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Topography of forming and resorbing cells on endosteal surfaces of the rabbit humerus by double-staining with *in situ* hybridization and tartrate-resistant acid phosphatase reaction: a new model to study the bone reaction to loading

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Since the first investigations made by Wolff, it has been known that bone adapts to mechanical load. The mechanisms which guide the reaction of osteoblasts and osteoclasts to load are still insufficiently known. *In situ* hybridization (ISH) allows the detection of intracellular gene transcripts. Therefore, the ISH technique was further developed to allow the detection of pro-alpha 1 (I) collagen gene transcripts on undecalcified bone surfaces. Additionally, this new technique was combined with the tartrate resistant acid phosphatase technique. The combination of the two methods allows the detection of forming and resorbing cells on the same undecalcified bone surface. In addition, a new animal model was developed to study the reaction of bone to mechanical load. This model mimics the situation of bone implants (e.g. hip prosthese), which is a static situation which is dynamically loaded by the action of the patient.

1. Introduction

Since the first observations in 1869 [1], it has been known that bone reacts to a change of load with adaptation of mass and architecture. With higher loading due to training in sports, bone formation occurs [2]. Bone resorption results from weightlessness, e.g. in space flight, and thus low loading, as well as in the case of confinement to bed [3, 4]. The insertion of implants is often followed by adaptation of the bone trabeculae, depending on the shift of load. The implant surface determines the bone structure. It has been shown that smooth surfaces (roughness RT 1 μm defined according to DIN 4768 and DIN 4762/1E (Rauhtiefe), Deutsches Institute für Normung, 10787 Berlin, Germany) were integrated by development of a bony frame, whereas rough surfaces (roughness > 50 μm) led to direct contact of the bone trabeculae [5]. This is known from hip prostheses: in the case of loosening of the stem the bone reacts by forming a "cage" around the implant. This formation is not seen in tight, anchored stems. The remodelling following implantation seems to be created as a result of the static situation, but the conditions are dynamic because of the movement and actions of the patient. The mechanisms leading to the activation of bone cells have not yet been clarified. Investigations performed to answer this question were done with histological preparations, representing sections of the pertinent tissues. With this technique it is only possible to demonstrate the relation of bone cells with each other in a very small frame, even a series of sections being no more than a strip. Another disadvantage of most of the studies performed was that specimens had to be decalcified to enable conventional sectioning. A further improvement can be expected by surveying the topographical arrangement of cells on surfaces over a greater area.

The model presented here displays endosteal surfaces and the various cells for morphological and histological investigation.

Double staining of tartrate-resistant acid phosphatase reaction (TRAP) for the demonstration of osteoclasts, and *in situ* hybridization (ISH) using a cDNA probe of pro-alpha 1 (I) collagen for the existence of osteoblasts, demonstrates the spatial distribution of these cells.

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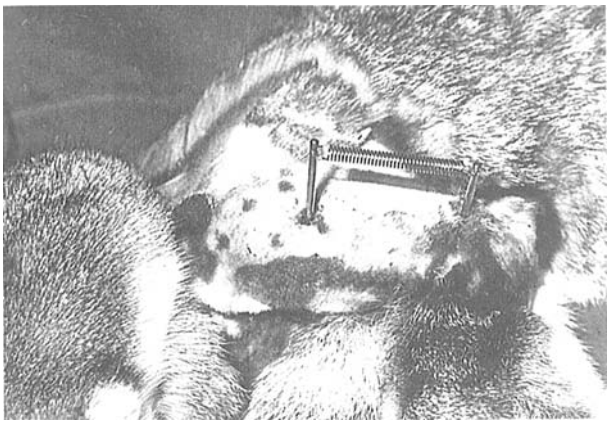

Figure 1 Left humerus of chinchilla rabbit with Schanz' screws and tension spring between; 7 days after operation (tension spring used in pre-trial).

