The effects of single chemotherapeutic agents on the growing skeleton
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Document Version
Publisher’s PDF, also known as Version of record

Publication date:
2003

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Longitudinal growth

As children treated for childhood malignancies, rats receiving chemotherapy and their diet matched controls all showed a significant reduction in growth in weight and height. The rats receiving MTX or doxorubicin in this study had an additional decrease in tibial length that can not be explained by reduced food intake alone, but must be a side effect of the chemotherapeutic agent itself. Cases of diarrhea were scarce and it is unlikely that malabsorption was a contributing factor to the growth retardation in the drug treated rats.

Although growth persists during MTX treatment, the final length is decreased. The effect is less pronounced than in rats treated with doxorubicin but nevertheless significant.

Cisplatin is well known for its nephrotoxic effect, the accompanying hypomagnesemia affects the skeleton. It causes cessation of bone growth with a decrease in activity of osteoblasts and osteoclasts, decreased bone formation and osteopenia.\textsuperscript{15,20} Cisplatin has a cytotoxic effect on growth plate chondrocytes in cell cultures.\textsuperscript{24} One would expect with all these results to see some influence of cisplatin on length growth. The absence of such an effect in this study can be possibly explained by the relatively low dose cisplatin.

Malnourished rats have smaller livers with a decreased dihydrofolate reductase content. This causes decreased excretion of methotrexate into bile and increased plasma concentrations and toxicity of the drug.\textsuperscript{9,13,14,19} Doxorubicin is inactivated by glycosidase, an enzyme found mainly in the liver and kidney.\textsuperscript{16} Compared to the diet matched controls liver weight in the doxorubicin treated rats was increased, this may well be due to parenchymal degeneration and swelling.\textsuperscript{5} Decreased liver function may increase the serum levels of doxorubicin and the effect of this drug on bone. It is possible that the additional effects caused by malnutrition are the reason we were able to show an effect on length growth of methotrexate and doxorubicin in this study.

In rats treated with cisplatin there was a periodical increase in weight after each dose. Cisplatin is nephrotoxic and the weight increase seen in these rats is due to fluid they accumulate after injection of the drug. Kidney weight in the cisplatin group was reduced compared to the diet matched controls, a sign of nephrotoxicity of cisplatin.

In this experimental rat model, doxorubicin and methotrexate decrease length growth in the rat tibia by respectively 18\% and 5\%. In contrast cisplatin does not affect length growth at all.

In chapter four the histological basis of this decrease in length growth is further clarified.\textsuperscript{31}

Histology of the growth plate and metaphysis

The results in the different diet control groups in this study were not uniform for every parameter but it was clear that the number of hypertrophic chondrocytes per column was increased by malnutrition. Combined with the decreased height of this column, this suggested a smaller cell size of the hypertrophic cells and growth retardation. This is in accordance with the retarded growth length found in these rats.\textsuperscript{13} The effect of malnutrition on trabecular volume is not uniform in the different diet control groups either. The appearance of the metaphysis in the different diet control groups does not seem to differ much from the ad libitum group. Long and possibly less well organized trabecula appear.

When looking at the histomorphometry results the trabecular bone volume is increased in one diet
control group and decreased in another, making it difficult to draw a clear conclusion on the effect of malnutrition on trabecular formation.

Doxorubicin causes thinning of the growth plate and a disturbed columnar arrangement of chondrocytes after treatment. A column of chondrocytes can be seen as a unit resulting from the proliferation of one stem cell. The reduced number of cell columns in the doxorubicin treated rats implies that doxorubicin inhibits stem cell proliferation. A further inhibitive effect on chondrocyte replication can be deducted from the reduced number of proliferating chondrocytes. As chondrocytes are the basis for the formation of trabecula, it is logical that the number of trabecula and trabecular volume in the doxorubicin treated rats is reduced. Doxorubicin not only inhibits stem cell and chondrocyte replication which results in growth retardation in these rats, bone formation is inhibited as well. The excess of the fat cells in the marrow could reflect a selective differentiation of stromal precursor cells down an adipogenic, rather than an osteogenic pathway. This may contribute to the reduction in trabecular bone volume seen in these animals.

