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Expression of antagonists of WNT and BMP signaling after non-rigid fixation of osteotomies

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ABSTRACT

Delayed fracture healing and non-unions represent rare but severe complications in orthopedic surgery. Further knowledge on the mechanisms of the bone repair process and of the development of a pseudoarthrosis is essential to predict and prevent impaired healing of fractures. The present study aimed at elucidating differences in gene expression during the repair of rigidly and non-rigidly fixed osteotomies. For this purpose, the *MouseFix*™ and the *FlexiPlate*™ systems (AO Development Institute, Davos, CH), allowing the creation of well defined osteotomies in mouse femora, were employed. A time course following the healing process of the osteotomy was performed and bones and periimplant tissues were analyzed by high-resolution X-ray, MicroCT and by histology. For the assessment of gene expression, Low Density Arrays (LDA) were done. In animals with rigid fixation, X-ray and MicroCT revealed healing of the osteotomy within 3 weeks. Using the *FlexiPlate*™ system, the osteotomy was still visible by X-ray after 3 weeks and a stabilizing cartilaginous callus was formed. After 4.5 weeks, the callus was remodeled and the osteotomy was, on a histological level, healed. Gene expression studies revealed levels of transcripts encoding proteins associated with inflammatory processes not to be altered in tissues from bones with rigid and non-rigid fixation, respectively. Levels of transcripts encoding proteins of the extracellular matrix and essential for bone cell functions were not increased in the rigidly fixed group when compared to controls without osteotomy. In the *FlexiPlate*™ group, levels of transcripts encoding the same set of genes were significantly increased 3 weeks after surgery. Expression of transcripts encoding BMPs and BMP antagonists was increased after 3 weeks in repair tissues from bones fixed with *FlexiPlate*™, as were inhibitors of the WNT signaling pathways. Little changes only were detected in transcript levels of tissues from rigidly fixed bones.

The data of the present study suggest that rigid fixation enables accelerated healing of an experimental osteotomy as compared to non-rigid fixation. The changes in the healing process after non-rigid fixation are accompanied by an increase in the levels of transcripts encoding inhibitors of osteogenic pathways and, probably as a consequence, by temporal changes in bone matrix synthesis.

Introduction

The regeneration of bone during fracture healing is a process in which pre- and post-natal developmental programs take place in a highly coordinated manner [1]. Despite the complexity of the process, delayed healing or the formation of cartilaginous pseudarthroses is rare at 2.5% for non-unions and 4.4% for delayed unions in tibial fractures [2]. Though low in number, these complications present severe cases in orthopedic practice due to the debilitating effects on the affected patients and the required extensive restorative surgery.

The formation of a pseudo-joint can be brought about mechanically, i.e. by unstable fixation of an otherwise inconspicuous fracture or by reduced physiological healing capacity due to diseases such as diabetes, inflammatory conditions, and age [3–5]. To improve prediction and treatment of the course of fracture repair, detailed knowledge of the underlying cellular and molecular processes is essential. In the past, four parameters have been defined to be critical for functional bone repair, (i) the local and temporal availability as well as the concentrations of the signaling molecules present at the repair site, (ii) adequate progenitor cells, (iii) the scaffold/extracellular matrix, and (iv) the mechanical stability [6]. When these preconditions are met, the process, mirroring embryonic developmental steps, will proceed to the successful repair of the fractured bone [1,7–9]. Several

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clinical and pre-clinical studies focused on the conditions leading either to normal or delayed healing [10,11]. Clinical studies, however, usually lack sufficient numbers of samples and are hampered by a heterogeneous collective. Animal studies, on the other side, frequently use fracture models with fractures that differ within and between the groups [12].

Investigations on the cellular processes during fracture healing revealed a fixed order of distinct developmental processes to take place [3]. The first step in the healing cascade is characterized by an inflammatory response, formation of a blood clot and attraction of leucocytes. Subsequently, angiogenesis is initiated and mesenchymal progenitor cells are attracted to the site of injury [13,14]. Depending on the stability of fracture fixation, either endochondral or membranous bone formation, both followed by remodeling of the immature woven bone and replacement by lamellar bone are initiated [15,16]. Each of these stages of the repair process is characterized by a specific microenvironment generated by the participating tissues and cell lineages. Inflammatory growth factors dominate during the initial phase of fracture repair. These are subsequently replaced by vasculogenic factors, by factors acting on chemotaxis and differentiation of mesenchymal progenitor cells and lastly, to initiate remodeling of the primary bone, by factors coupling bone formation and resorption [3].

Recently, the AO Development Institute developed an internal fixation system optimized for mouse femora [17]. The system allows the creation of highly reproducible femoral osteotomies with defined edges and an accurate realignment of the bone ends. Furthermore, by using the two different fixation systems, *MouseFix*™ and *FlexiPlate*™, the latter with only one fourth of the mechanical top/bottom stiffness as compared to the former, one component of the key parameter mentioned before, the mechanical stability, could be modified. With the *FlexiPlate*™ System, a delay in bone repair and formation of a stabilizing cartilaginous callus was observed previously [18].

Using these internal fixation systems, we aimed at elucidating the gene expression during healing of osteotomies in mouse femora. The data suggests that an overshooting induction of inhibitors of WNT and BMP signaling pathways may contribute to the observed callus formation and the differences in the healing process of bone defects after rigid and non-rigid fixation.

