Effect of the Antineoplastic Agent Methotrexate on Experimental Heterotopic New Bone Formation in Rats

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ABSTRACT

Heterotopic new bone formation was induced by implanting pieces of demineralized bone matrix in the abdominal wall of 22 growing Sprague-Dawley rats. The animals were divided into three groups and were given, 24 hr after initiation of the bone induction process, a single i.v. injection of methotrexate, 100 or 250 mg/kg body weight, or placebo, followed after 2 hr by leucovorin rescue.

A slight and transient arrest in weight gain was noted in the methotrexate-treated animals. New bone formation during 3 weeks after implantation was analyzed by the amount of ash in implants, and as a measure of bone formation at the end of the experiment, short-time incorporation of 46Ca and [3H]proline was used. The ash content of implants was reduced by 56 and 68% in the two methotrexate groups. Uptake of both nucleotides was greatly reduced in heterotopic bone, whereas metaphyseal and diaphysealibia and teeth were not affected.

The results indicate a pronounced inhibition of methotrexate on bone induction, persisting for at least 3 weeks. Methotrexate reduces the bone-forming potential, with possible consequences for the success of limb-saving surgery and fracture healing.

INTRODUCTION

Recent advances in adjuvant chemotherapy in the treatment of osteogenic, chondrogenic, and other bone tumors have stimulated efforts to perform limb-saving surgery rather than amputations. The bone defects left after local tumor resection often require reconstructive surgery, and this has caused new problems, since in many cases, chemotherapy will have to be administered during the bone-healing period. Chemotherapeutic agents are known to cause weakening of bone, leaving the patients more vulnerable to fractures (8, 10). Fracture healing has been reported to be delayed or even completely inhibited under these circumstances (5).

Fracture healing involves differentiation and proliferation of osteoprogenitor cells (2, 9). Many chemotherapeutic agents used in adjuvant tumor treatment are known to exert effects on rapidly proliferating cells, and a delaying influence by such agents on wound and fracture healing might therefore be expected (3, 12).

As part of a larger project to investigate how effects of adjuvant chemotherapy on new bone formation might be modified, we have in the present study analyzed the effects of methotrexate on experimental heterotopic bone formation. We have utilized the well-established method of inducing heterotopic new bone formation by implanting pieces of demineralized bone matrix in soft tissues of rats (13). With this method, the complete sequence of events involved in new bone formation can be studied. The heterotopic bone is formed by the host tissue cells that proliferate and differentiate under the influence of a bone morphogenetic protein originating from the implanted bone matrix (13).

MATERIALS AND METHODS

To prepare cortical bone matrix implants, femora and tibiae were collected from growing male Sprague-Dawley rats (200 g) (Anticimex, Stockholm, Sweden). The bones were cleansed of soft tissues and demineralized in 0.6 M HCl at 4° for 24 hr. The metaphyses and bone marrow were removed. The bones were cut to approximately the same size, defatted in 1:1 chloroform:methanol at 20° for 1 hr, washed in cold water for 24 hr, freeze-dried, and weighed. The implants had a mean dry weight of 11 mg (range, 9 to 13 mg). The pieces of bone matrix were implanted in small muscle pouches created in the abdominal wall of 22 male Sprague-Dawley rats (175 g). There were 6 implants per animal. The animals were divided into 3 groups: a control group of 6 animals and 2 treatment groups of 8 animals. Surgery was performed in neurolptic analgesia (Hypnorm Vet; Leo, Helsingborg, Sweden). The animals were weaned regularly.

Twenty-four hr after implantation, methotrexate (Lederle, Cyanamid Nordiska AB, Sweden) treatment was commenced. In ether anesthesia, the inferior epigastric vein was dissected free, and the animals were given an i.v. injection of methotrexate (100 or 250 mg/kg body weight) or of NaCl (0.9 g/liter). The wounds were closed with one suture. Exactly 2 hr after the first injection, all animals were given an injection of leucovorin (3 mg/kg body weight) in the same vein, also in ether anesthesia. Leucovorin was then given in the same amount s.c. after 6, 24, and 48 hr.