Methotrexate is the one chemotherapeutic agent that is most frequently associated with skeletal symptoms and side effects. The height of the proliferating layer and the number of proliferating chondrocytes were not affected by administration of methotrexate. The height of the hypertrophic layer and the number of cells in it were even increased. The rats treated with methotrexate did however exhibit growth retardation. This is not due to changes in the growth plate itself but a consequence of the reduction of trabecular volume. The decreased trabecular bone volume may be due to diminished action of osteoblasts as described by some authors, an increased recruitment of osteoclasts as described by others or a combination of the two mechanisms. In this study bone metabolism parameters were not measured.

Methotrexate is known to cause myelosuppression in rats. The increase in the number of megakaryocytes in the bone marrow of the rats treated with methotrexate in this study could be reactive repopulation after cessation of treatment. The last injection of methotrexate was given 1 week prior to harvesting of the bones and apparently the bone marrow needs limited time to recover from this dose of MTX.

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Both the appearance of the metaphysis and the histomorphometry results show a decrease in trabecular volume and bone content caused by CDDP.

Doxorubicin inhibits proliferation of growth plate chondrocytes leading to growth plate thinning and longitudinal growth retardation. The growth retardation caused by methotrexate is a consequence of the inhibitive effect of methotrexate on the trabecular zone. Cell proliferation in the growth plate

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itself is not inhibited by methotrexate. Cisplatin decreases the height of the proliferating layer of growth plate chondrocytes, but growth plate height itself is not affected. The trabecular volume of the proximal tibial metaphysis is decreased by all three chemotherapeutic agents. Only part of the effect of the chemotherapeutic agents on the growth plate and metaphysis can be explained by the treatment induced malnutrition.

Chapter four illustrates the complexity of the effects polychemotherapy treatment on the growing skeleton. In chapter five the biomechanical consequences of the histological effects become more clear.

**Biomechanical properties**

The resistance of the growth plate to shear forces is determined by the intercellular matrix and not by the chondrocytes. The weakest area is the zone of hypertrophic chondrocytes. In this layer, the intercellular matrix content is the lowest. With increasing growth rate, the growth plate widens, because of an increase of the hypertrophic zone, and therefore becomes more vulnerable to shear forces. Chemotherapeutic treatment may induce malnutrition causing reduced tibial length growth, which may be more pronounced by doxorubicin or methotrexate treatment. In chapter four it was shown that methotrexate caused an increase in the height of the growth plate, but cisplatin did not affect it. Doxorubicin was the only chemotherapeutic agent that caused reduction of growth plate height. Under normal circumstances this would increase the shear strength of the growth plate. The shear strength of the growth plate in the current study was reduced by a decreased surface area and altered maximum shear stress. The surface area of the growth plate is decreased in size by the treatment-induced malnutrition. In the rats treated with doxorubicin, this was caused by fewer rows of chondrocytes, indicating an inhibition of chondrocyte proliferation. Why doxorubicin and methotrexate increase growth plate surface area compared with the malnutritioned groups is unclear. From the altered maximum shear stress it can be concluded that apparently the quantity or quality of the matrix produced by the growth plate chondrocytes is affected. From the results of the current study it is unclear whether this is caused by a change in organic matrix or a change in calcification. There is no explanation for the fact that in the diet control group for the rats treated with doxorubicin the maximum shear stress is increased significantly compared with the ad libitum group. Malnutrition in this group seems to increase the maximum shear stress of the growth plate, in contrast to the other diet control groups in which malnutrition decreased the maximum shear stress as expected. Irregularity of the growth plate is an additional factor influencing the resistance of the growth plate to shear forces. Focal chondrocyte necrosis caused by malnutrition or chemotherapy could potentially explain some of the results. But the study of serial sections of the growth plate as described in chapter four showed this not to be a significant factor.

