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Biotechnology, material sciences and bone repair

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Summary: It is very likely that the need for bone substitutes will increase in the next decade. The present substitutes are generally bioactive and osteoconductive. Glassy or ceramic materials have been used up to now to act as a guide for bone healing tissue and were shown to admit bone apposition at their surface, probably due to the epitaxial growth of carbonated apatite crystals on it. Different forms of bone substitutes have been developed which do not show exactly the same properties. The reaction of bone tissue against bioactive material debris shows major differences from that of polymers or metals. *In vitro* models were developed to study the interface between bone cells and extracellular matrix, and the surface of bioactive material. Biotechnology makes available some morphogenetic proteins or growth factors in large quantities for combination with osteoconductive material which then can become osteoinductive. Bioartificial bone tissue constituted by a primary osteogenic cell line immobilised at the surface of osteoconductive materials

made it possible to obtain osteogenic materials. The ideal bone biomaterial is still to be engineered. The combination of material sciences and molecular biology will help to optimise the next generation of material surfaces.

Key words: Bone repair – Biotechnology – Material science – Bioartificial – Osteoconductive – Osteoinductive

The use of bone substitutes in human surgery has dramatically increased over the last decade. These materials have up to now been used to guide and expand the bone healing tissue, to become integrated within it and then subjected to the same remodeling process as the actual bone.

Several factors have led to an increased need for bone substitutes. Modifications in the regulations concerning bone banking have resulted in a reduction in allogenic bone tissue availability which has, together with autologous bone, been considered up to now as the substitute of choice. The potential viral contamination of such materials has imposed the centralisation of bone-bank management and increased the number of rejection criteria for bone contributed to such banks. This centralisation means that most of the *res nullius* which were recovered, stored and

used in the same surgical department is no longer available. Synthetic or semi-synthetic materials do not expose the patients to the same risk of infection which exists for allogenic bone, even though this risk is low.

The necessity for bone reconstruction after prosthetic revision is also increasing the need for bone substitutes. The corrosion products released by arthroplastic materials activate the macrophages, which in turn activate the osteoclasts. Both osteoclasts and macrophages are then responsible for the osteolytic processes observed in the adjacent bone.

Development of the use of substitute materials has greatly evolved over the last decade, although bone replacement by exogenous material is not of course new. Post-trepanation cranioplasties were probably the initial surgical interventions involving bone substitutes. The earliest materials of this kind were found in a Peruvian cranium dating from 2000 BC [24]. Such materials were made of gold or silver. Plaster of Paris was used in 1892 by Dreesmann to fill the cavities created by bone infections, which were frequent at that time. In a few cases this was subsequently degraded and replaced by bone. In 1969, Larry Hench from the University of Gainesville showed that silicate phosphates did not trigger inflammatory reactions

Table 1. Classification of the different biomaterials used as bone substitutes**Osteoconductive materials:**

Mild inflammatory reaction. Allow the apposition of osteoblasts at the material surface

Osteoinductive materials:

Induce the differentiation of mesenchymal cells in osteoblasts

Osteogenic materials:

Carry osteogenic cells at their surface

Bioactive materials:

Designed to elicit or modulate biological activity

when implanted into bone but became bound to the latter [16]. In 1981, Jarcho demonstrated that the apposition of bone to the surface of calcium phosphate ceramics gave no or only a mild foreign body reaction [18].

The framework within which bone substitute research has evolved was set at this date. Subsequent years have seen a series of small steps leading to an optimisation of materials and the arrival of biotechnology and genetically engineered products in this field.

Classification of bone substitutes

Surgeons and biologists have issued a very different classification to that used by material scientists, based on relationships existing between the bone tissue and the material once implanted (Table 1).

Osteoconductive materials are designed to provide a framework which guides the bone healing tissue. The bone

is in contact with the material without any interposition of cells or tissue. This class of materials contains the calcium phosphate ceramics which consist of hydroxyapatite (HA) and/or beta tricalcium phosphate (β -TCP) and calcium phospho-silicate glass (Table 2). Their value depends on the ability of implanted sites to provide a healing tissue containing pre-determined osteoblast stem cells for subsequent colonisation. These materials have been qualified as bioactive, which means that they are designed to elicit or modulate biological activity [30] of the surrounding tissues. With regard to bone replacement materials, however, bioactivity is directly related to the term bone-bonding. The nature of the biological reaction occurring between bone tissue and these materials is not clear.

