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Responses of bone to titania–hydroxyapatite composite and nacreous implants: a preliminary comparison by *in situ* hybridization

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The effect of two biomaterials on bone formation *in vivo* by *in situ* hybridization, was compared by using RNA probes complementary to collagen α1(I) RNA, osteonectin RNA and osteocalcin RNA. Holes were drilled into the midshafts of rat femurs. Titania–hydroxyapatite composite (THA) or nacre cylinders were implanted and the bone–implant regions collected 14 days after operation. Cuboidal osteoblasts, intensely labelled with the three probes, were seen to be lining the newly formed bone surrounding the THA implant. Between the implant and the new bone, a layer of un-labelled, apparently non-osteogenic cells was observed. By contrast, the nacre implant was bonded to the newly formed bone without any soft tissue interference. Osteoblasts lining the distal surface of the newly formed bone were stained with all three RNA probes, although weaker than in the THA sample. Some of the osteoblasts were flattened. We concluded from the appearance of the osteoblasts that the bone formation in the nacre samples had progressed beyond the phase of maximal synthetic activity. Around the THA implant, the labelling indicated that bone-forming activity was still high. It was concluded that the bioactivity of nacre was higher than that of THA.

1. Introduction

Understanding of the mechanisms underlying cell/tissue–material interactions is important for material choice and implant design. It is well known that different biomaterials induce different tissue responses. Two categories of hard tissue replacement materials, bioactive and inert, were defined according to the histological observations of the resulting tissue–material interface. Bioactive material forms a direct bond between bone and material without an intervening layer of soft tissue, whereas inert material has no direct bone bonding [1]. Recently, it has been demonstrated that calcium carbonate-based materials, such as coral and nacre, can also form a tightly welded interface with bone without soft tissue interference [2–4]. The reasons for the different tissue reactions are not yet understood.

In general, new bone formation comprises a complex sequence of events that begins with the recruitment and proliferation of mesenchymal precursors of osteoblasts, followed by cell differentiation, synthesis and organization of an extracellular matrix, and ultimately matrix mineralization. Osteoblasts undergo significant morphological changes. Early osteoblasts have an irregular structure. During the polar secretion of bone matrix, many cuboidal osteoblasts are arranged side by side on the matrix surface. As protein secretion reduces, osteoblasts flatten progressively, ending up as a continuous layer of bone-lining cells. To analyse the biological effects of biomaterial on new bone formation, markers to recognize members of the osteoblasts lineage are useful [5, 6].

In the present study, the expression of three osteoblast-related genes, collagen α1(I), osteonectin and osteocalcin, was monitored in osteoblasts at the interfaces of bone to THA and nacreous implants. The aim was to compare the bioactivity of the implant materials by the expression of osteoblast-related genes in the adjacent bone, in order to draw conclusions about the mechanisms of bone–implant interactions.

2. Materials and methods

2.1. Materials

Dense THA (85% TiO_2 and 15% $Ca_{10}(PO_4)_6(OH)_2$ composite), was sintered with a relative density of 99.8%. The nacre (shell of margaritifera, fresh-water pearl mussel), was composed mainly of calcium carbonate, with less than 2% organic matrix. THA and nacre cylinders, 2 mm diameter and 3 mm high, were

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prepared by ultrasonic machining. The THA cylinder was cleaned in an ultrasonic bath in ethanol and sterilized in an autoclave. The nacre cylinder was sterilized by high voltage to avoid the degeneration of organic components.

2.2. Animal and tissue preparation

Slightly oversized holes were drilled into the midshafts of male rat femurs, and THA or nacre cylinders were implanted with a light press-fit. The rats weighed 250 g. Fourteen days after the surgery, the rats were killed. The bone–implant regions were collected, fixed in 4% paraformaldehyde for 24 h and decalcified in 12.5% EDTA for 10 days at 4 °C. The specimens were dehydrated and embedded in paraffin. The implants were removed with great care before the paraffin hardened. Sections of 7 μm thickness were cut along the long axis of implants, mounted on glass slides coated with aminopropyl triethoxysilane (Sigma, St Louis, MO, USA) and processed for *in situ* hybridization. Some sections were stained with haematoxyline–eosin. Only sections having a cavity width close to the diameter of the implants were analysed.

