A HISTOLOGICAL INVESTIGATION ON EARLY TISSUE RESPONSE TO TITANIUM IMPLANTS IN A RAT INTRAMEDULLARY MODEL

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KEY WORDS

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Miniature grade 1 titanium screws were inserted in the tibias of 12 mature Wistar rats. Animals were sacrificed at 4, 7, and 21 days postimplantation. A combination of two morphological approaches was used to examine the tissue response to implants. One part of the prepared tissue specimens was routinely embedded in paraffin after a weak decalcification in EDTA. In this case, the implant was mechanically removed before embedding. The remainder of the specimens were embedded in polymethylmethacrylate resin, and ground sections were realized according to Donath's method. New bone formation occurred soon after implantation. Initially, on the fourth day, the presence of osteoid could be observed near the implant surface. On the seventh day, well-mineralized bone tissue was apposed directly on the implant surface. On the 21st day, the bone tissue became a highly organized lamellar bone.

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INTRODUCTION

Brånemark *et al*,[1] has shown that, in humans, osseointegration is achieved 3 or 4 months after implantation and that remodeling takes place over a 1-year period under functional conditions. The rat is an interesting experimental model because the bone formation around the implant is achieved in 28 days.[2,3] Most investigations have focused on long-term osseointegration, reporting that, in the rat, 3 weeks after implantation, the bone–implant interface is characterized by a mature bone directly in contact with the implant.[3–5] In contrast, less information is available regarding the biological events occurring during the early stages of osseointegration. The mechanism through which bone formation occurs on the titanium surface remains controversial. Davies *et al*,[6] showed, by *in vitro* experiments, that bone deposition could begin on the titanium surface. Other authors like Sennerby *et al*,[7] concluded that bone formation could begin to develop toward the implant.

In the rat model, implants are generally inserted in the diaphysis of long bones such as the tibia or the femur. In this case, the implant is in contact with cortical bone, cancellous bone, and bone marrow. Bone formation around the titanium implants in the cortical compartment was described as a sim-

ple remodeling of the preexisting bone.[8] Bone formation around the titanium implant in the medullary compartment was reported as a trabecular repair process or as a *de novo* bone formation occurring after differentiation of new osteoblasts from bone marrow undifferentiated mesenchymal cells.[8]

The purpose of our study was to examine the temporal evolution of cellular and matrix events occurring on the implant surface during the early stages of osseointegration. We focused our investigations on bone formation around the implant in the medullary compartment. The cortical response to the implant is not taken into account.

Materials and Methods

Implants

Commercially pure grade 1 titanium screws, 3.0 mm long and 2.0 mm in diameter (Nobel Biocare, Göteborg, Sweden) were inserted in the tibia of twelve 14-week-old male Wistar rats (Iffa Credo, l'Abresle, France). During experimentation, animals were housed two per cage. They were maintained on a laboratory diet plus water *ad libitum.* The experimental protocol was approved by the local authorities (accreditation number 04930).

Surgical protocol

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium at 50 mg/kg (Sanofi, Paris, France). The hind limbs were shaved and disinfected. A 10-mm-long skin incision was made just below the knee. Muscular dissection was then carried out to expose the anterio-medial part of the tibia diaphysis. The periosteum was carefully removed from the implantation site with a periosteal elevator. A transcortical hole was drilled with a dental handpiece (Wasserman, Germany) fitted with a round cutting bur (size 014, Maillefer, Switzerland) at 1000 rpm. Profuse irrigation was used to cool the surgical site. The hole was tapped to the exact dimensions of the threading with a hand tap (Nobel Biocare, Göteborg, Sweden), and the implant was screwed down in the hole. Primary fixation was confirmed. Closure of the wound site was realized with Vicryl 4/0 (Ethicon, Neuilly, France) for the muscular layer and with American silk 4/0 (Peters, Bobigny, France) for the skin.

The procedure was repeated on the second hind limb. Rats were closely observed, but no antibiotics were given to the animals. The rats remained mobility free.