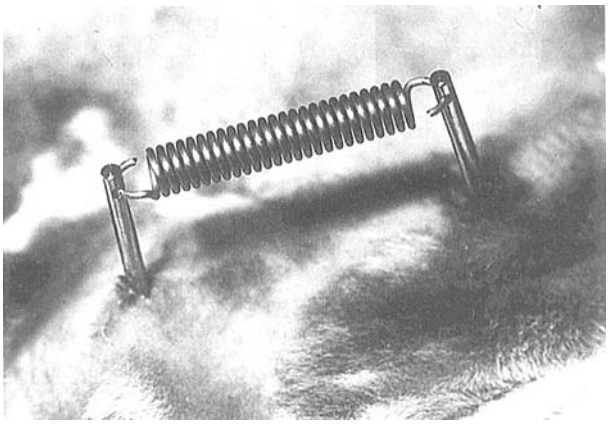

Figure 2 Closer view of another chinchilla rabbit with tension spring acting with 30 N force on the bone (tension spring used in trial).

2. Materials and methods

Female chinchilla rabbits, body weight at the beginning of the experiment between 3.200 and 4.350 kg, from the stock of the Central Animal Laboratories of the Freie Universität of Berlin were anaesthetized with Ketamine and Xylazine (Ketanest® 50 mg/ml, Parke, Davis Co., 10587 Berlin, FRG, and Rompun® 2%, Bayer, 51373 Leverkusen, FRG); in a ratio of 2:1 at a dosage of 1 ml per kg body weight (intramuscular). With a 1.5 mm drill and using a drillgauge strictly from the lateral side via a stab incision, drill holes were placed into the metaphysis of the proximal and distal humerus at a distance of 45 mm. Selfcutting Schanz' screws with a diameter of 2.5 mm made of alloy CrNiMb (ISO 5832-1, DIN 17443, ASTM F138, F139) (Synthes-Mathys Co, 2544 Bettlach, Switzerland) were screwed into the drill holes. The screws had a 10 mm long thread at one end, while at the other end they had a drill hole; their total length was 35 mm. A stainless steel tension spring (Gutekunst & Co., 72555 Metzingen FRG) was attached to the Schanz' screws of one humerus; the contralateral humerus was equipped only with screws but lacked the spring. The tractive power of the spring was chosen according to previously performed *in vitro* investigations in which the forces acting directly on the bone were determined to be 30 N (Figs 1 and 2). *In vitro* the rabbit humerus fractured when a force of 52 to 73 N was applied by the Schanz' screws. Between the screws a maximum strain of 1300 μstrains was produced on the bone surface opposite the spring, and − 1300 μstrains on the surface beside the spring, measured by strain gauges. This load was shown in previous *in vitro* investigations to induce bone formation [6, 7]. The additional force disturbs and changes the physiological distribution of forces at the bone and should lead to cellular reactions.

The animals were housed singly on hay in cages. They were fed standard dry food pellets (Altromin®, Altromin Spezialfutterwerke, 32791 Lage, FRG) as well as water *ad libitum*. They tolerated the trial for 7 days without problems. The housing conditions were standardized with circadian day and night rhythm, light for 12h, 60 lux, and soothing background music was used. The roolm temperature was 20 °C and humidity 55%. The animals were moving normally in the cage; they ignored the Schanz' screws as well as the external fixator.

2.1. Preparation of specimens

After 1, 3, and 7 days the animals were sacrificed. Under anaesthesia (as above) the humerus was prepared after incision of the skin down to the lateral intermuscular septum. The meta- and epiphyseal, proximal and distal parts of the bone were not touched, in order not to disturb the blood circulation. Indeed, during the whole explantation arterial blood came out of the central medullary vessel. After removing the tension spring from one side, the segment with the screws was excised with a diamond disc saw which was rinsed with physiological saline solution at room temperature. Between the Schanz' screws, at about 14 mm distance from them, smaller segments of the compact cortical bone were excised and taken from the lateral and medial aspect and immediately snap frozen in liquid nitrogen. The collection of the probes was performed as quickly as possible in order to minimize possible reactions resulting from autolysis due to arrest of blood circulation. The animals were euthanized in deep narcosis by inhalation of CO_2.

