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ALENDRONATE DECREASES TRACP 5B ACTIVITY IN OSTEOARTHRITIC BONE

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Abstract
The activity of a tartrat-resistant acid phosphatase 5B (TRACP 5b), a marker of osteoclast function, was quantified in osteoarthritic bone specimens from patients treated with Alendronate.

Prior to total hip replacement, 12 patients were randomized in a bisphosphonate and a control group. The bisphosphonate group received daily oral Alendronate for 50 days before operation. After operation, the femoral heads were harvested. Samples of the anterior femoral head (A1) and the intertrochanteric area (A2) were taken, analyzed with an immunoassay and stained for TRACP 5-positive-cells.

The immunoassay revealed that TRACP-5b activity of the bisphosphonate group was significantly increased in A1 compared to A2, but not of the control group. Bisphosphonate treatment decreased enzyme activity compared to the controls: 0.41 U/mg vs. 0.31 U/mg in A1 and 0.26 U/mg vs. 0.18 U/mg in A2 ($p<0.05$). The histological examination shows significantly less TRACP-positive cells in bisphosphonate-treated bone sections, confirming the results.

Our data suggest that bisphosphonates reduce TRACP 5b activity in the intertrochanteric area rather than in the anterior femoral head. Consequently, they are more effective in areas of well-supplied bone than in osteoarthritic bone tissue.

Key words: Bisphosphonate; osteoarthritis; TRACP 5b; bone; osteoclast

INTRODUCTION

Bisphosphonates are frequently used in diseases with pathologic bone turnover such as osteoporosis and tumor-induced hypercalcaemia and inhibit bone loss successfully (Berenson et al. 1999, Felsenberg et al. 1998). In bone diseases like osteoarthritis first studies examine a possible benefit of a bisphosphonate treatment (Cohen 2004, Fujita et al. 2001, Hayami et al. 2004, Spector 2003). To evaluate these treatments, pathologic bone samples have to be critically examined to supply evidence of an effect of biphosphonates in bone.

Fujita showed that 80 patients with degenerative joint disease benefited from an Etidronate therapy over a 12 day period (Fujita et al. 2001). A dose related improvement in subjective pain as well as a decrease of subchondral bone mineral density was observed. However, decreased stiffness in the subchondral bone might reduce the progression of osteoarthritis by exposing the overlaying cartilage to less shear stress (Burr et al. 2003, Imhof et al. 1999, Radin et al. 1986). In a canine cruciate deficiency model of osteoarthritis, bisphosphonate treatment was shown to decrease the turnover of subchondral bone. At the same time, it did not affect the severity of cartilage changes (Myers et al. 1999). In contrast, Hayami and collegues ascertain a chondroprotective effect of Alendronate in a similar animal model (Hayami et al. 2004).

Bisphosphonates are known to increase the bone mineral density and reduce fracture risk in diseases with pathologic bone turnover (Black et al. 1993, Felsenberg et al. 1998). Aminobisphosphonates inhibit the farnesyl pyrophosphat synthase of the mevalonic pathway, whereas Etidronate and Clodronate mimic inefficient analogs of GTP. The osteoclasts absorb these drugs while dissolving the bone, slowing down their activity and culminating in apoptosis (Rogers et al. 1999).

Osteoclast activity can be assessed by the measurement of the tartrat-resistant acid phosphatase 5 (TRACP 5) which is produced by osteoclasts and macrophages (Ballanti et al. 1997, Halleen et al. 2000). Isoenzyme 5b is specific to active, bone-resorbing osteoclasts whereas isoenzyme 5a is solely produced in active macrophages (Halleen et al. 2001). Serum levels of TRACP 5b are increased in osteoporosis and tumor metastasis of bone (Halleen et al. 2001) and seem to be inversely correlated to bone mineral density (Scarnecchia et al. 1991). The measurement of TRACP 5b levels in blood samples is useful for monitoring the bisphosphonate treatment. Patients with osteoporosis who received 5mg oral Alendronate per day showed a decrease of 40% serum activity compared to a placebo group (Halleen et al. 2001).

To obtain information about the local effect of bisphophonates on TRACP 5b activity in osteoarthritic bone, we quantified enzyme activity and examined the bone samples histologically. The first objective was to examine the distribution of TRACP 5b to the osteoarthritic femoral head. Secondly, we compared os-

teoclast activity of bisphosphonate treated bone with control samples to elucidate if the blood supply and bone turnover is sufficient enough to incorporate bisphosphonates in osteoarthritic bone.

Material and Methods

Patients

Before undergoing a total hip replacement 12 patients were randomized, after formal consent, in two different test groups.