Methods

Surgical procedures

Female 12 week old *C57Bl/6J* mice (Charles River, Sulzfeld, Germany) were used in this study, which was approved by the local Committee for Animal Experimentation (Bernese Committee for the Control of Animal Experimentation, Bern, Switzerland, permit number 103/07 to MOM) and conducted in accordance with its regulations. The mice were anaesthetized by subcutaneous injections of a mixture of fentanyldihydrogencitrate (50 μg/kg body weight), medetomidine hydrochloridum (500 μg/kg body weight) and climazolame (5 mg/kg body weight). During the surgical procedure, the animals were placed on a heating pad to prevent hypothermia. After shaving and disinfection, a longitudinal incision in line with the femur was made on the lateral thigh. The interval between the *vastus lateralis* and the *biceps femoris* was developed to expose the bone. Subsequently, the *gluteus superficialis* tendon was detached from the *trochanter tertius*. The *MouseFix*™ or *FlexiPlate*™ Systems (AO Development Institute, Davos, CH) were then fixed to the femur with 4 interlocking screws. A mid-femur osteotomy, width 0.22 mm, was created between the 2 central screws using a Gigli saw. Care was taken not to harm the periosteum. Thereafter, the wound was closed.

The experimental groups, each with an $n=3$, were defined as *MouseFix*™ ± osteotomy, and *FlexiPlate*™ with osteotomy. Animals were sacrificed 3 days and 1, 3, and 4.5 weeks after surgery.

X-ray and MicroCT analysis

After surgery and after excision of the femora at the end of the experiment, the integrity of the surgical site was documented by high resolution X-ray (MX-20, Faxitron X-Ray Corporation, Edimex, Le Plessis, France). The tissues assigned to histological analysis were fixed in paraformaldehyde (PFA, 4% in PBS) for 24 h and subsequently transferred into 70% ethanol for MicroCT analysis (MicroCT40, Scanco, Bruettisellen, CH), using the software that was provided by Scanco with the purchase of the MicroCT40 system. For measurements, the long axis of the femur was oriented orthogonally to the axis of the X-ray beam. The X-ray tube was operated at 70 kVp and 114 mA, the integration time was set at 200 ms. Bone repair was evaluated in the area between the two central screws close to the osteotomy. To distinguish between woven and lamellar bone, the tissue was segmented into 3 tissue types based on their greyscale, i.e. <200 for soft tissues, between 200 and 360 for tissues with low mineralization (cartilage callus, woven bone) and >360 for tissues with high mineralization (lamellar bone), according to Gongroft et al. [18]. The analysis was performed at highest resolution with a voxel size of 6 μm.

Histology

After MicroCT analysis, the sections were embedded in MMA as described previously [19]. Thereafter, sections, which were subsequently ground down to approx. 200 μm, were prepared using an annular saw (Leica SP1600, Leica Microsystems, Glattbrugg, CH). The sections were polished and stained using McNeal's tetrachrome [20,21]. Microphotographs were taken on a Nikon Eclipse E800 microscopy system (Nikon Inc., Switzerland, Egg, CH).

Quantitative RT-PCR

To analyze the cellular composition of the repair tissues, quantitative RT-PCR was performed, using pre-synthesized Assays-on-Demand (AoD, Life Technologies/ABI, Rotkreuz, CH). After excision, the tissue of interest was stored in RNAlater (Qiagen, Basle, CH) until use. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Basle, CH) according to the recommendations of the manufacturer. After reverse transcription with MMuLV RT (Roche Diagnostics, Rotkreuz, CH), PCR was performed on an ABI PRISM 7500 System (ABI, Rotkreuz, CH). The reaction mixes contained Taqman Fast Universal PCR Master Mix (ABI, Rotkreuz, CH) and cDNA between 5 ng and 10 ng. The mixes were preincubated for 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 5 sec at 95 °C and 15 sec at 60 °C each. The data was evaluated using the sequence detection software SDS V2.3. The following AoD were employed: COL1A1 (Mm00801666_g1), COL2A2 (Mm01309565_m1), COL10A1 (Mm00487041_m1), ACAN (Mm00545794_m1), ALP-1 (Mm00475834_m1), BGLAP (BGP, Mm03413826_mH), CSF-1R (cFMS Mm01266652_m1), F4/80/EMR-1 (Mm00802529_m1), MYH2 (Mm01332564_m1), GUSB (Mm00446953_m1). The expression of transcripts encoding the members of the WNT family of growth factors was also assessed by quantitative RT-PCR, the respective AoD used in this analysis are listed in Table 3. To compare the transcript levels, the values obtained from rigidly fixed *MouseFix*™ tissues after 3 weeks were set as "1", and the relative expression levels were calculated against *MouseFix*™ 3 weeks.

Low density arrays

Custom made low density arrays (LDA), which were loaded with pre-selected AoD, were used to analyze transcript levels of 9 housekeeping genes for standardization and 87 genes of interest (Suppl. Table 1). Before performing the LDAs, the quality of the RNA was controlled on an Agilent 2100 Bioanalyzer (Agilent, Basle, CH). The PCR