2.2. Probe preparation

For the *in situ* hybridization a 1.3 kb fragment of pro-alpha 1 (I) collagen cDNA was subcloned into plasmid pGEM 1 (Promega Biotec, 69117 Heidelberg, FRG). After linearization of the plasmids with either Hind III or Eco R I restriction endonuclease, T7 or Sp6 RNA-polymerase was employed to obtain transcripts of antisense or sense strands. The probes were labelled with (^{35}S) UTP, specific activity 1.2 to 1.4×10^9 cpm/μg [8]. The RNA probes were adjusted to 50–200 bases length by a controlled alkali hydrolysis.

2.3. Tissue preparation

The rabbit humerus segments stored in liquid nitrogen were immersed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7,0, and thawed. The fixation was done for 20 min, there-

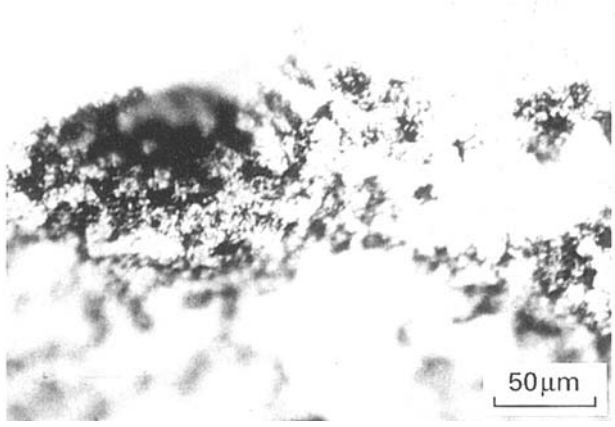

Figure 3 Cluster of cells on medial endosteum with positive signal for pro-alpha 1 (I) collagen in ISH, 3 days after beginning of loading with the tension spring.

after the specimens were rinsed three times for 5 min in PBS, washed once in PBS, then dehydrated for 2 min each, in ethanols of increasing concentration (30, 70, 90, 100% ethanol in diethylpyrocarbamate (DEPC) treated water). The bone segments were put into correspondingly marked biopys cassettes (Tissue Tek®, Miles, Hampshire, England No. 4170). Thereafter the tissue was dried in air at room temperature for 1 h.

2.4. In situ hybridization

Air-dried bone segments were immersed in 0.2 N HCl for 20 min and washed in DECP-H_2O for 5 min. After digestion with 0.125 mg/ml Pronase (Boehringer, 68305 Mannheim, FRG) in PBS for 10 min they were rinsed for 30 s in 0.1 M glycine/PBS and fixed for 20 min in 4% paraformaldehyde/PBS. After rinsing again for 3 min in PBS, bone pieces were acetylated in a freshly prepared solution of acetic anhydride, diluted 1:400 in 0.1 triethanolamine, pH 8.0, for 10 min. Dehydration in graded ethanols followed a washing step in PBS for 5 min, then again air-drying.

Hybridization mixture, 25 µl (50 µl formamide/10 µl 100 mM diothiothreitol (DTT)/10% dextran sulfate/10 mM Tris-HCl, pH 7.5/10mM $NaPO_4$, pH 6.8/0.3 M NaCl/50 mM EDTA/1 × Denhard's solution (0.002% Ficoll 400/0.002% polyvinylpyrrolidone/0.002% bovine serum albumin)/0.2 mg/ml yeast tRNA), containing 2×10^5 cpm of ^{35}S labelled RNA probe, was applied to each bone piece. The bone pieces were fixed on hollow grinding slides, the endosteal surface facing upwards. Hybridization was performed for 18 h at 50 °C in a sealed humid chamber. The hollow grinding slides were covered with RNase-free coverslips.

