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Observation of the bone matrix structure of intact and regenerative zones of tibias by atomic force microscopy

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Atomic force microscopy (AFM) was used to comparatively study the structure of the bone matrix of rat tibia from an intact region with that from regions submitted to surgical injury. We used young male adult rats (Wistar), with corporal masses between 250 and 300 g. Each injury was provoked by drilling a 1.5-mm-diam hole in one cortical tibia surface. The healing course was monitored at 8 and 15 days after the injury. Atomic force microscopy images, at different magnifications, allowed the identification of the time dependence of the osteoblast activity, measured by the increase in the area of neoformed primary bone and in the organization of the collagen fibers of the bone matrix. Characterization of the natural recovery of the damaged bone tissue by AFM is potentially of great importance because it allows the comparison of natural recovery processes with those induced by medicines and other therapeutic procedures. © 2001 American Vacuum Society.

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I. INTRODUCTION

Morphological studies of the natural bone remodeling process, especially after bone injury, have great scientific importance for the understanding of the regeneration processes that involve the action of osteoblasts (bone-forming cells) and of osteoclasts (bone-resorbing cells) in the synthesis of bone tissue. Such processes are highly complex since they are related to as yet not well-understood hormonal regulating and signaling mechanisms, which can accelerate or even completely interrupt bone regeneration.1–4 Several studies are in progress to promote bone regrowth using implants,5 hormonal treatment,6 genetic therapy,7 and laser radiation.8 Thus, it is of utmost importance to have a morphological microscopic model of the natural bone recovery process for a better evaluation of such therapies, and also to obtain new insights into natural bone regrowth mechanisms.

In this work, we studied the natural recovery of damaged bone tissue using atomic force (AFM) and scanning electron (SEM) microscopies. Atomic force microscopy provides a new tool for the investigation of biological samples, with no need for staining, coating, dehydration, or a vacuum environment, and opens up the possibility of structural investigation of the bone tissue under near physiological conditions with excellent resolution.9–12 Here we made a comparative AFM analysis of the structure of bone matrix of rat tibia at an intact non-surgically-injured region and at regions submitted to surgical injury.

II. EXPERIMENTAL PROCEDURE

The experiment was performed using 36 male Rattus norvegicus albinus, lineage Wistar with body weights in the range of 250–300 g and 75 days old. After anesthesia with sodium pentobarbital, a small incision was made in the skin of the animals to expose the antero-medial surface of the tibia. A hole of around 1.5 mm in diameter was then pierced, using a dentistry burr, in one cortical tibia surface. The animals of each group were sacrificed with an overdose of anaesthetic 8 or 15 days after the injury. The injured tibias were then immediately removed, dissected, and fixed with a 10% formalin solution for 24 h. After decalcification, the injured areas of the tibias (n = 18) were selected and dehydrated through an ascending ethanol series, dried by the critical point method using liquid CO2, and coated with gold in a sputter coater for subsequent scanning electron microscopy (SEM) analysis using a Jeol JXA 840 A SEM at a typical accelerating voltage of 20 kV. For AFM studies, the injured tibias had the unaffected cortical portion of the bone removed with a razor blade and discarded. The remaining portion containing the bone site undergoing neoformation was then fixed on a metal wafer. To allow comparison, the AFM analysis (Nanoscope II AFM) was performed both on intact nondamaged tissue regions and on postinjury regenerating tissue.

III. RESULTS AND DISCUSSION

Figure 1(a) shows a low-magnification SEM wide-view image taken of the central area of the tibia where the injury was made, after eight days of recovery. One may observe that the callus (an immature primary bone tissue formed temporarily to seal the lesion) is in the process of filling the surgically made hole with new tissue, and that a few wide gaps between the callus and the intact bone still exist. In our samples, only one surface of the tibia was injured, to preserve the bone marrow stem cells, responsible for bone-
producing osteoblasts. Further magnifications of the callus interface to intact bone are presented in Figs. 1(b) and 1(c) 8 and 15 days after injury, respectively. In both images the intact area is in the lower part of the figure. One may see that the regeneration of the bone tissue is occurring with an increase in the formation of ordered collagen fibers. Some well-defined collagen fibers and osteoblastic cells are seen in the upper part of Fig. 1(c). Bone consists largely of type I collagen proteins (>$90\%$) and other proteins containing a mineral phase of substituted hydroxylapatite, but since our samples were demineralized there are no hydroxylapatite components in these images.

Three-dimensional details of the surface features were revealed by AFM. Figures 2(a)–2(c) show typical AFM images taken on the intact bone tissue, at different magnifications. One may observe the almost parallel organization of the collagen bundles and details of the periodic collagen bands that exist along the longitudinal axis of the fibers in all images. These effects are due to the staggered arrangement of collagen fibril units which constitute the type I collagen fibers of bone matrix.

Figures 3(a)–3(c) show typical AFM images taken of the regenerating bone tissue, 8 days after injury. Apparently the bone-recovering processes were in progress but the usual
mature bone matrix organization was not achieved at this stage of repair. The healing process is better observed in the magnified image of the tissue presented in Fig. 3, where well-oriented collagen fibers are clearly seen.

Fig. 3. AFM images taken at the regenerating primary bone surface 8 days postinjury. Apparently the usual bone matrix organization has not been reached at this stage of recovery (a), (b). In (c), however, the regeneration of the tissue is at an advanced stage as indicated by the presence of well-oriented collagen.

Fig. 4. AFM images showing the improved orientation of the bone matrix collagen fibers 15 days after injury. (a) “Rows” of collagen fibers are observed in low-magnification images. Observe in (b) and (c) details of the three-dimensional orientation of the fibers. (b) The border of the fibers between the “rows.” (c) A high-magnification image of the parallel bundles of mature collagen fibers.

Figures 4(a)–4(c) show typical AFM images taken of the regenerating bone tissue, 15 days after injury. Large “rows” of collagen fibers are observed on the top surface of the cortical tissue, indicating that the animal’s natural bone re-

modeling is reaching a structure close to that of the intact bone tissue. Figure 4 shows a three-dimensional AFM image taken at the lateral border of the fibers, showing their orientation. Further magnification of this structure shows that the collagen fibers are organized in parallel arrays, close to the bundles exhibited by mature collagen fibers. The typical cross-section height profile of such fibers is presented in Fig. 5(a), and its two-dimensional Fourier domain analysis is shown in Fig. 5(b). Such results show that the statistical diameter of the fibers is 159 nm, in good agreement with literature values (124–170 nm).

The use of AFM at molecular resolution allowed us to observe topographical natural bone-recovery, which advances our knowledge of this process and permits correlation with the three-dimensional architecture obtained by SEM macroviews. This study revealed that AFM can be an additional valuable technique to study structural changes in collagen organization, such as those which occur under physiological conditions (growing or aging), pathological conditions (trauma, diseases), or under hazardous or beneficial exogeneous agents, influencing the bone remodeling process.

IV. CONCLUSIONS

Using scanning electron and atomic force microscopies we compared the reorganization pattern of injured bone tissue 8 and 15 days after surgical injury with the matrix organization of intact bone tissue. Atomic force microscopy images at high spatial resolution in three dimensions revealed the macromolecular transition in morphologies from the injured bone matrix to the characteristic mature structures. Lamellaelike structures were observed but it was not possible to observe the periodic collagen band usually present in aged fibers.

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