2.3. Synthesis of riboprobes

Probes were labelled with digoxigenin (DIG) by using an RNA labelling kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions. Fragments of rat collagen α1(I), osteonectin and osteocalcin cDNAs were selected from rat tooth and femur cDNA libraries, constructed in lambda ZAP® (Stratagene, La Jolla, CA, USA), by using oligonucleotides specific for published rat sequences [7–9]. Selected phages were converted to plasmids by superinfection with ExAssist helper phages; inserts were sequenced to ensure their identity. Antisense RNA probes were generated by the transcription of linearized inserts of rat collagen α1(I), osteonectin and osteocalcin cDNA by T7 RNA polymerase. A probe for negative controls was produced by transcription of the osteonectin cDNA by T3 RNA polymerase.

2.4. *In situ* hybridization

Paraffin sections were dewaxed with xylene, re-hydrated, treated with proteinase K (20 $\mu g\,ml^{-1}$) for 30 min at 37 °C and post-fixed in 4% formaldehyde for 5 min. The fixation was stopped by 0.2% glycine. Thereafter, the sections were washed once with phosphate-buffered saline (PBS) and treated with 0.2 M HCl for 10 min at room temperature (RT), then washed with PBS and treated with triethanolamine and acetic anhydride (2.66 ml triethanolamine in 200 ml water, plus 0.5 ml acetic anhydride, added together with the slides) for 10 min at RT. After acetylation, the slides were washed twice with PBS and prehybridized in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0 (2 × SSC), 50% formamide at 52 °C for 60 min. Each section was covered with 100 μl hybridization buffer (50% formamide, 10% dextran sulfate, 10 mM tris-HCl, pH 8.0, 0.3 M NaCl, 5 mM EDTA, pH 8.0, 0.1 × Denhard's solution, 1 $mg\,ml^{-}$ tRNA), containing 0.5 $ng\,\mu l^{-1}$ DIG-UTP-labelled RNA probe, which had been heated at 98 °C for 3 min. The slides were incubated in a humid chamber overnight at 52 °C. After hybridization, the specimens were washed once with 4 × SSC, then incubated with 50% formamide in 2 × SSC for 30 min at 52 °C to remove non-perfect hybrids. The slides were washed with TNE (10 mM tris-HCl, 500 mM NaCl, 1 mM EDTA). The specimens were treated with RNase A (20 $\mu g\,ml^{-1}$) for 30 min at 37 °C in TNE, and then washed with TNE at 37 °C for 5 min. The slides were incubated with 2 × SSC and 0.1 × SSC three times for 10 min at RT. The presence of DIG-labelled RNA was revealed through phosphatase-coupled anti-DIG antibody fragments (Boehringer Mannheim protocol).

3. Results

3.1. Histological observations

Sections through the bone region that had contained the implants were stained with haematoxylin–eosin. For both materials, we observed massive formation of new bone, which to a great extent covered the implants. No major damage to the implant bone interface was noted (Fig. 1a and b).

3.2. *In situ* hybridization

3.2.1. THA-implanted bone

On the newly formed bone surrounding the THA implant, cuboidal osteoblasts were observed. These were intensely labelled with collagen type I, osteocalcin and osteonectin probes, indicating that osteoblasts were in an active stage and new bone formation was still in progress around the implant. The osteoblasts covered the newly formed bone matrix on all sides,

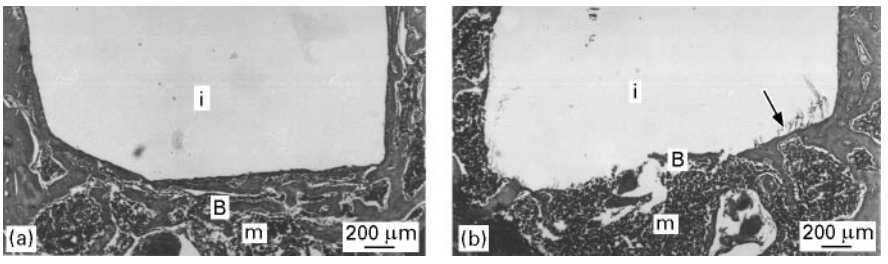

Figure 1 Haematoxylin–eosin staining of sections through femoral bone 14 days after implantation of (a) THA and (b) nacre cylinders. New bone (B) formed around implants (i). Organic nacre component (arrow) was embedded in bone matrix. (m) Bone marrow.

that is both the side facing the implants as well as the distal side. Nearest the implant, a layer of spindle-shaped cells was observed. These cells were not labelled by any of the osteoblast markers tested, which indicated that they represented cells with no bone synthesis activity. Bone marrow cells appeared to express osteonectin and osteocalcin mRNA (Fig. 2, THA column).