Body weight is a significant determinant of bone mineral density and bone strength. Intramembraneous bone formation is responsible for the width growth of long bones and the increase of cross sectional area. The latter also is influenced positively by body weight. Malnutrition not only causes growth retardation and a decrease in bone volume but also has been shown to affect the bone mineral content. The combination of impaired architectural arrangement of bone and reduction of bone mineral content decreases bone functional strength.
Rats receiving chemotherapeutic treatment feel sick and eat less, resulting in malnutrition and weight reduction, causing reduction of total femoral and tibial diaphyseal surface area and therefore a decrease of the maximum bending moment. The altered ratio between cortex and medulla, reflected in the decreased bending resistance, was observed in all groups.

In bone the maximum bending stress of bone increases linearly with increasing bone mineral content. The authors may assume that because the maximum bending stress was unaffected in the diet control groups, the bone mineral content in this study was not decreased by malnutrition. Doxorubicin clearly affected the geometrical and biomechanical properties of the growing lower limb of the rat. The maximum bending moment needed to break the femur was decreased significantly because of a decreased bending resistance. The bending stress was increased compared with the diet control group, indicating that bone mineralization was not inhibited by doxorubicin.

As described earlier, methotrexate is known to cause osteopathy in children who are treated with a long term dose for acute lymphoblastic leukemia or in children who are treated with a high dose for osteosarcoma. In chapter five methotrexate did not affect the maximum bending strength although femoral bending resistance was decreased. The other factor determining bending strength, (the maximum bending stress), did not differ significantly from the diet control group.

In chapter five it was demonstrated that the maximum bending moment was not affected by cisplatin; therefore the risk of bone fracture was not increased by this chemotherapeutic agent alone, although the bending resistance was reduced. Similar to doxorubicin the femoral bending stress was increased in rats treated with cisplatin, indicating that bone mineralization was not inhibited by cisplatin.

Treatment with doxorubicin, methotrexate, or cisplatin reduces growth plate shear strength, because of a reduction in surface area and maximum shear stress. These effects are in part explained by the treatment-induced malnutrition, however the single chemotherapeutic agents also affect the skeleton. Doxorubicin and cisplatin reduce maximum shear stress of the proximal tibial growth plate suggesting a change in quantity or quality or both of the intercellular matrix produced by chondrocytes but the exact mechanism cannot be determined from these results. Methotrexate has no effect on growth plate maximum shear stress.

Treatment with doxorubicin, methotrexate, or cisplatin reduces the diaphysis maximum bending moment, therefore there is an increased risk of bone fracture. This is caused by a reduction in bending resistance reflecting the reduced cortex/medulla ratio of the diaphysis. Two factors are responsible for these effects, chemotherapy-induced malnutrition and the accompanying reduction in body weight and the direct effect of the chemotherapeutic agents on the skeleton. Malnutrition causes a decrease in maximum bending moment. The exact target of the chemotherapeutic agents within the bone cannot be determined from the results of the current study. Doxorubicin caused a decrease in bending resistance and bending moment of the lower limb. Both methotrexate and cisplatin caused changes in geometrical and biomechanical properties of the femur that led to a reduced bending resistance. A striking finding is that none of the chemotherapeutic agents in this study inhibited bone mineralization. Doxorubicin and cisplatin increased the maximum bending stress of the bone, indicating a higher mineral content. Methotrexate did not affect bone mineral content.
Fracture pattern
The final objective of this study was to investigate whether the changes in growth plate and metaphysis morphology and strength resulted in an aberrant fracture pattern after shear loading of the proximal tibial growth plate. Fracture is easier when the growth plate is wider. Although Salter first proposed that the fracture line in epiphyseal injury invariably runs through the hypertrophic zone, it was later shown that more often the pattern of failure after the application of shear forces runs through different layers of the growth plate. In chapter six it was demonstrated that in healthy growing male Wistar rats the pattern of failure in epiphyseal injury runs mainly through the transitional zone between proliferating and hypertrophic chondrocytes in the growth plate. There are intact columns of proliferating chondrocytes that are resistant to the shear force and the pattern of failure may vary throughout the growth plate. This pattern is not altered by treatment induced malnutrition as can be concluded from the fracture pattern in the diet control groups. Treatment with doxorubicin does however change the pattern of failure after shear loading of the growth plate. In the doxorubicin treated rats the pattern of failure ran largely through the trabecular zone. Hardly any trabecula were present to resist the shear force causing the fracture. Treatment with doxorubicin weakens the metaphysis in such a way that it appears far less resistant to shear forces than the growth plate itself. This type of fracture is less favorable. Separation of the epiphysis from the metaphysis and its vasculature will result in growth retardation or cessation and accompanying deformities of the long bones.

