16 New Composite Material: PLLA and Tricalcium Phosphate for Orthopaedic Applications-In Vitro and In Vivo Studies (Part 1)

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β-tricalcium phosphate (β-TCP) is an osteoconductive, absorbable material, which, in porous ceramic form, is highly suitable for implants used in bone reconstruction, or as bone substitutes. Tricalcium phosphate, used for many years to replace or complement autologous bone in bone grafts, has proved its clinical effectiveness in many indications [1–7]. Like any ceramic material, however, these implants have a brittle fracture behaviour. They break suddenly, without any prior plastic strain, which restricts the clinical applications for these materials to surgery with low stress levels.

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On the other hand, biodegradable polymers from aliphatic polyesters of alpha-hydroxy acid derivatives, such as polylactic acid (PLA), evidence a modulus of elasticity closer to that of natural cortical bone and can retain high strength over time [8]. But these materials can induce unspecific inflammatory tissue response [9–15] resulting in delayed bone healing / fusion or osteolytic reactions [9–21].

To overcome the disadvantages of these different materials, composite materials of calcium phosphates and polyesters have been developed and evaluated in-vitro or in-vivo [22–32]. Such composite materials tend to increase phenotypic expression of osteogenic cells, reduce foreign-body reaction and improve bone healing in a dose-dependent manner as compared to pure polymer materials. Various calcium phosphate (HA or β-TCP) contents have been evaluated, ranging 5–60 % (w/w) depending on the study. A low mineral content (10 % w/w) seems to induce a moderate inflammatory reaction and does not evidence significant improvement of bone response in the sheep femur as compared to pure PLLA [33] since higher HA ratios improves bone healing and lowers inflammatory response of bone tissue in the same model. Similar results are reported concerning PLLA/TCP composites (70/30 w/w) in the dog mandible and tibia [34]; such implants are progressively replaced by normal bone tissue without adverse reaction. Significant improvements of in the extend and

D.G. Poitout (ed.), *Biomechanics and Biomaterials in Orthopedics*,
DOI 10.1007/978-1-84882-664-9_16

quality of bone healing are also reported about composite materials containing up to 60 % (w/w) of calcium phosphate mineral in the rabbit or rat [35, 36].

The objective of this research was to carry out an *in vitro* and *in vivo* study of the biological performance of PLLA/TCP composite materials with increasing mineral contents (0–60 % w/w) in order to estimate the influence of the β-TCP ratio on human osteogenic cells behavior and bone tissue response, then further to evaluate the scope of potential applications in bone surgery.

Material and Methods

Samples

The β-TCP granules were prepared by granulation and sintering at 1000 °C in air of a β-TCP powder free of HA or calcium pyrophosphate by infrared spectrometry and X-Ray diffraction, then further sieved to <125 µm particles. Appropriate w/w mixtures of β-TCP and PLLA (Purac Biochem, Gorinchem, The Netherlands) were prepared by mixing in air at 60 °C ± 5 °c for 24 h then processed by injection molding in the desired shape and size. Resulting composite samples were sterilized by gamma irradiation (25 KGr).

The pure β-TCP ceramic samples (SBM, Lourdes, France) had a total porous volume of 50 % (±5 %) with pore size ranging 5–400 µm, and were free of HA or calcium pyrophosphate by infrared spectrometry and X-Ray diffraction.

In Vitro Study

The *in vitro* study consisted of an evaluation of inflammatory potential by assaying the IL-1α secreted by monocytes, then cell proliferation (counting) and phenotype expression (PAL and I collagen) in human osteogenous cells in contact with samples of PLLA/TCP composite materials (e = 2 mm / ϕ = 10 mm) containing 0 %, 30 %, and 60 % (w/w) β-TCP, respectively.