After hybridization the bone segments were washed for 5 h at 54 °C in a solution of (0.1 M Tris-HCl, pH 7.5/0.1 M $NaPO_4$/0.3 M NaCl/50 mM EDTA/1 × Denhardt's solution/10 mMDTT), changing the solution after the first hour. To decrease background activity, bone pieces were digested for 30 min at 37 °C with 20 µg/ml RNase A in (0.1 M Tris-HCl, pH 7.5/1 mM EDTA/0.5 M NaCl). Further washing with the same buffer, but without RNase was performed for 30 min under the same conditions, then rinsing 15 min in 2 × SSC (1 × SSC = 150 mM NaCl/15 mM sodium citrate) and 15 min in 0.1 × SSC. Bone pieces were dehydrated in graded ethanols containing DEPC-H_2O and 0.3 M ammonium acetate before overnight air drying.

For autoradiography the bone pieces were dipped into Ilford G5 nuclear emulsion (Ilford, Mobberley, Chesire, UK), diluted 1:1 in 0.6 M ammonium acetate at 42 °C. After 2 h of drying in the vertical position they were stored in light-proof boxes containing potassium hydroxide at 4 °C for 2 days. The exposed bone pieces were developed for 2.5 min in Kodak developer (Kodak, Hemel Hempstead, UK), diluted 1:1 in distilled water, rinsed in 1% acetic acid, fixed in Kodak fixer, diluted 1:4 with distilled water for 3 min and washed with water for 20 min.

2.5. Combined histochemistry/*in situ* hybridization

Tartate-resistant acid phosphatase staining (TRAP) was also performed on endosteal surfaces. The method was based on established histochemical techniques [9, 10], varied by shorter incubation time (see below). It was shown that best results were obtained using first histochemistry, then *in situ* hybridization. Fresh bone pieces, or after storage in fluid nitrogen, were put into Lillie's formaldehyde solution 4%, pH 7.4 for 30 min, then rinsed for 1 min in acetate buffer. The incubation was performed according to [10], but only for 10 min, then rinsed in DEPC H_2O for 1 min and fixed in 4% paraformaldehyde/PBS, rinsed again in 3 × PBS and 1 × PBS each for 5 min. The bone segments were dehydrated in graded ethanols (30, 70, 90, 100%, each 2 min) and dried in air for 30 min. Afterwards hybridization with an incubation in 0.2 N HCl for 20 min was commenced and performed as described above.

3. Results

The endosteal surfaces of rabbit humeri displayed cells which synthesized mRNA for pro-alpha 1(I) collagen. The quantity and distribution of these cells was different according to the varying intervals of the trial, i.e. 1, 3, and 7 days. Additionally there were differences in bones loaded by the force of the spring, those which were only operated on and instrumented by Schanz' screws, and non-operated animals. At the beginning of the trial a more disseminated distribution of the cells was observed, after 3 days the cells were lying in clusters (Fig. 3). The reaction of humeri with screws but without a spring was the same, only weaker and delayed (Fig. 4). After 3 days the intensity and distribution of signals in non-loaded humeri were similar to those in the loaded situation after only 1 day, and after 7 days, unloaded reaction resembled the 3 days loaded reaction.

Double-staining with TRAP and ISH for pro-alpha 1(I) collagen provided a specific topographical relation of the two cell populations, osteoblasts and osteoclasts. Thus, in areas which showed positive signals for the production of pro-alpha 1(I) collagen, cells with positive evidence of tartrate-resistant acid