After 20 days, the rats were given 40 μCi L-[3H]proline and 5 μCi 46Ca/kg body weight in a single i.v. injection as described above. Twenty-four hr later, the animals were sacrificed by decapitation. Implants, tibiae, and teeth were recovered; dissected free from adhering tissue; and used for estimation of ash and dry weight and activity of the 2 nucleotides.

Two implants from each group were prepared for histological examination. After demineralization, they were stained with hematoxylin:eosin and azure. The purpose was to provide evidence that ash and isotope activities represent new bone formation, not to histologically compare the groups.

Three implants from each animal were ashed in a muffle furnace, and ash weights were determined. The ash was then dissolved in 3 ml 1 M HCl and counted in a liquid scintillation counter after addition of 10 ml Aquasol. The remaining 3 implants were incubated in 0.6 μM HCl for 6 hr at 4° to solubilize the mineral. The soft piece of matrix (containing less than 2% of the 46Ca activity) was washed thoroughly in distilled water and put in a counting vial, and 0.2 ml of perchloric acid and 0.2 ml of hydrogen peroxide were added. After the samples were heated to 70°...
for 1 hr, 15 ml of a scintillation solution containing toluol:Cellulose (2:1) and 6 g of PPO/liter of toluol were added, and the samples were counted for determination of $^3$H activity. The supernatant containing the mineral was added to 10 ml of Aquasol and counted. The teeth and tibiae were treated in the same manner; one-half of them were ashed, and the rest were incubated in 0.6 M HCl. The tibiae were first divided into metaphysial bone (from the physis to below the tibial tuberosity) and diaphysial bone (from below the tibial tuberosity to just above the distal physis), and the amounts of ash and activity of the radionucleotides were calculated separately for these 2 portions of bone.

Liquid scintillation was performed in an Intertechnique L 2000 counter. All nucleotide activities are expressed as the percentage of activity of given dose ($\mu$Ci/kg body weight). Specific $^{45}$Ca activity designates percentage of given dose/g ash.

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

RESULTS

A slight weight arrest was noted in all groups the first 3 to 4 days after the operation (Chart 1). The control group and the methotrexate (100 mg/kg body weight) group showed a more rapid weight gain between the fifth and tenth postoperative day than did the methotrexate (250 mg/kg body weight) group. This difference was significant ($p < 0.01$). Body weights became normal within 2 weeks after the initiation of treatment.

The ash content of teeth of the methotrexate-treated animals did not differ significantly from that of the controls, nor was the specific activity of $^{45}$Ca or the ratio of $^{45}$Ca to $^3$H activity affected (Chart 2).

The specific activity of $^{45}$Ca and the $^{45}$Ca:$^3$H activity ratio were not affected significantly by the treatment when calculated for either the metaphyses or the diaphyses of tibiae (Chart 3).

Histological examination of implants revealed newly formed bone trabeculae juxtaposed to the matrix implant (Fig. 1). The bone was colonized by bone marrow. Cartilage was seen in the

The amounts of ash in the implants from the methotrexate-treated animals were 44 and 32% in the 100- and 250-mg/kg body weight groups, respectively, as compared to the controls (Chart 4). This difference was significant at the $p < 0.01$ level. The total $^{45}$Ca activity of the implants was reduced to the same degree. Thus, the specific activity of $^{45}$Ca was approximately the same in all groups.

The absolute activity of $^3$H of the 2 methotrexate groups was 50 and 62% of that of the control group (Chart 4). This difference was significant at the $p < 0.01$ level. The ratio of the absolute activity of the 2 nucleotides ($^{45}$Ca:$^3$H) in the methotrexate-treated groups was approximately one-half of that of the controls. This was attributed to the less pronounced decrease in $^3$H activity induced by the treatment. The difference in ratio was significant ($p < 0.01$).
DISCUSSION

Methotrexate, a folate antagonist, is one of the most widely used chemotherapeutic agents in the treatment of human cancers. With "rescue" techniques that use leucovorin to protect normal tissues against lethal damage, it has been possible to use very high doses of methotrexate in the treatment of tumors, such as osteosarcoma, that do not respond to lower doses (6, 7, 11). High-dose methotrexate therapy can be associated with a number of acute toxic reactions, especially in the pulmonary and gastrointestinal organs, and long-term methotrexate treatment has been reported to cause severe toxic effects, such as liver cirrhosis, osteoporosis, and spontaneous fractures (8). With widened indications for adjuvant high-dose chemotherapy, increased knowledge of the effects of methotrexate on normal tissue becomes imperative.