Preparation of decalcified sections

Animals were sacrificed in groups of four at 4, 7, and 21 days after implantation. They were deeply anesthetized by the same procedure as described above and fixed with an intracardiac perfusion of fixative solution of 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. All of the tibias were excised and immediately immersed in the same fixative solution for 48 hours at 4°C. After rinsing, 500-μm-thick sections of the bone-implant block were done with a low-speed saw (Buehler, Lake Bluff, Illinois) at 6000 rpm. The bones were decalcified in 4% ethylenediaminetetraacetic acid (Carlo Erba, Rodano, Italy) for 2 weeks at 4°C. The implant was mechanically removed. Bone embedding was performed using a routine paraffin-embedding procedure. Then 8-μm-thin sections were cut on a microtome (Jung, Heidelburg, Germany) with a stainless steel blade. The staining procedure was done with Masson's trichrome stain.

Preparation of undecalcified sections

After euthanasia, specimens were fixed with 80% ethanol for 2 weeks at 4°C. Then the tibias were dehydrated using a graded ethanol series and were embedded in polymethylmethacrylate resin. Undecalcified 20-μm-thick sections were prepared using the Exakt cutting-grinding system.[9] The sections were stained with Giemsa and basic fuschin for light microscopy.

Results

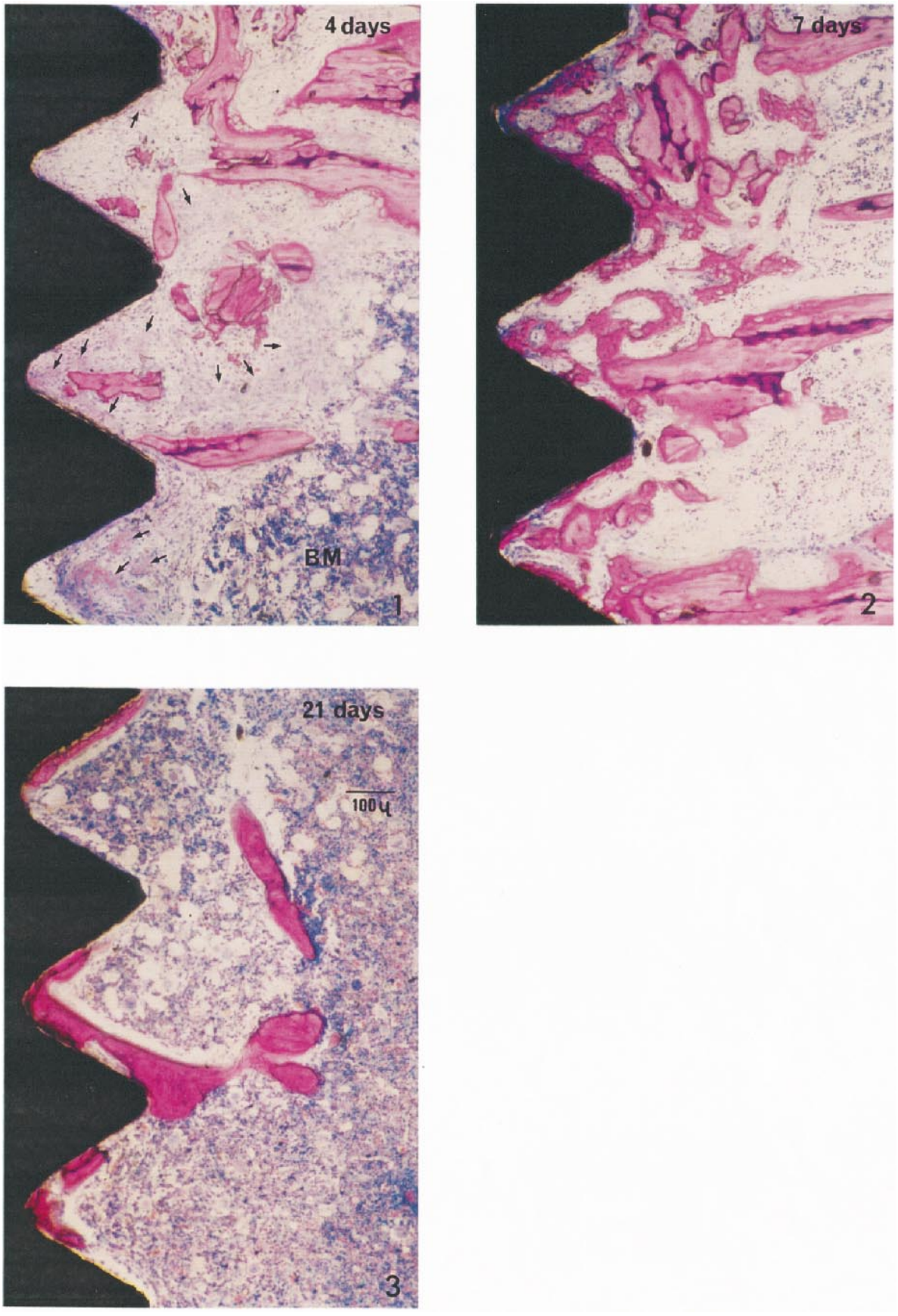
Clinical results

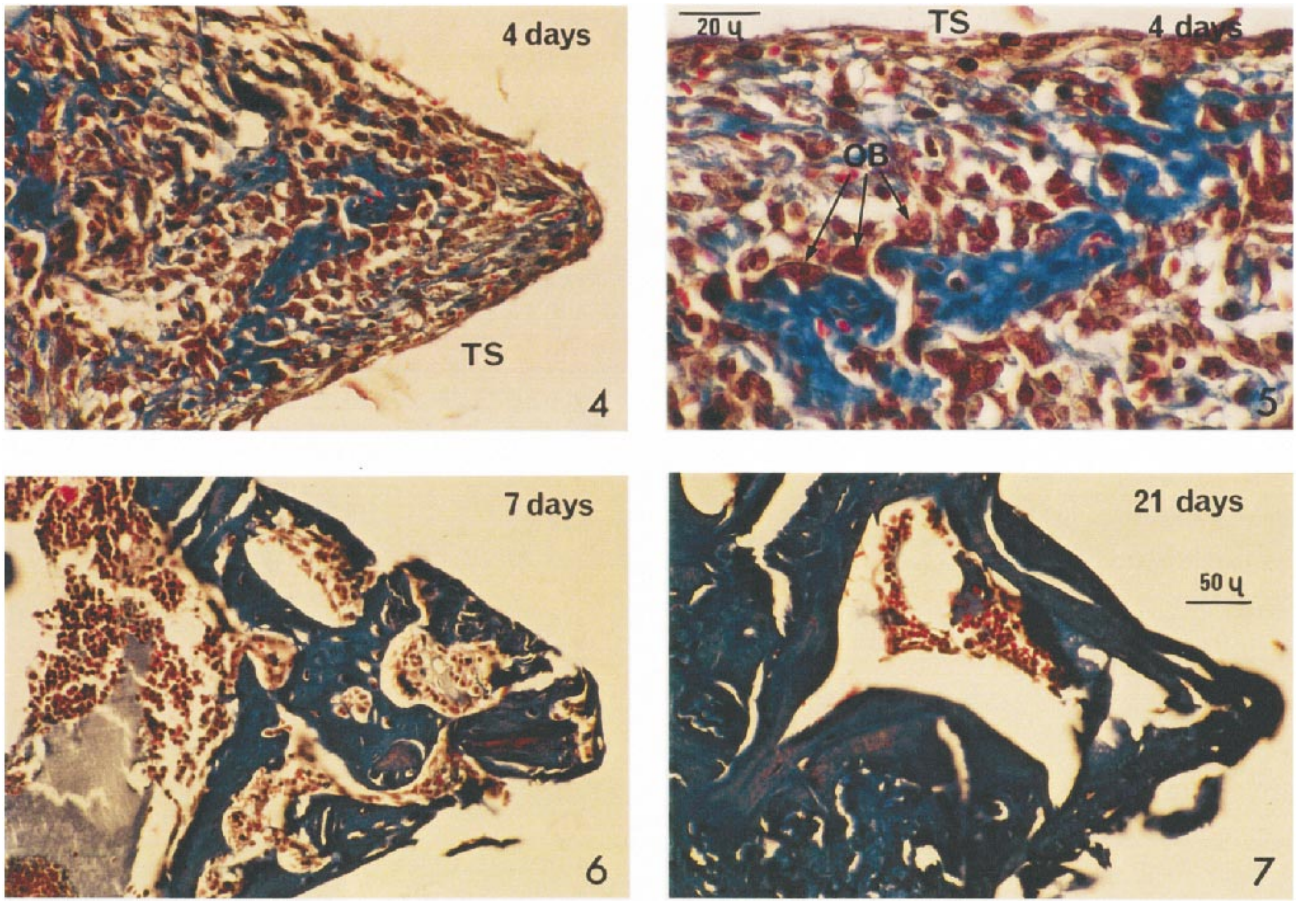
All animals recovered from surgery without any complications. At the time of sacrifice, any inflammatory reaction was observed. A radiograph of each tibia was taken. The top of the implant was inserted in cortical bone while the base of the implant was in contact with cancellous bone and bone marrow.