The bisphosphonate group (Age: 63 ± 11yrs, m = 2, f = 4) received oral 10 mg/d Alendronate over a period of 50 ± 11.2 days before operation. The control group (Age: 70 ± 7 yrs, m = 2, f = 4) did not receive any bone affecting drugs two years before operation.

The patients` physical condition, compliance and possible side effects were observed carefully. All patients took their medication reliably and side effects like gastrointestinal disorders or fever were observed in one patient. After operation, the harvested femoral heads were immediately stored at -80 °C.

Preparation of the Femoral Heads

Specimens were taken out of two defined areas of the femoral head (A1 and A2) (Fig. 1). A1, the femoral head area and A2, the intertrochanteric area. Thereafter, these two bone pieces were sawed in bone chips with a size of 10 mm x 15 mm x 3 mm. The bone chips were either prepared for the histological examination or were grinded with a bone mill to 80-100 µm bone particles after detaching the cartilaginous or synovial parts of the bone specimen. To control particle size, bone flakes were sieved (pore size = 0.1 mm) and controlled for homogenous size under light microscopy and oversized flakes were excluded.

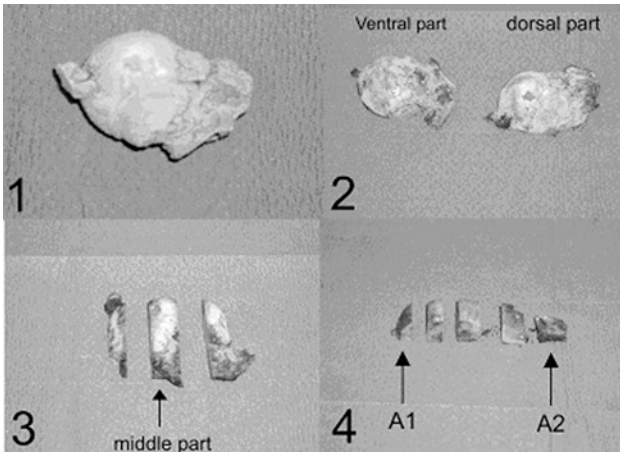

Fig. 1. Method to take the biopsies from the femoral head: 1. After operation, the harvested femoral heads were immediately stored at -80 °C and processed frozen later 2. The head was divided in a dorsal and a ventral part, the latter was used for further samples processing 3. Samples were taken from the middle of the ventral part 4. Arrows label the two areas A1 (Anterior femoral head) and A2 (Intertrochanteric Area) the biopsies were taken from.

The semi quantitative Mankin Score was used to assess the presence and severity of osteoarthritis. This scale is based on the scoring of four parameters: cartilage integrity, cellular features and the intensity of Safranin O present in the matrix and the integrity of the tidemark. The score is progressive (0-14) in that the higher the score the more severe the disease (Mankin et al. 1971). Three sections from each sample were scored by two blinded observers (ATM and HR).

Bone-TRACP5b-ELISA

The bone powder was weighed to 20 – 40 mg portions. Eight specimens were collected perchance from the femoral head area and the intertrochanteric area, respectively. Each sample was incubated under intermittent shaking with 500 µl 0.9% isotonic saline for 10 min at room temperature and was then left for 7 days at 4 °C. The samples were centrifugated at 2500 x g for 4 min at room temperature and 100 µl of the supernatant was analyzed with the TRACP5b BONE ELISA-Kit (Medac Diagnostica, Wedel). To assess the influence of particle size on TRAP 5b activity, we compared enzyme activities in supernatants of differently sized particles from the same bone area using different sieves (pore size = 0.1mm, 0.5 mm and 1mm).

Histochemistry

The prepared bone chips were fixed in 1.4% buffered paraformaldehyde for 24h at 4 °C and dehydrated in ethanol and embedded with methylmethacrylate in plastic jars for 24h at 4 ° C. As a control, samples of a potato and of a heat-denaturated bone tissue were processed. The sections (5 mm) were cut dry at 25 °C on a Polycut E (Reichert) (1.5 mm/s). The slices were pressed for 14 days at 37 °C, and then the acrylat was removed in 100% methoxymethylacetate for 1h.