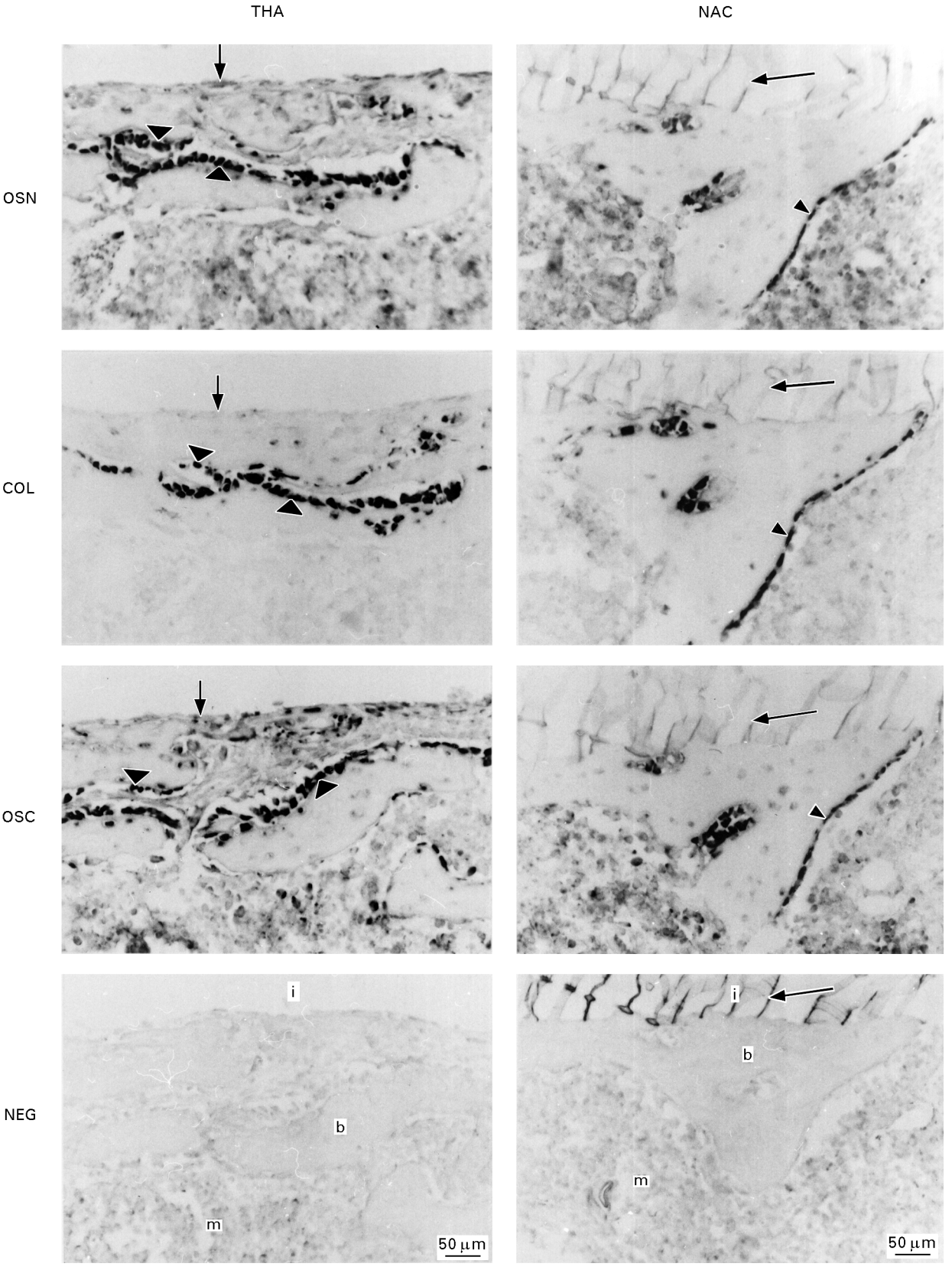

Figure 2 In situ hybridizations using probes for osteonectin RNA (OSN), collagen α1(I) RNA (COL), osteocalcin RNA (OSC), negative control representing the osteonectin mRNA sequence (NEG). Cuboidal osteoblasts (large arrow head) are visible on the bone matrix (b) in relation to titania-hydroxyapatite implant (THA), and a layer of non-osteogenic cells (short arrow) in between the newly formed bone and THA. Newly formed bone (b) was directly bonded with the nacre implant (NAC), organic nacre (long arrow) component was embedded in the bone matrix. Flattened osteoblasts (small arrow head) spread on the surface of newly formed bone. (m) Bone marrow.