Four days postimplantation

Undecalcified sections (Fig 1) showed bone debris resulting from the drilling procedure on the implant site. Numerous empty osteocytic lacunae either with degenerated osteocytes or without osteocytes were recognized in this preexisting bone. The tissue facing the implant consisted of a cell-rich connective tissue clearly distinguishable from the bone marrow tissue. The discrimination between these two tissues was based on the differences in location, shape, and staining pattern of cells. The absence of polymorphonuclear cells and multinucleate cells suggested that no acute inflammatory response had taken place in the medullary compartment. Unmineralized tissue, which could be seen as being osteoid, was noted in the vicinity of the implant surface. Sometimes, some forms of mineralized matrix could be observed but never directly on the implant surface.

On the decalcified sections (Figs 4, 5), the presence of newly formed bone around the implant was evident. Islands of osteoid were noted along the implant (Fig 4). These osteoid sites were characterized by a collagen-based matrix lined by cuboidal-shaped osteoblasts (Fig 5). In these osteoid islands, some well-developed osteocytes could be observed in large lacunae. While osteoblastic cells were revealed within this forming bone, they were not found on the implant surface. The microgap between this newly formed bone and the implant threads was filled by a cell-rich connective tissue (Fig 4). A fine and sparse organic matrix appeared to mediate the attachment of these mes-





FIGURES 4–7. Decalcified sections. FIGURE 4. At 4 days postimplantation, newly formed bone could already be observed along the implant. The islands of newly formed bone were enclosed in a cell-rich tissue. TS = titanium screw before removal (magnification ×185). FIGURE 5. At higher magnification (×590), the newly formed bone appeared as a collagen-based matrix (stained with aniline blue) surrounded by osteoblast cells (OB). Some osteocytes could be observed inside these islands of newly formed bone. FIGURE 6. At 7 days postimplantation, the bone around the implant was woven bone. Trabeculae formed a randomly oriented scafflold (magnification ×185). FIGURE 7. At 21 days postimplantation, a lamellar bone was present on the implant surface. Thin trabeculae lay parallel with the implant surface (magnification ×185).

enchymal-like cells. The implant was generally covered by one or several layers of flattened cells (Fig 5).

Seven days postimplantation

Undecalcified sections (Fig 2) showed an important increase in the amount of the bone around the implant. The bone fragments with empty osteocytic lacunae were still present. This preexisting bone was not resorbed. The newly formed bone was, however, clearly mineralized and was apposed directly on the implant surface. This newly formed bone showed a net-like structure of thin bone trabeculae containing osteocytes in lacunae. This bone tissue, however, was not fully organized as lamellar bone. The newly formed bone, which was distinguishable from the preexisting bone by its differential staining pattern, was deposited on the preexisting bone. Mineralized bone was discontinuously found close to the implant surface.

Decalcified sections (Fig 6) illustrated the progression of bone formation around the implant. The osteoid islands observed during the previous stage (fourth day) had increased in diameter and joined together. At 7 days, the newly formed bone looked like woven bone: the trabeculae formed a ran-

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FIGURES 1–3. Undecalcified sections. FIGURE 1. Bone marrow response 4 days after implantation. Numerous debris of preexisting bone could be observed. The cell-rich tissue facing the implant was clearly distinguishable from the bone marrow tissue (BM). Islands of osteoid (arrows) were noted in the neighborhood of the implant (magnification ×97). FIGURE 2. At 7 days postimplantation, there was an important amount of bone around the implant. The newly formed bone developed on the preexisting bone (magnification ×97). FIGURE 3. At 21 days postimplantation, a thin layer of mature bone remained on the implant surface. The bone debris resulting from the drilling disappeared (magnification ×97).

domly oriented scaffold that confined large and numerous intratrabecular bone marrow areas. The trabecular surface was lined with osteoblasts. Large lacunae containing well-developed osteocytes were observed. At the level of light microscopy, the bone matrix was in close contact with the implant surface. Osteoblasts were rarely observed directly on the titanium surface. The prominent microcirculation associated with the early phases of healing was no longer apparent.