In brief, the cell proliferation tests were carried out using human osteogenous cells from bone marrow, as these composite materials are destined for use in bone implants. Cells were seeded at an initial density of 5000 cells.cm^{-2} in slide wells (Iscove's modified Dubelco medium + fetal calf serum at 10 % v/v) containing material samples (36 wells per material) then incubated for increasing periods, up to 27 days. Cells were counted in Malassez cells following trypsinization of the cell layers. Alkaline phosphatase (PAL) was assayed by colorimetry, using p-nitrophenyl phosphate substrate, after centrifuging the suspensions and freezing the cell pellet. To study collagen synthesis, the wells containing the samples were seeded at an initial density of 40.10^3 cells.cm^{-2} then the slides were incubated for 48 h, changing the medium (IMDM + 1 % SVF) after 24 h. Extra- and intracellular collagen was assayed separately by colorimetry (Kit Biocolor S2000). Monocyte-macrophages from human peripheral blood were put in contact with the various materials to study their inflammatory potential (3.4.10^6 cells/well, HAM F12 + 10 % v/v SVF and antibiotics). This cell/material contact may induce cell activation, causing them to synthesize and secrete cytokines, particularly IL-1α, indicative of an inflammatory reaction. The density of cells in contact with the material was assessed after 24–72 h in culture, using the protocol described above. The quantity of extra- and intracellular IL-1α secreted by the cells was also assayed (ELISA, Cayman chemical Interleukin 1-α kit). The culture well was the negative control and the culture medium containing 10 µg.ml^{-1} lypopolysaccharides was the positive control.

In Vivo Study

The *in vivo* study was carried out using cylindrical implants de composite materials (l = 8 mm / ϕ = 6 mm) composed of composite materials containing 0 % or 60 % β-TCP and pure β-TCP, respectively. The implants were inserted in femoral sites in rabbits, using the Kathagen protocol.

Each animal received a 60 % implant and either a 0 % or a 100 % implant in the contralateral femur so that the materials could be compared with one another. Five animals were examined for each material and implantation period, giving a total of 30 animals. They were sacrificed by an overdose of anesthetic 1, 3, and 6 months after implantation. The rabbit femurs were fixed in a 4 % formaldehyde solution for several weeks. Each femur was cut transversally above and between the condyles so that the implant could be cut transversally. The fragments were then washed in tap water and demineralized in a 5 % EDTA diNa solution. The implants containing PLA were dehydrated in increasingly concentrated ethanol solutions then dissolved in chloroform for a few hours. The femur fragments were then put into absolute ethanol for 24 h. They were immersed in HEMA solution for 48 h, then in HEMA solution containing the activator for 24 h. Once the blocks had set, 5 µm slices were cut and colored using Giemsa solution.

Results

In Vitro Study

The results showed, first of all, that the composition of the materials tested had no significant impact (Mann & Whitney) on IL-1α secretion in the intra- or extracellular compartments. The values measured for each material were comparable to those of the negative control and ten times less than those of the positive control, irrespective of the incubation period. Similar results were obtained for monocyte proliferation. None of the materials studied had any cytotoxic effects.

The results of the proliferation of the osteogenous cells from bone marrow (CO) are shown in Fig. 16.1, which represents the variation in the number of CO depending on the culture time for the different materials studied.

Cell density increased not only over time, but also with the percentage β-TCP in the composite material, which had a significant impact (Mann & Whitney) after 3 days in culture. For example, at the end of the test (27 days), the number of osteogenous cells present on the 60 % material ($61{,}000 \pm 3500$ cells.cm^{-2}) was 220 % higher than on the 0 % ($27{,}300 \pm 300$ cells.cm^{-2}) and 120 % higher than on the 30 % material ($42{,}500 \pm 3600$ cells.cm^{-2}). The PAL activity was comparable for all the materials, but the percentage β–TCP in the composite had a significant impact on type I collagen synthesis. The quantity of collagen fixed in the extracellular matrix, assessed per well (Fig. 16.2a) or per 10^6 cells (Fig. 16.2b), increased with tricalcium phosphate content, reaching a maximum at 60 % mineral content.

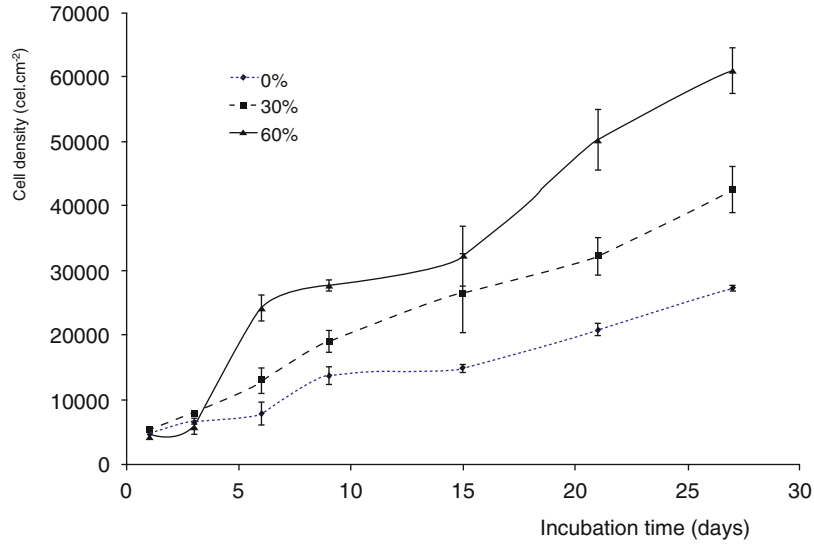

Fig. 16.1 Variations in the density of human osteogenous cells on the surface of various composite materials in function of the incubation period