In the methotrexate and cisplatin treated rats the pattern of failure runs through all layers of the growth plate and the border with the trabecular area. The metaphysis is less resistant to shear forces due to the reduced trabecular volume but this does not result in a type of fracture as seen after treatment with doxorubicin. Both in the methotrexate and the cisplatin treated rats the hypertrophic layer (the weakest zone in the growth plate) is increased in height and the shear stress of the growth plate is reduced. The latter suggests an altered quantity or quality of the growth plate matrix but this does not result in an altered pattern of failure.

In conclusion, chapter six shows that doxorubicin affects the pattern of failure after shear loading of the proximal tibial growth plate. This is due to a reduction in trabecular volume and results in a fracture type more prone to healing difficulties and subsequent growth deformities. Methotrexate and cisplatin do not alter the pattern of failure after epiphyseal injury.

Future perspectives
This study provides insight into the effect of three single chemotherapeutic agents, doxorubicin, methotrexate and cisplatin on the growing skeleton of the rat. Doxorubicin, methotrexate and cisplatin affect the growing rat skeleton in the three areas of most importance: the growth plate, metaphysis and cortex. The conclusions mentioned above are only applicable to the tested doses of the chemotherapeutic agents and the rat skeleton. The observed effects are short term and it remains unclear whether long term effects would be similar. We do however feel that these results may contribute to an explanation for the ever more frequently reported decrease in bone mineral density after completion of chemotherapeutic treatment for childhood cancer. It is clear that not only the effect of the chemotherapeutic agents themselves, but also treatment induced malnutrition exerts an
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important effect on the growing skeleton. All the more reason to be aware of the nutritional state of the pediatric cancer patient. We feel these results warrant long term follow up of children treated with chemotherapeutic agents, with special attention for skeletal complications.

Close monitoring of length growth and bone mineral density during and after chemotherapeutic treatment is necessary. In concurrence with the improving survival rates there is a growing number of reports on decreased bone mineral density after chemotherapeutic treatment for childhood cancer.

We therefore feel it warranted to investigate the possibility and value of the screening of children, treated with chemotherapeutic agents, at the occurrence of a fracture. Total body magnetic resonance imaging (MRI) might be the most appropriate diagnostic tool in detecting further (asymptomatic) skeletal abnormalities, including epiphyseal injuries. When children treated with chemotherapeutic agents and especially doxorubicin, suffer epiphyseal injury after minor trauma, the fracture pattern may be less favorable. Extra care should be taken to recognize healing deformities and longer immobilization may be necessary to allow these fractures to heal and to decrease the risk of growth deformities. The usefulness of screening for skeletal abnormalities and necessity of suppletion of hormonal factors needs to be investigated. Intervention studies are required to see whether suppletion of vitamin D, phosphate and calcium can prevent an increased morbidity from fractures not only in the period directly following treatment but also in later life.