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Gene expression and protein activity in bone-bonding and non-bonding PEO/PBT copolymers

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The formation of a reactive carbonate-apatite surface is generally considered a critical step for bone-bonding. However, the contribution of proteins in the establishment of bone-bonding is debatable. In the current study, osteocalcin, osteopontin and β-actin mRNA expression and alkaline phosphatase activity were therefore determined in different porous substrates loaded with marrow after subcutaneous implantation for 2 weeks. Two polyethylene oxide (PEO)/polybutylene terephthalate (PBT) copolymers were used for this purpose. First, an 80/20 PEO/PBT which generates a carbonate-apatite surface and bonds to bone rapidly. Second, a 30/70 PEO/PBT which previously did not show bone-bonding up to 1 year postoperative. Bone had formed within the pores and occasionally at the calcified surface of the 80/20 materials. In contrast, 30/70 materials did not calcify and bone was not found in the pores. Despite these morphological differences, gene expression and protein activity was similar in 80/20 and 30/70 PEO/PBT materials. These results suggested that bone-bonding of PEO/PBT copolymers is more likely to depend on the formation of a carbonate-apatite surface than on a specific influence of a bioactive material on osteogenic cells.

1. Introduction

A common feature shared by acknowledged bone-bonding biomaterials is the postoperative formation of a carbonate-containing apatitic surface layer [1–4]. This reactive layer is, despite a lack of detailed information on events occurring at the interface, considered to be a critical determinant for bone-bonding. Indeed a direct relation between the rate of carbonate-apatite formation and bone-bonding seems to exist [1, 5–7]. Largely speculative is the role of proteins in the bone-bonding process.

Proteins adsorb to implant surfaces, upon insertion in body fluids, and possibly coprecipitate when carbonate-apatite crystals are postoperatively generated [2]. It has been reported that serum proteins delay the rate of crystallization in biomimetic experiments [8] and, simply due to a high affinity for the mineral phase, inhibit mineralization *in vivo* [9]. In contrast, it was demonstrated that specific proteins are involved in the induction of and during mineralization processes [10, 11]. Furthermore, osteopontin, osteocalcin and sugar residues were reportedly present in the natural lamina limitantes and cement lines of bone [12]. The presence of these proteins in the electron-dense layer at hydroxyapatite interfaces indicated mechanistic similarities in the establishment of such structures and suggested an active, organic role in the process and establishment of bone-bonding [13].

Because of the latter suggestion we intended to investigate the role of proteins in bone-bonding by assessing the influence of different substrates (bone-bonding and non-bonding) on gene expression (1) and protein activity (2). For this purpose two polyethylene oxide (PEO)/polybutylene terephthalate (PBT) proportions were used. On the one hand, an 80/20 PEO/PBT which postoperatively generates a reactive carbonate-apatite surface layer with which bone becomes integrated resulting in bone-bonding [14, 4]. On the other hand, a 30/70 PEO/PBT which did not calcify and subsequently displayed non-bonding behaviour up to 1 year of evaluation [6]. Porous discs of these two PEO/PBT proportions were, with or without marrow cells, implanted at heterotopic sites in rats and the bone formation process morphologically evaluated. Gene expression (1) was analysed by Northern blotting. The cDNA probes used for hybridization included osteopontin, which possesses a high affinity for mineral and is thought to play a role in cell attachment [10, 11] and osteocalcin, considered to be exclusively synthesized by osteogenic cells [15, 16, 11]. The expression of these two genes was quantitatively related to the expression of β-actin, a protein filament

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generating cell movement and attachment and present in various non-muscle cell types. Furthermore, alkaline phosphatase content (2), a marker of osteoblast activity [17] involved in the induction of mineralization [18], was measured in the implants.

2. Materials and methods

2.1. Materials

In this study polyether/polyester copolymers (Polyactive^R, HC Implants bv, Leiden) composed of a soft block, polyethylene oxide (PEO), and a hard block, polybutylene terephthalate (PBT) were used. Two PEO/PBT proportions, 80/20 and 30/70 PEO/PBT, were examined in the form of porous discs (diameter 5 mm, thickness 1.5 mm) which were sintered from granular starting material. These discs had a pore size of 300 $\pm$ 150 μm and an interpore connection of 150 $\pm$ 50 μm. A Phillips S525 scanning electron microscope was used to evaluate the pore structure of both implant types (Fig. 1).