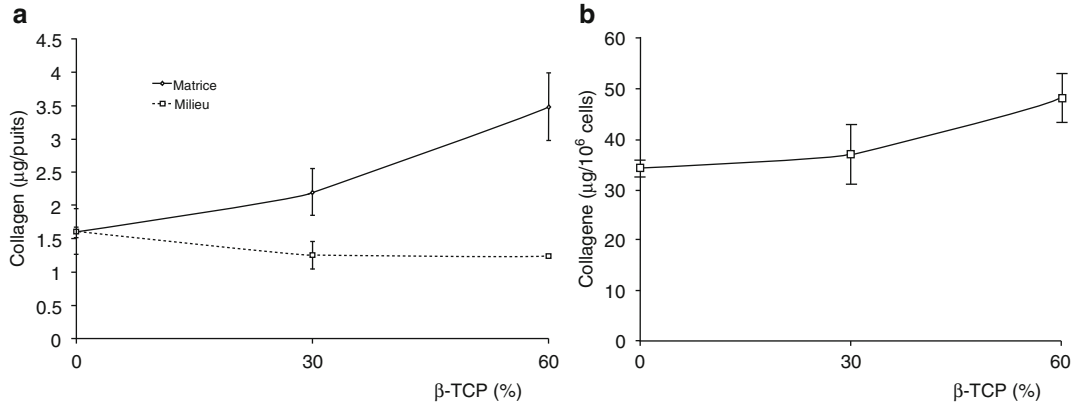

Fig. 16.2 (**a**) Assay of type I collagen per well as a function of the β–TCP content of the materials studied. (**b**) Assay of type I collagen in the extracellular matrix on the basis of 10^6 cells, as a function of the β–TCP content in the materials studied

In Vivo Study

The pure PLLA implants did not appear degraded, even after the longest implantation time. They were surrounded by conjunctival tissue containing large numbers of macrophages. After 6 months, some of the macrophages had phagocytized particles that appeared crystalline, but the implant maintained its structure. There were few bone trabeculae in direct contact with the implants, although, after 6 months, there was some ossification in contact with the material, separated by a fine layer of a few µm of metachromatic material. There were still some macrophage cells and collagen at the material interface.

Very soon after implantation, bone tissue was detected in direct contact with the composite implants. This tissue showed residues of cartilaginous matrix and was probably formed by chondroid ossification (Fig. 16.3).

At 6 months, the implant showed signs of degradation. Loose conjunctival tissue containing bone trabeculae infiltrated the implant, breaking it up in certain cases. It should be noted that these areas contained few macrophages. Their appearance was no different to that observed with porous ceramics of pure β-TCP. The bone tissue was in direct contact with the implant. After 6 months, some implants had broken up. The fragments were relatively homogeneous in size, i.e. a few hundred µm. A web of bone trabeculae had formed between the fragments (Fig. 16.4).

A few macrophages containing polymer debris were observed, but in a very limited number. More generally, the surface had been eroded, with widespread pits several hundred microns deep. Ceramics particles had been released and were trapped in the trabeculae or medullary cavities.

Discussion

The results of the *in vitro* tests indicate that adding increasing quantities of tricalcium phosphate to the lactic acid polymer stimulates both the proliferation of human osteogenous cells and the synthesis of the extracellular matrix. Cell density increased with the percentage of β–TCP in the composite, showing that cell proliferation was promoted by the presence of β–TCP. The results of the collagen assays in the extracellular matrix showed clearly that the quantity of collagen per cell increased in the same way, indicating that the β–TCP in the polymer matrix stimulated cell activity. The composite material with the highest mineral content (60 % by mass) showed the best behavior. We know that healing and consolidation of the bone by endochondral ossification are directly linked to the recruitment and phenotype expression of osteogenous cells at the site.