Demonstration of the acid phosphatase was carried out using simultaneous Azo Dye methods: Burstone`s complete medium was prepared by dissolving Naphtol-AS-BI-Phosphate (Sigma) in N,N-dimethylformamide, followed by addition of 0.2 M acetate buffer (pH 5.0), 50 mM L(+)-tartratic acid disodium salt, Fast Red Violet LB (Sigma) as the coupling agent and two drops of 2% $MgCl_2$ (Burstone 1959, Cole et al. 1987). The tissue sections were incubated for 3h at 37°C. The slides were washed in running water for 30 min, dried by room temperature and counterstained with 1 % Fast Green FCF (Sigma). Coverslips were mounted with Eukitt (ProSciTech) and preparations were examined for presence or absence of a reaction product. The TRACP-positive cells were counted under light microscopy (Figs. 2 and 3) and the sections were characterized regarding to bone structure.

Alternative Staining (Pentachrome / Safranin O / Hematoxylin and Eosin): Sections of three samples of the femoral head area and intertrochanteric area were dyed with a Pentachrome stain to visualize bone structure and new build osteoid (Fig. 3): One femoral head showed a lack of new build osteoid – as a marker of metabolically and functionally active bone – and was excluded from the study. Additionally sections of

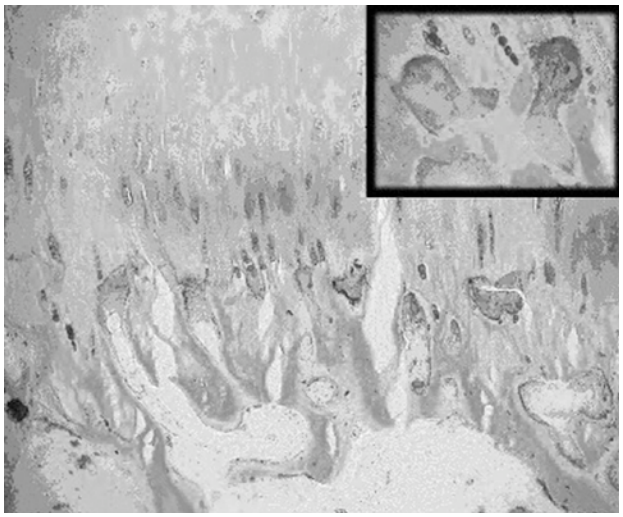

Fig. 2. Biopsies were taken from the Anterior Femoral Head and the Intertrochanteric Area and embedded in methylmethacrylate. TRACP 5-positive cells are stained red, here in the subchondral bone of the Anterior Femoral Head taken from a patient of the control group. Magnified TRACP-positive cells are shown in the black box (Burstone`s Media, 1% Fast Green FCF, 20x / 40x, LM).

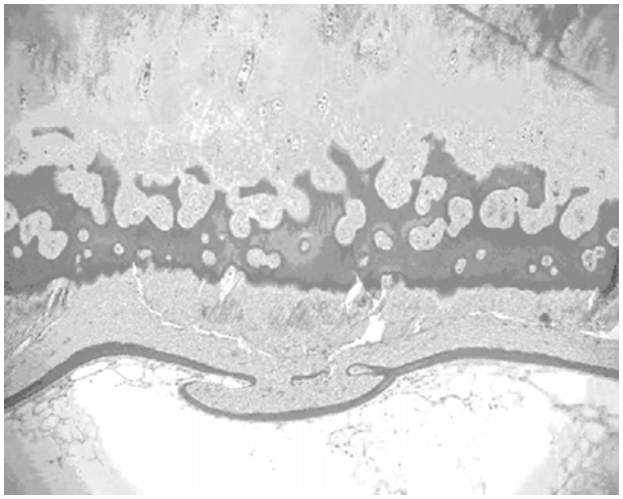

Fig. 3. After embedding, cutting and staining with Pentachrom Stain sections of the subchondral bone plate show a new build osteoid as a sign of anabolic bone turnover. Femoral heads without new build osteoid were excluded from study (Pentacrom Stain, 30x, LM).

three samples of femoral head area and intertrochanteric area were stained with Safranin O and hematoxylin/ eosin to assess the severity of osteoarthritis according to the Mankin scoring system (Mankinet al. 1971)].

Statistical Analysis

Assuming a non-gaussian distribution, non-parametric tests (Mann-Whitney U test, Wilcoxon test) were applied (SPSS statistical program, version 11.5, SPSS Inc. Chicago, USA).

Results

Examination of the Femoral Heads

All femoral heads showed macroscopic signs of osteoarthritis as areas of cartilage decay, osteophytes and a rare bone structure. Histological examinations supplied these observations of an osteoarthritic bone with rare trabecular structure, thin shape of compacta, and a cartilage injury. The examination of the control group revealed a Mankin score of 10.38 ± 1,74 (Mean value ± SDM) and in samples of the bisphosphonate group a score of 9.36 ± 1.26 (Mean value ± SDM) was detected. There was not any significant difference between the Mankin score of the Control Group and the bisphosphonate-treated group.