2.2 Surgical procedure

Details of the surgical method have been extensively described [19]. Briefly, the femora and tibiae of male syngeneic Fischer rates were dissected. The bone marrow was hydrostatically forced into phosphate buffered saline solution, disaggregated and centrifuged to obtain a cell suspension containing 5×10^8 cells/ml. The porous PEO/PBT discs were either soaked into this suspension or left untreated and then subcutaneously implanted in the back of rats, according to the following schedule: $12 \times 80/20$ with marrow (80 +), $8 \times 80/20$ without marrow (80 −), $12 \times 30/70$ with marrow (30 +) and $8 \times 30/70$ without marrow (30 −). A total of 40 implants was evaluated at 2 weeks.

2.3. Microscopy

For microscopical analysis eight implants (4×80 + and 4×30 +) were processed. These implants were fixed in Karnovsky's fixative (4% paraformaldehyde, 1.5% glutaraldehyde in sodium cacodylate buffer, pH = 7.4), dehydrated in graded series of ethanol and embedded in methyl methacrylate (MMA). Undecalcified histological sections were cut on a modified innerlock diamond saw and stained with methylene blue and basic fuchsin.

2.4 Northern blotting

Six 80 + and 30 + implants each (four intact implants and half of four more implants—the second half was used for the alkaline phosphatase assay) were employed for the gene expression study. As negative controls, six implants each of 80 − and 30 − were used, and as positive control, total RNA isolated from rat cancellous bone (CB). Total RNA from the PEO/PBT implants was extracted by conventional guanidine isothiocyanate/cesium chloride density gradient methodology. Denatured total RNA was electrophoresed with 1.1% agarose-formaldehyde gels and transferred to a nylon membrane. Hybridization was carried out in Quickhyb solution (Stratagene, CA) and the membrane was then exposed to Kodak X-Omat film with an intensifying screen. Prior to exposure, labelled membranes were scanned in a BAS 1000 image analyser (Fujix) to obtain quantitative information on gene expression.

2.5. cDNA probes

The preparation of cDNA probes has previously been reported in detail [20]. Briefly, oligonucleotide primers corresponding to the protein sequences were synthesized by a DNA snythesizer. Concomitantly, messenger RNA (mRNA) was purified from rat cancellous bone by oligo-dT immobilized latex beads and used for the synthesis of cancellous bone cDNA by reverse transcriptase. The oligo nucleotide primers and the cancellous bone cDNA library were employed to amplify the cDNA probe by the polymerase chain reaction. The amplified cDNA was then purified by gel electrophoresis and ligated to plasmid DNA. Ligated DNA was transfected into a competent cell (Ecoli JM 109), extracted and analysed. The cDNA probes were

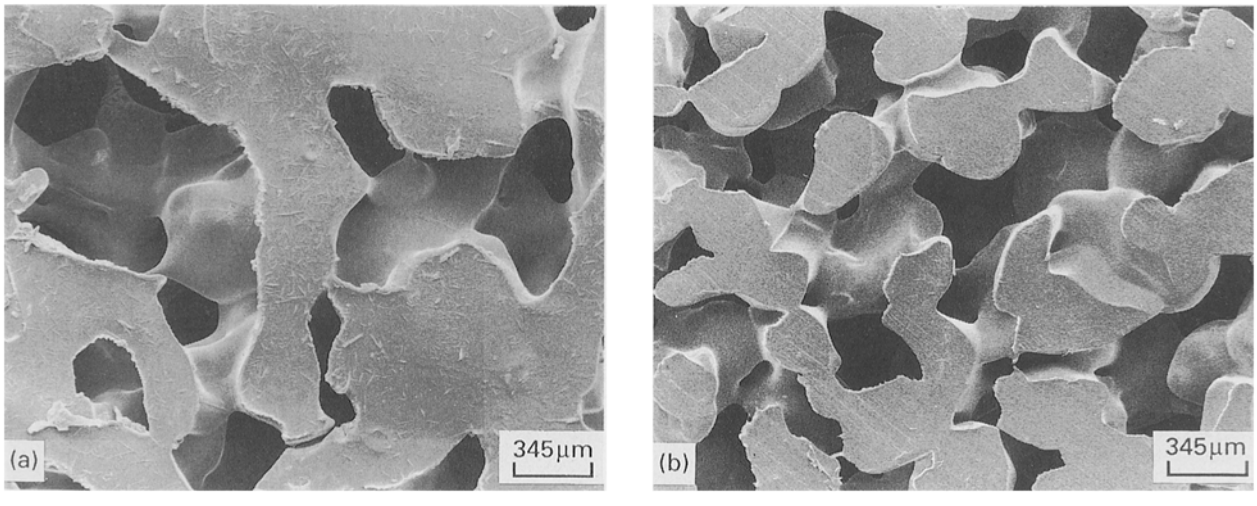

Figure 1 Pore structures of 80/20 (a) and 30/70 (b) implants.