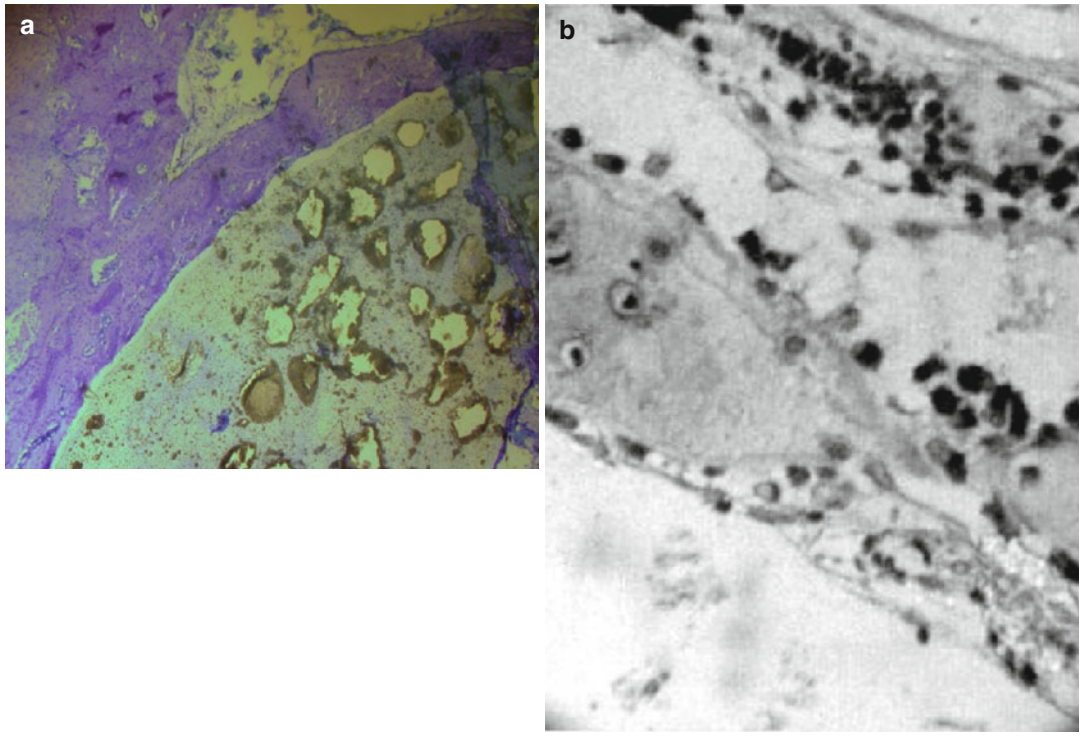

Fig. 16.3 (**a**) Beginning of the composite degradation process. Many implants still have the same texture as PLLA. Toluidine blue. (**b**) Detail of bone trabeculae in contact with the composite. There is no sign of a reaction to a foreign body. Toluidine blue × 40

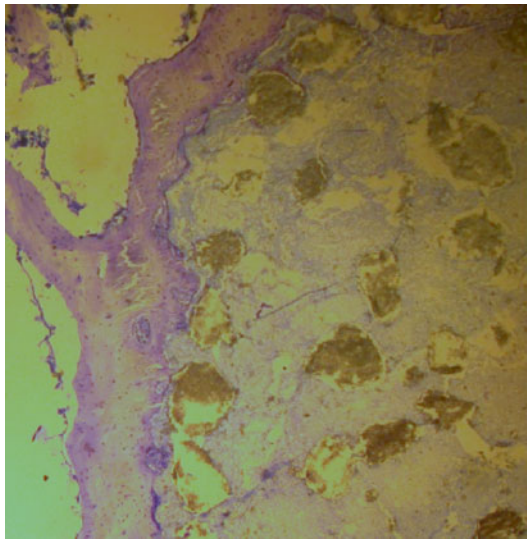

Fig. 16.4 Irregular composite surface after 3 months. There are even calcium phosphate particles released from the matrix and integrated into the bone tissue. Toluidine blue × 300

Composite materials thus seem more favorable for bone consolidation than pure polymer materials and have been shown *in vitro* to improve biological behavior. These results confirm hypotheses on PLA/TCP [26–28, 37] materials and, more generally, the absorbable polyester/calcium phosphate composites presented in the literature [22–25, 29–32, 38], where the presence of minerals in the polymer matrix promoted the proliferation and phenotype expression of osteogenous cells *in vitro*. These findings were also corroborated by the histological examinations, which indicated clearly that pure PLA implants were systematically surrounded by a layer of conjunctival tissue containing large numbers of macrophages, irrespective of the length of implantation, whereas bone tissue grew in direct contact with composite implants during the first few months and matured gradually until the end of the study period with no marked macrophage activation. The composite material under

investigation is thus more favorable for bone consolidation *in vivo* than pure PLA and has identical properties to those of pure β–TCP ceramics.

