B. Detail showing circular trabecula of new formed bone covered with osteoblasts and containing osteocytes (N), remnant of implanted bone matrix (I), and highly vascularized connective tissue with bloodforming cells (M). ×160.

were in an anabolic state 19 days after methotrexate treatment, recovering from the weight loss seen 4 days after treatment. The increase in net bone formed could be the result of this anabolic state. It is, however, questionable whether this recovery from the methotrexate treatment can account for 30 per cent higher ash content, and furthermore, one would expect higher ^{45}Ca activity if new bone formation was occurring more rapidly. Another possible explanation is that methotrexate treatment gives a lower rate of bone resorption, resulting in increased net bone. Heterotropic bone is subject to rapid turnover, at a similar rate as metaphyseal bone of young rats (Bauer et al. 1984), so that reduced bone resorption could lead to a considerable increase in bone mass. Osteoclasts and their precursors of the monocyte phagocyting system are very sensitive to irradiation, whereas osteoprogenitor cells are relatively resistant (Friedenstein et al. 1971, Owen 1978). Methotrexate may also inhibit

the osteoclast precursor cells, whereas the undifferentiated fibroblasts that are induced by BMP would not be affected if methotrexate is given before commencement of the bone-induction process.

Bone resorption in the present study was assessed as the rate of elimination of isotope activity after a single dose of ^{45}Ca as previously described (Bauer et al. 1984). Metaphyses and implants showed rapid elimination of the tracer as a sign of high turnover, whereas diaphyses showed very little resorption. Teeth, not subject to resorption, showed increase in ^{45}Ca activity as a sign of reuse of the isotope liberated by resorption (Bauer et al. 1984). However, no differences in rates of bone resorption in any of the bone compartments studied could be detected 30 days after methotrexate treatment, although reduced bone resorption may have been present at an earlier stage. Because mineralization and bone resorption are not fully developed in the induced bone until 2 weeks after implantation, bone resorption in earlier stages of bone induction is difficult to measure in this model.

In conclusion, bone formation is sensitive to inhibition by methotrexate in normal bone (Nesbit et al. 1976, Friedlander et al. 1984, Pelker et al. 1985), and especially in conditions where recruitment of new bone-forming cells is required, such as fracture healing (Cohen et al. 1975, Hajj et al. 1981, Friedlander et al. 1983) and heterotopic bone (Nilsson et al. 1984). Our experiments confirm these findings and show that bone cells are sensitive to inhibition even when the bone-cell differentiation is well under way. Although we cannot satisfactorily explain the net increase in bone formation seen when methotrexate is given before implantation of bone matrix, it is evident that a less detrimental effect of the antineoplastic drug on bone can be expected when given before or a substantial period after a treatment, such as bone grafting that poses increased demands on bone-forming capacity.

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