labelled with ^{32}P by using a multirandom primer labelling kit (Amersham) and ^{32}P-dCTP and used for Northern blot hybridization. Complementary DNA of OC was supplied by Dr P. A. Price and cloned after the ligation to plasmid DNA. For the Northern blot of β-actin, the amplified cDNA after polymerase chain reaction was used.

2.6. Alkaline phosphatase activity

Per material, with and without marrow cells, four times half an implant (the complimentary parts of the implants used for Northern blotting) was homogenized and centrifuged. The supernatant was assayed for ALP activity using p-nitrophenyl phosphate as a substrate. ALP activity was expressed as μmol per implant after 30 min of incubation at 37 °C.

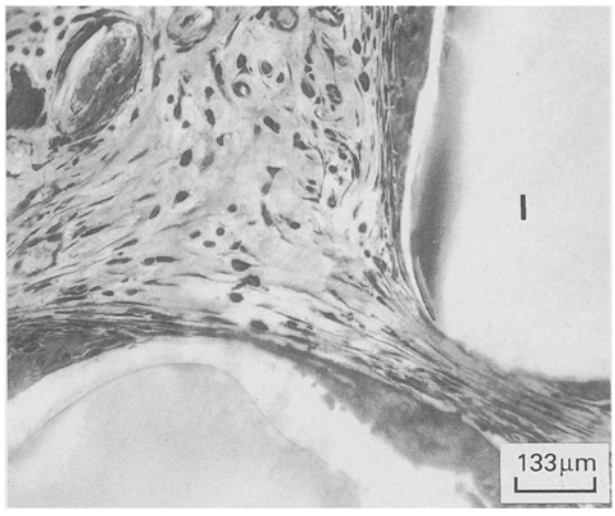

Figure 2 LM of a 30/70 implant (I) showing typical fibrous tissue ingrowth in the pores.

3. Results

3.1. Morphology

In the histological sections different observations were made for the 80/20 and the 30/70 implants. The latter material did not show calcification within its surface. Osteoblast-like cells appeared in the peripheral parts of the implants and in the centre of those pores, but bone formation was not evident. In general, the 30/70 implants were mainly filled up by vascularized fibrous tissue and some inflammatory cells (Fig. 2).

Within the outer surface of 80/20 implants, calcification was observed in the light microscopical sections (Fig. 3a,b). This calcification was composed of individual granules which occasionally fused to form dense structures. The occurrence of calcification was quite abundant and occupied large areas of the outer surface of the 80/20 implants. All 80/20 implants, with marrow, that were investigated for morphological purposes showed bone formation within the pore region. Bone formation processes varied from clustering of large cuboidal shaped osteoblast-like cells to areas of mature bone with osteoid and osteoblast seams (Fig. 3a,b). Bone formation was frequently observed in the vicinity of the calcified 80/20 surface but contact between these two compartments was restricted to a few locations (Fig. 3b).

3.2. Gene expression and protein activity

The yield of total RNA isolated from 80/20 and 30/70 implants with and without marrow amounted to 30–70 μg. 10 μg of each group was subsequently separated by gel electrophoresis and stained to visualize the 28S and 18S ribosomal bands as a screening of RNase-activity. Hybridization revealed a comparable expression of β-actin for both materials (80/20 and 30/70) with and without marrow and the control cancellous bone. A clear difference was observed in the expression of osteocalcin (Fig. 4): a prominent mRNA signal for the CB control, whereas in the 80 − and 30 − groups osteocalcin expression was absent. In both marrow implants (80 + and 30 +) osteocalcin mRNA was detected, although the signal was much weaker than the control (Fig. 5). When the expression of osteocalcin was related to β-actin expression it was calculated that the mRNA signal was similar for the