Quantification of TRACP 5-postitve Cells (Histochemistry)

More TRACP 5-postive cells (1.23 ± 0.56 cells (Mean value ± SDM) / Field Of Vision (FOV)) were counted in the anterior femoral head than in the intertrochanteric area of the control group 0.68 ± 0.27 cells/FOV. In contrast, the cell count in the bisphosphonate group showed 0.62 ± 0.20 cells/FOV in area 1 compared to 0.29 ± 0.20 cells/FOV in area 2 (Fig. 4).

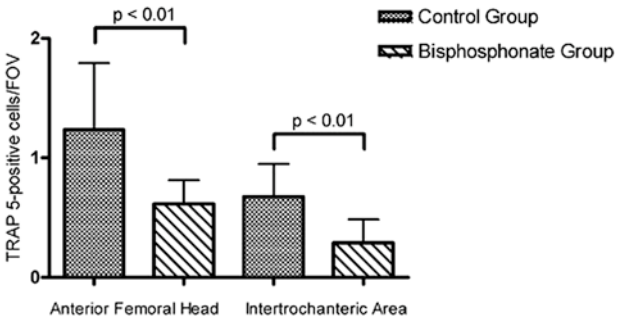

Fig. 4. The samples of both groups, taken from the Anterior Femoral Head and the Intertrochanteric Area, were stained for Tartrat-resistant Acid Phosphatase. The TRACP 5-positive cells were counted under light microscopy and the average number of TRACP-positive cells per field of vision (FOV) of the bisphosphonate-group (BP) was compared to the control group (C)
($p < 0.01$).

In the femoral head area the number of TRACP-positive cells was significantly ($p<0.001$) lower in bisphosphonate-treated-bone (1.23 ± 0.56 cells/FOV) than in bone of the control group (0.62 ± 0,20 cells/FOV). In the intertrochanteric area samples of bisphosphonate-treated bone revealed a number of 0.68 ± 0.27 TRACP-positive-cells per FOV compared to 0.29 ± 0.20 cells per FOV in the control group ($p<0.001$).

TRACP 5b Activity in Bone (Immunoassay)

A variability of about 20% was observed in TRACP 5b activity varying in particle size of the bone flakes

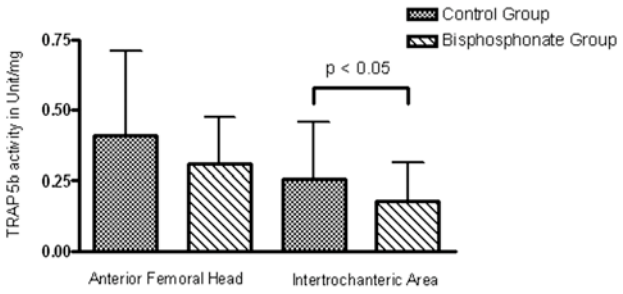

Fig. 5. The samples of both groups, taken from the Anterior Femoral Head and the Intertrochanteric Area, were grinded, incubated with isotonic saline and the supernatant was analyzed with an ELISA for TRACP 5b. TRACP 5b activity is specified in Unit/mg of bone, enzyme activity of the bisphosphonate group is compared to the control group ($p < 0.05$).

(0.1mm, 0.5 mm and 1mm). But repeated measurements of bone samples of the same area and of a homogenous particle size using light microscopy control showed only intra-assay variations about 3-5%. In our control samples – cartilage, heat treated bone and potato – no enzyme activity was detectable.

The analysis of our patient samples showed a homogenous distribution of TRACP 5b activities in arthritic human femoral heads. In the control group, an enzyme activity of 0.41 U/mg bone was measured in the anterior femoral head and 0.26 U/mg in the intertrochanteric area (Fig. 5). A significant difference ($p<0.001$) in enzyme activity between area A1 (0.31 U/mg) and the intertrochanteric area (0,18 U/mg) was observed in the bisphosphonate group only. Comparing these test groups, a decrease of TRACP 5b activity was observed under bisphosphonate therapy: The anterior femoral head showed a TRACP 5b activity of 0.41 U/mg in the control group and 0.31 U/mg in the bisphosphonate group. A significant decrease from 0.26 U/mg to 0.18 U/mg was observed in the intertrochanteric area (Fig. 5).

Discussion

Bisphosphonates are potent inhibitors of osteoclast function. They might be considered as a therapeutic option in the treatment of degenerative bone diseases (Fujita et al. 2001), but there less known about their suggested curing effect in osteoarthritis. For this purpose it has to be elucidated if tartrat-resistant acid phosphatase 5b, a well-known marker of osteoclast function, follows a specific distribution pattern in osteoarthritic femoral heads and if administration with bisphosphonates is an effective treatment.