We have chosen to study the effects of methotrexate on bone metabolism. In earlier studies, the effect of methotrexate on fracture healing in rats was investigated. A single injection of methotrexate did not produce any negative effects, whereas weekly injections, although not producing any detectable changes in femoral bone, delayed fracture healing as assessed by both histological and mechanical methods (5). The dosage was, however, low (1 mg given to mature rats). In another study, Adriamycin or methotrexate did not influence incorporation of autogenous bone grafts in dogs, but new bone formation and bone resorption were reduced (1). A negative effect of antineoplastic agents on wound healing has also been reported (3). These investigations indicate that any antitumor drugs administered at therapeutic dose levels at the time of operation may decrease wound strength and act at different stages of the wound-healing process.

In the present investigation, methotrexate was administered as a single i.v. dose, counteracted after 2 hr by leucovorin. This mode of treatment was designed to simulate the treatment of patients with osteosarcoma (6, 11). A sign of the systemic effect of the treatment was that the normal rapid weight gain of the animals was arrested in the group treated with 250 mg of methotrexate/kg of body weight. However, the animals had regained their weight within 10 to 14 days. Teeth, which are known to be resistant to toxic effects, exhibited no significant change in incorporation of the 2 nucleotides, nor in dry or ash weights, that could be attributed to the methotrexate treatment. The same finding with no evident effect of the antineoplastic agent was noted in orthotopic bone with either rapid (metaphyses) or slow (diaphyses) metabolism. As expected, the former exhibited a much higher uptake of the nucleotides, but the ratio of the nucleotides remained constant in the portions of bone studied, indicating that they represent good markers for organic and inorganic components of bone.

Following implantation of bone matrix in muscle pouches, host mesenchymal cells proliferate and differentiate into chondroblasts. Cartilage is seen after 7 days, and new bone, after 10 days (13). The new bone is colonized by hematopoietic cells and osteoclasts, so that within 3 weeks after implantation, an ossicle is formed. A bone morphogenetic protein, released from the bone matrix implant, is reported to be the inductor of bone morphogenesis (13). In the present investigation, methotrexate was given 24 hr postimplantation, during the proliferation and differentiation stage of bone induction. The ash content of implants was decreased with both doses of methotrexate, which is in agreement with the effect of ionizing radiation on heterotopic bone formation (4). The bone induction process is extremely radiosensitive 2 days postimplantation. The radiosensitivity declines rapidly, so that bone formation is refractory to inhibition by irradiation after the first 7 days after implantation.

The implants do not contain any mineral at implantation. Thus, ash weight gives a measure of the sum of new bone formation and bone resorption during the induction process. \(^{45}\)Ca and \(^{3}\)H activities in the bone 24 hr after injection of these isotopes give the sum of incorporation and elimination of the nucleotides during this period. With this short time, activity may be taken as a measure of incorporation. The ash weight, as well as the incorporation of \(^{45}\)Ca, greatly reduced by methotrexate treatment at both dose levels, and they were decreased to the same degree; thus, the \(^{45}\)Ca specific activity was unchanged. The uptake of \(^{3}\)Hproline was also affected by the treatment but to a lesser extent, and as a consequence, the \(^{45}\)Ca:\(^{3}\)H ratio in the implants was decreased to about one half in the methotrexate groups as compared to the control group.

The results indicate a pronounced effect of a single high dose of methotrexate on experimental heterotopic bone formation, while no effects on bone metabolism in the teeth or orthotopic bone could be detected. The effect of the treatment seems
to persist for at least 3 weeks, since incorporation of both $^{45}$Ca and $[^{3}H]$proline was affected at the end of the experimental period. A less pronounced effect on the uptake of the latter isotope was noted, and this could be interpreted as a specific inhibiting effect by the antineoplastic agent on osteoblastic differentiation.

REFERENCES


Fig. 1. Photomicrographs of bone matrix implant from control animal 3 weeks after implantation. a, area of new bone formation with bone trabeculae (B) and demineralized implant (I). x 80. b, area of chondroosteoid formation with cartilage (C) and implant (I). x 150. Demineralized sections. H & E and azure.
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