This result is not particularly surprising if we consider the properties of the materials in the composite separately. We know that pure lactic acid-based polymers are not cytotoxic or inflammatory *in vitro* [10–13, 39, 40] but are capable of causing an inflammatory reaction *in vivo* [9–13, 15], as confirmed by clinical observations [16–21]. Pure β–TCP, on the contrary, promotes bone healing by osteoconduction, becoming surrounded by healthy bone tissue containing active osteogenous cells, with no intermediate fibrous layer. It is, thus, conceivable that composite materials such as those we studied have a "hybrid" biological behavior that becomes closer to that of pure tricalcium phosphate as the mineral content increases. What mechanisms are involved?

Pure lactic acid polymer implants are absorbed slowly, over several years, depending on the volume of the implant [19, 41]. The main mechanism is internal hydrolysis [42], resulting in the sudden release of partially degraded polymer and monomer particles [9] at the end of the resorption process, which acidifies the peri-implant region [43]. During this phase, large numbers of active macrophages, containing particles resulting from hydrolytic degradation, are found within fibrous inflammatory tissue on the implant surface [10–15, 19]. If no macrophage activation mechanism is established it may be assumed that the monomer concentration [9, 43] (drop in pH) and the number of fine "phagocytizable" [44] particles contribute to this reaction. We observed significant resorption of composite material implants, and thus of polymer, without any inflammation. At 6 months, the surface was eroded to a depth of several hundred micrometers but there were only very few macrophages, some of which contained polymer particles. It cannot, therefore, be considered that activation of the cells during resorption of the polymer matrix was solely due to the presence of these particles. We know that the acidification that accompanies the degradation of lactic acid polymers may also set off an inflammatory reaction [43]. In a systematic *in vitro* study, Lin [28] measured the pH of solutions containing a variety of PDLLA/TCP composite materials. The pH stabilized at 2.3 for a pure polymer material and 5 for all the materials containing mineral, which shows the effect of β–TCP on the acidity of the solution. This observation was interpreted by the author as a result of the slower degradation kinetics of composite materials as compared to pure polymers. However, other authors have confirmed our histological finding that degradation kinetics increased with a higher mineral content [23, 26, 38], which leads us to observe that β–TCP causes an increase in both the degradation kinetics of the composite material and the pH of the solution. This may be interpreted by noting that the TCP particles increase the permeability of the material, resulting in a more homogeneous hydrolysis of the polymer matrix in a liquid medium, whereas hydrolysis of pure polymer materials starts from the surface. β–TCP is alkaline in solution and may neutralize the acid functions of the hydrolyzed polymer chains. It is also soluble in an acid medium and dissolves in the hydrolyzed polymer, thus buffering the acidification of the medium. This may also account for the high level of osteoblast activity observed, as human osteogenous cells are stimulated by the dissolution products of materials containing calcium and phosphate ions, like β–TCP [13, 30]. More generally, osteoblast cells activity and healing time may be linked to the concentration of dissolved calcium and phosphate ions and, thus, the dissolution kinetics of ionic materials. This interpretation accounts for our observations and is in agreement with widespread findings in the literature.

Conclusion

This study showed that adding increasing percentages of β–TCP to a lactic acid polymer matrix stimulated the proliferation of human osteogenous cells and synthesis of the extracellular bone matrix in a dose-dependent manner.

In vivo results indicate that, in comparison with pure PLA, tricalcium phosphate-containing composite materials had faster degradation kinetics, caused less inflammatory reaction, and promoted contact osteogenesis.

The composite material containing 60 % β-□TCP demonstrated a similar performance to pure tricalcium phosphate bone grafts in terms of osteogenesis and is apparently compatible with the production of intra-osseous implants for obtaining bone fusion or healing. Further studies are necessary to evaluate the ability of such a composite material to retain sufficient mechanical strength overtime for providing safe correction or stabilization of the implanted bone fragments.

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