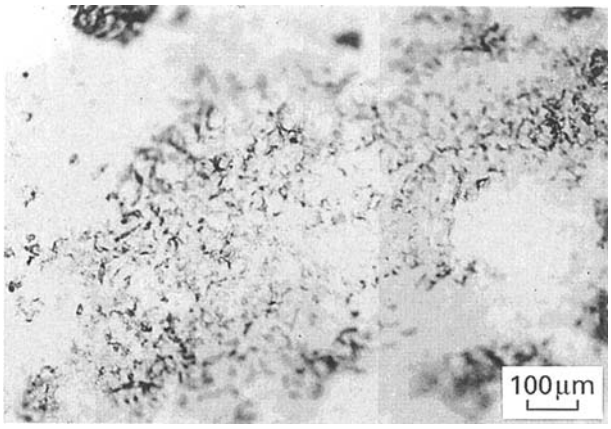

Figure 4 Weaker reaction of cells with positive signal for pro-alpha 1 (I) collagen in ISH on lateral endosteum 3 days after operation but without disturbing the forces at the humerus by use of a tension spring.

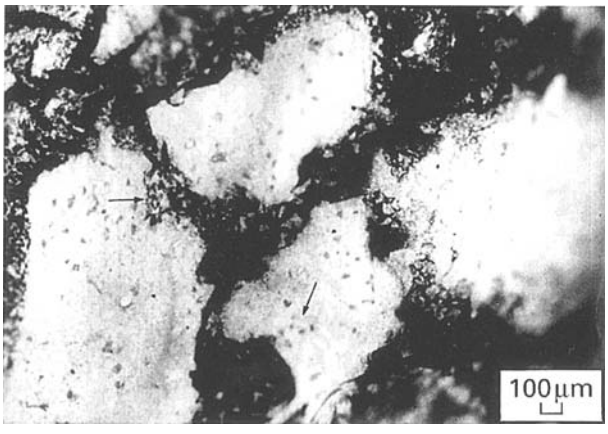

Figure 5 Combined staining of endosteal surface with ISH for pro-alpha 1 (I) collagen (cells with strong positive signal in black areas) and TRAP (single cells with positive signal in light areas) (arrows). No evidence of both cell types (osteoblasts and osteoclasts) in the same area.

phosphatase were never found (Fig. 5). Therefore there are areas of resting cells, areas with resorption being active or inactive, and areas with bone matrix and deposition. This is in accordance with the accepted view of endosteal surfaces.

4. Discussion

In earlier studies the distribution of macrophages on implant surfaces was described [9] and it was found that the performance of implant material and the reaction of the bone was not sufficiently well known to explain the reactions of the bone cells to a change of loading and the resorption of calcium-phosphates contained in implant materials. If a strain of more than 1000 µstrain is produced, the bone reacts in the form of new bone formation [11]. After osteotomy and resection of one of the forearm bones the load on the remaining bone is changed. This bone reacts by altering its mass and geometry according to the new loading situation [12]. The mechanisms leading to a coordination of the bone cells among themselves are not fully understood. Therefore, new techniques should be developed for better insight into these processes. Additionally, in a cell culture system it can be demonstrated that osteoblasts and osteoclasts work in groups or cohort groups and that single cells of both types do not mix [13]. The combination of ISH and TRAP for osteoblast and osteoclast determination corresponds with these *in vitro* findings.

The same type of reaction but smaller in extent in the only instrumented humerus (not disturbed by a tension spring) has a parallel in another investigaton, in which an operation on only one of the tubular bones led to a similar but weaker reaction in the contralateral bone [14].

For the first time this model demonstrates the inner surface of the rabbit humeri and the topographical arrangement of cells in the case of a controlled disturbance of the force acting physiologically on the bone. The establishment of ISH and TRAP on these surfaces alone or in combination as double-staining allows identification of osteoblasts and osteoclasts on the same undecalcified surface. Similar studies on transverse sections of embryonic not-yet-calcified bone have recently been published [15]. TGF β1 gene expression in normal human fracture healing was demonstrated after decalcification of 24 h formaldehyde-fixed specimens, embedded in paraffin [16]. The method presented here, however, clearly points beyond that, as it is possible to study topographically the inner surface of the mature completely calcified bone without previously decalcifying it. The method allows quantification of the cellular reaction by means of histomorphometry using nearly native undecalcified bone surfaces.

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