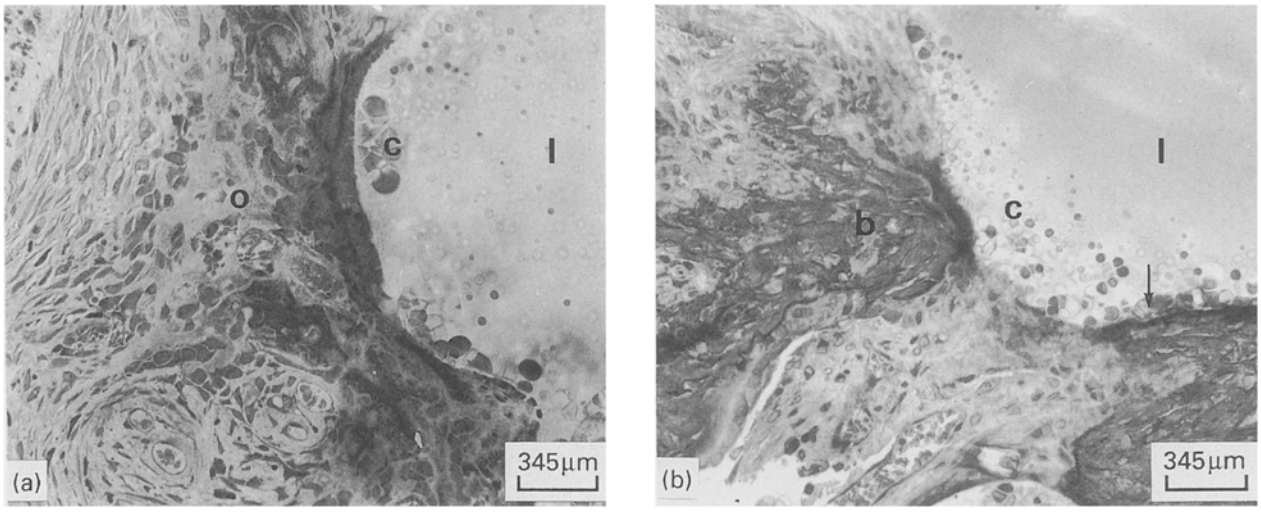

Figure 3 Histology of an 80/20 implant (I) showing the calcification (c) within the material surface and the different stages of mineralization, mainly osteoid (o) in A and bone (b) in B, in the pores (a, b). Note the contact in B Between bone and calcified surface (arrow).

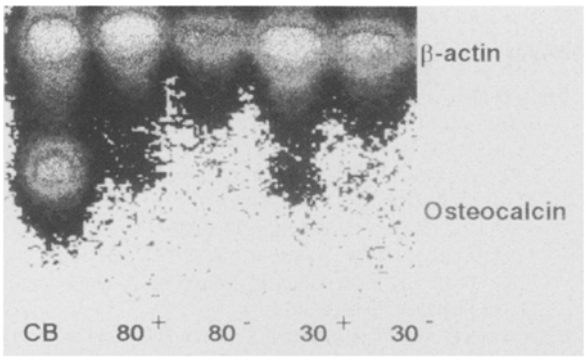

Figure 4 Expression of β-actin and osteocalcin.

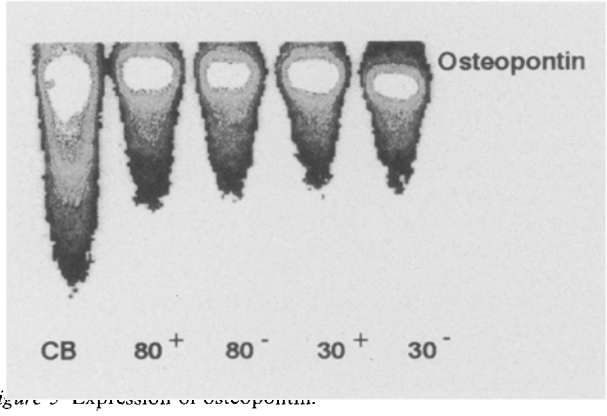

Figure 5 Expression of osteopontin.

80 + and 30 + implants: both 8%. Osteopontin mRNA was most prominent for the cancellous bone control. A prominent and comparable signal for the expression of osteopontin was observed in both implant types with and without marrow cells (Fig. 5). Alkaline phosphatase activity was similar in the 80/20 and 30/70 implants without marrow cells (1.0 μmol/implant). Higher activity was measured in the implants soaked in marrow; 2.6 and 2.8 μmol/implant for the 80/20 and the 30/70 implants, respectively.