Twenty-one days postimplantation

Observation of undecalcified sections (Fig 3) revealed that the implant was surrounded by a thin layer of mature bone. The preexisting bone fragments had been completely resorbed. On the 21st day, the amount of bone around the implant was less important than that observed on the seventh day. However, the layer of newly formed bone close to the implant surface became thicker and lay parallel with the titanium surface. The bone marrow returned to its place and filled all the spaces between the bone trabeculae.

Decalcified sections (Fig 7) showed that the newly formed bone had changed into lamellar bone. The bone structure was highly organized: thin parallel layers of lamellar bone had formed on the implant surface. The osteocytic lacunae were smaller than those from the seventh day. Their long axes were almost parallel to the trabeculae orientation. Some osteocytes could be observed on the bone–implant interface.

DISCUSSION

This investigation on the early events of the osseointegration process adds additional information to data previously collected in rat tibial models. Despite some limitations, this nonfunctional loaded transcortical model has been widely used and can be considered as a standard for testing the biological response to an alloplastic material.[10]

At 4 days, a fine network of osteoid was observed around the implant. These findings confirm those found by Fujii *et al*,[3] and Nanci *et al*,[11] which showed that newly formed bone could already be observed from 5 days. From our results, this new apposition appeared to occur toward the implant surface. Bone formation started with the appearance of osteoid bone, and then gradual formation of mineralized matrix appeared. The initial calcification did not occur on the implant surface. This showed that osteoblasts had not been activated by a direct contact with the titanium surface. According to the study of Neo *et al*,[12] this mode of bone formation suggests osteoconduction. But it has been proven that a single mechanical injury to the bone marrow compartment without implantation could cause a *de novo* bone formation and a substantial increase of cancellous bone.[13,14] This bone reaction was transient and diminished with time.[13,14] Trauma to the marrow compartment might induce osteogenic signals that are local and systemic in nature.[4] Schmid *et al*[15] suggested that opening the vessel-rich medullary spaces would facilitate capillary sprouting and enhance vascular access into the area to be regenerated. For the jaw bone tissue, Lundgren *et al*,[14] demonstrated that, in rabbits, mechanical intervention consisting of drilling of the cortical and cancellous bone resulted in a substantial modification in the bone tissue morphology. The authors observed an increase in the number of bone trabeculae per cancellous bone unit. In short, the drilling procedure enhanced bone formation. In the absence of implants, this newly formed bone would disappear. However, when an implant was inserted in the receptor bone defect, it acted as a stake and remained encapsulated by a thin bone tissue layer.

On the fourth day, one or several layers of slender cells were observed along the titanium surface. These cells could be putative osteoprogenitor cells. The nature of these cells remains to be found by further investigations.

On the seventh day, mineralized, newly formed bone was deposited from top to bottom of the implant surface. This bone appeared to be woven bone. On the 21st day, the bone tissue had changed into lamellar bone. The amount of bone tissue observed around the implant decreased between the 7th and the 21st days. However, the trabeculae became thicker and lay parallel with the implant surface. These observations were consistent with those found by Takeshita *et al*.[13] This demonstrates that the newly formed bone undergoes a remodeling process. In the present study, the implants were not directly loaded, but important mechanical stresses were transmitted to the implant region of the tibia while the rats were moving.

CONCLUSION

In the jaw, the cortical bone is an important region for the implantation prognosis because it ensures the primary stability of the implant. The present study documents the important biological role of the cancellous bone and bone marrow regions. A *de novo* bone formation and apposition around titanium implant can originate from the bone marrow, irrespective of the cortical region. This data could suggest that, from a biological point of view, a low density jaw bone (D4 type) is not an obstacle for osseointegration of dental implants.

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