The local determination of TRACP 5b activity in bone tissue of patients treated with bisphosphonates and of control samples results to a similar decrease of enzyme activity as demonstrated in literature analysing serum samples: A bisphosphonate therapy over a short period (3-5 days) leads to a decrease of 10-20% TRACP 5 activity using a colorimetric assay (Martinez et al. 1997, Pedrazzoni et al. 1995). TRACP 5b-isoenzyme detected with an immunoassay is decreased within a range of 40% in serum samples of osteoporotic patients treated with 5mg/d Alendronate for a period of 3 months (Halleen et al. 2001). The detection of the isoenzyme 5b with a specific antibody is an improvement in using serum TRACP as a marker of bone resorption (Halleen et al. 2000). It has been shown that total serum TRACP 5 activity is an osteoclastic marker of weak sensitivity (Ballanti et al. 1997). Likewise, Napthol-AS-BI-phosphate, a substrate for acid phosphatases, has a high affinity to isoenzyme 5b and can be used for specific biochemical assays and histological examinations of TRACP 5b (Janckila et al. 2001).

This study demonstrates that TRACP 5-positive cells are elevated in biomechanically and inflammatory stressed areas like the anterior femoral head in osteoarthritis. In comparison a smaller cell number of TRACP 5 -positive cells can be found in the intertrochanteric area. Other investigators observed in histochemically stained sections of weight bearing areas various changes of osteoarthritic bone. Metabolic parameters such as TRACP 5 -positive cells were increased in osteoarthritis and these changes were correlated to the severity of cartilage lesions (Reimann et al. 1979). Under Alendronate therapy the number of TRACP-positive cells is decreased in anterior femoral head as well as in the intertrochanteric area. In presence of Alendronate the number of osteoclasts is decreased in cell cultures and osteoclasts undergo finally apoptosis (Benford et al. 2001, Halasy-Nagy et al. 2001).

However, our data reveal that under Alendronate therapy TRACP 5b activity was significantly decreased in the intertrochanteric area only. Alendronate might be rather effective in the well-supplied intertrochanteric area than in the anterior femoral head. Bisphosphonates are delivered into the bone by blood supply and regions with extended parts of well-supplied cancellous bone take up a higher amount of these drugs (King et al. 1997). The anterior femoral head is more affected by osteoarthritis and the degenerative changes lead to a minor physiological blood supply (Ghosh et al. 2001). A limited blood supply results in a low bisphosphonates delivery in these areas. According to our data a significant difference of TRACP 5b activity between the anterior femoral head and the intertrochanteric area was only observed in patients receiving bisphosphonate therapy. These data support the thesis that TRACP 5b activity might be decreased by Alendronate in the intertrochanteric area and leads to a difference of enzyme activity compared to the anterior head area where Alendronate is less effective.

Under a 12-month bisphosphonate therapy a dose related improvement of subjective pain affected by osteoarthritis was observed (Fujita et al. 2001). Our data confirm this author suggesting that relief of pain can not be explained by a local effect of bisphosphonates on osteoclasts of osteoarthritic bone. It is more likely that bisphosphonates modulate inflammatory reactions of the joint which are perceived by osteoarthritic patients with less pain. A chondroprotective effect of bisphosphonates is controversially discussed (Hayami et al. 2004, Myers et al. 1999), but Zoledronate and Aledronate were recently reported to reduce cartilage damage caused by intraarticular injections of chymopapin in the knee joint or in an anterior cruciate deficiency model (Hayami et al. 2004, Muehleman et al. 2002).

We could not observe a significant difference in the Mankin Scores of the Control and the bisphosphonate group. This can be explained by a minor supply with bisphosphonates in osteoarthritic bone, as discussed above. It has been suggested that bisphosphonates should be administered in higher doses for chondroprotection than used for treatment in osteoporosis (Hayami et al. 2004).

Our study concerning local effects of bisphosphonates on osteoarthritic bone has to be enlarged to obtain more valid information. Under bisphosphonate treatment a significant decrease of TRACP 5b activity was observed only in well-supplied bone areas, but not in degenerative regions of the examined femoral head. Thus Alendronate seems not be appropriate to treat osteoarthritis locally by inhibiting osteoclasts but it is more likely that it modulates systemic inflammatory reactions which accompany osteoarthritis. The method described is an approach to evaluate osteoclast function under bisphosphonate treatment in bone tissue affecting osteoarthritis and can help to establish new concepts of anti-resorptive treatments in degenerative bone diseases.

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