3.2.2. Nacre-implanted bone

The nacre implant was bonded directly to the newly formed bone, without interference by cells. In contrast to THA implant, the bone matrix was not covered completely by osteoblasts. The region facing the implant was free of cells, but elements of the organic nacre components were attached and incorporated into the newly formed bone matrix. This indicates that the newly formed bone matrix was directly bonded to the implants; and integrated matrix between bone and nacre had formed at the interface. Many osteoblasts on the distal bone matrix surface showed a flattened morphology, which indicates decreasing synthetic activity. All the osteoblasts and bone-lining cells were stained with all three probes, but the general staining intensities were lower than those of the THA samples. Again, bone marrow cells expressed osteonectin and osteocalcin mRNA (Fig. 2, NAC column).

4. Discussion

The study of bone formation on the surface of implants was earlier conducted on a histological and histomorphometric basis. New bone formation had been examined and quantified, and bone tissue/cells had been stained histologically and histochemically [10, 11]. Owing to the persistence of the protein products, these methods do not provide information about the activity of the individual cells. The advent of molecular biological techniques has opened the possibility to demonstrate the expression of osteoblast-related mRNAs in the interface of biomaterial and bone [2, 12, 13] and to propose several models of bone formation and fracture repair [14–19]. These experiments have provided valuable insight into the molecular mechanisms associated with new bone formation, with or without the context of implants. It is well known that bone tissue behaves differently in the vicinity of different materials. For example, if bioactive ceramics are implanted in porous form or adjacent to bone, bone will deposit from the pore wall to the centre. Adjacent to inert materials, bone deposition starts from the centre [20].

In the present study, we demonstrate the reactions of individual cells. The occurrence of osteoblasts in the tissue and their morphology in the implant-associated bone depended on the nature of the material. Around nacre, positively stained osteoblasts were found only on the distal surface of newly formed bone, or enclosed in newly formed bone matrix. Adjacent to THA, active osteoblasts were found between the implant and bone matrix. Apparently, bone matrix was deposited on the nacre directly, but not on the THA. The analysis of pure TiO_2 ceramic implant resulted in observations similar to those for the THA interface (data not shown). The morphological appearance of osteoblasts and the level of the marker expression indicated that bone formation around the nacre was more advanced than around the THA. Newly formed bone associated with the nacre implant contained many osteoblasts with a flattened morphology, typical for stages developing into bone lining cells. The THA sample contained mainly early-stage osteoblasts. Collagen α1(I) osteocalcin and osteonectin RNAs were expressed at somewhat lower level than in the THA samples.

At the surface of the THA, there was a layer of non-osteogenic cells between implant and bone. These cells did not exhibit any signal with any of the probes, whereas osteogenic cells in the surrounding regions stained strongly with all the probes. By contrast, on the surface of the nacre implants, there was no such non-osteogenic cell layer. Nacre formed a direct bond with the bone, as evinced by organic nacre components incorporated into the bone matrix. The observed differences of implant surfaces suggest that nacre provides a substrate suitable for depositing bone matrix. The THA surface behaved inertly with no direct bone–implant bonding. Taken together, we conclude that the bioactivity of nacre is higher than that of THA.

In situ hybridization is shown to be a useful method for studying the bioactivity of materials on the cellular level. With the selection of suitable probes, this method could be used to screen the bio-performance of biomaterials and optimize their reactivity. Further experiments are being conducted on the expression-time spectra of different mRNA probes in context with implants.

5. Conclusion

RNA expression patterns were compared in cells/tissues adjacent to implants of THA and nacre, to demonstrate the potential of the *in situ* hybridization technique for studying the mechanisms of cell–material interactions *in vivo*. The different reactions indicate that the materials interact with the surrounding tissue and exert effects on both osteogenic and soft-tissue cells. An intimate bone–nacre interface, as well as other histological differences, support the conclusion that nacre is bioactive but THA is inert, with respect to bone formation at 14 days after implantation.

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