4. Discussion

The purpose of this study was to relate bone-bonding behaviour to gene expression and protein activity and thus investigate the role of specific proteins in the bone-bonding process. The materials studied are synthesized from the same chemical starting chemicals, do not contain calcium and phosphorus prior to implantation and possess therefore (preoperatively) comparable physicochemical characteristics. The absence of calcium and phosphorus is significant in view of the importance of a calcified, carbonate-apatite, surface for bone-bonding [1–3]. This may assure a similar affinity of proteins with both PEO/PBT surfaces initially and allows thus for comparative observations.

A series of experiments demonstrated that a direct relation exists between calcification of the PEO/PBT copolymer surface and bone-bonding [5–7]. The rate of calcification is dependent on PEO content in PEO/PBT copolymers. Calcification was not shown in 30/70 materials up to 1 year postoperative [6],

TABLE I Alkaline phosphatase activity in 80/20 and 30/70 implants with and without marrow

Implant	80 +	80 −	30 +	30 −
ALP activity (μ mol/implant)	2.6	1.0	2.8	1.0

whereas 80/20 implants calcified, and subsequently bonded to bone, rapidly [7]. We reported also that calcification behaviour positively influenced the rate of bone ingrowth into porous PEO/PBT copolymers [14]. In this heterotopic model these relations are evidently confirmed. Calcification and bone formation were not seen in 30/70 samples whereas these features were prominently present in the 80/20 samples.

The biochemical data showed that both the 80/20 as well as the 30/70 implants supported osteoblast phenotype expression and thus the osteogenic differentiation of marrow stem cells. The detection of osteopontin and osteocalcin mRNA and the measurement of elevated alkaline phosphatase activity, when the implants were combined with marrow cells, leads to that conclusion. This statement seems, for the 30/70 implants, in contradiction with the morphological absence of bone. However, bone formation was undoubtedly observed in those substrates at 3 weeks (unpublished data A.M. Radder). Osteopontin is also expressed in implants that were not soaked in marrow which underlined the reported capacity of other cell types to synthesize this protein [11]. Despite the fact that beside osteopontin, alkaline phosphatase is not exclusively synthesized by osteogenic cells either [21], these proteins can., because of their high expression in cells of the osteoblastic lineage, be considered as differentiation markers of the osteoblast phenotype [22].

The influence of such specific bone proteins at bone/implant interfaces has drawn considerable attention. Adsorbed proteins did influence the type of carbonate-apatite formed [23]. Osteopontin was found in the in vitro, initially deposited globular accretions [24] and both osteocalcin and osteopontin have been localized in the natural lamina limitantes and cement lines of bone tissue. In addition, acid glycoproteins as glycosaminoglycans have been detected at the hydroxyapatite interface in vitro [25]. *In vivo* experiments demonstrated further the presence of osteocalcin and osteopontin in the electron-dense layer at the bone/hydroxyapatite interface [13]. The above cited observations are indicative of mechanistic similarities in the establishment of cement lines in bone and electron-dense layers at biomaterial interfaces and point to an active role of proteins in that process. This study showed a similar gene expression and protein activity in bone-bonding and non-bonding PEO/PBT substrates, despite a difference in morphological bone formation processes. These findings suggest that, in the case of PEO/PBT copolymers in the present model, bone-bonding is more likely to depend on the formation of a reactive carbonate-apatite surface than on a specific interaction of a bioactive surface on the osteogenic phenotype. Apart

from the fact that only a limited number of acknowledged proteins have been investigated, this obviously does not necessary exclude the role of proteins in the establishment of bone-bonding. Proteins will adsorb at the surface of any implant material and may also coprecipitate with microcrystals during carbonate-apatite generation [2]. In addition, osteocalcin and osteopontin are known for their high affinity for apatitic surfaces [10, 11] and osteopontin further has a cell attachment sequence [26]. It may therefore be hypothesized that these proteins preferentially adsorb onto a carbonate-apatite biomaterial surface and interact with membrane receptors on osteogenic cells stimulating bone formation on that surface. Such a hypothesis could then serve as an explanation for the different behaviour with respect to bone-bonding of different PEO/PBT copolymers.

5. Conclusions

This study showed a comparable gene expression and protein activity in bone-bonding and (non)bone-bonding substrates of largely similar nature. This suggests that, in the case of PEO/PBT copolymers, bone-bonding is dependent on the generation of a carbonate-apatite surface layer, possibly in combination with the coprecipitation or incorporation of organic moieties, rather than on bioactive-substrate induced osteoblast phenotype expression.

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