Osteoinductive materials act on differentiation of the cells in their proximity. They are able to induce phenotypic evolution of multipotent stem cells or any mesenchymal cells towards an

osteogenic phenotype. Such materials can induce bone formation whatever the material environment and in addition induce ectopic bone formation.

The boundary between osteoconductive and osteoinductive materials is no longer clear. Ectopic bone formation has very recently been found to be induced by certain calcium phosphate ceramics or glasses [19, 23, 31]. This effect is inconstant, does not occur in all mammals [31] with the same material and is difficult to reproduce in certain species. It is probable that the inconstancy of this effect is linked to the variable characteristics of the materials used in such experiments. It is difficult to draw conclusions about the osteoinductive ability of these materials as precise characteristics are not given in the literature. This is particularly true of their surface properties, which may facilitate immobilisation of the morphogenetic proteins produced by the organism.

Relation between osteoconductive materials and bone

Bone tissue is a connective tissue containing a specific extracellular matrix. The proteins in this matrix monitor nucleation and growth of calcium phosphate crystals. Their activity results in the formation of a protein matrix mineralised with a poorly crystallised A or AB carbonate-apatite. The extracellular matrix is synthesised by mesenchymal cells (the osteoblasts) found at the surface of newly-forming bone. These cells are responsible for bone tissue structure and pattern and are under the control of both genetic factors and informative molecules synthesised in their microenvironment. Osteogenic stem cells are located in the bone marrow or in the proximity of osteoblasts, and even in extraosseous tissues such as the periosteum. They are derived from pluripotent progenitor cells (CFU-C) which then differentiate into fibroblasts, pericytes, chondroblasts or osteoblasts. Bone once formed can be remodeled depending on the mechanical stress to which it is subjected. The osteoclasts are multinucleate

Table 2. Comparative characteristics of different osteoconductive materials. They depend on the manufacturing process and the porosity in particular

Characteristics	Bioglasses	Coral	Ceramics	Cements
Composition	Na ₂ O, K ₂ O, MgO, CaO, Al ₂ O ₃ , SiO ₂ , P ₂ O ₅ , CaF ₂	CaCO ₃ , amino acids	Hydroxyapatite, tricalcium phosphate	Hydroxyapatite, di, tri, tetra or octo calcium phosphates
Phase	Amorphous	Crystalline	Crystalline	Crystalline
Density	3.07-2.08	Not given	2.9-1.5	Variable
Compressive strength (MPa)	1000-500	395-25	100-2	2-50
Shear strength (MPa)	45-215	0-60	Poor	0-20
Porosity	Variable	10-50% depending on the species	Variable	10-40%
Young's modulus (Gpa)	35-118	3-100	0.8	

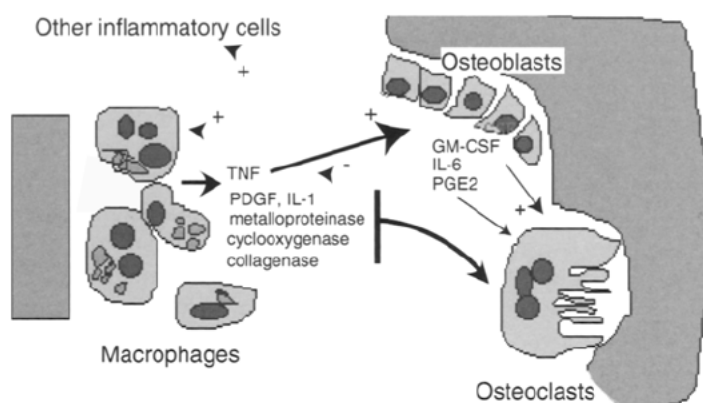


Fig. 1
Molecules synthesised by macrophages and their influence on the synthesis of osteoblasts and activity of osteoclasts

Table 3. Different stages of osteointegration of a calcium phosphate ceramic

<i>Stage 1:</i> Resorption of the post-operative hematoma and replacement by a loose connective tissue
<i>Stage 2:</i> Differentiation of osteoblasts from the fibroblast-like cells into the loose connective tissue
<i>Stage 3:</i> Immature bone formation at the surface of the material
<i>Stage 4:</i> Remodeling of the immature bone and the ceramic and replacement of this latter by mature bone

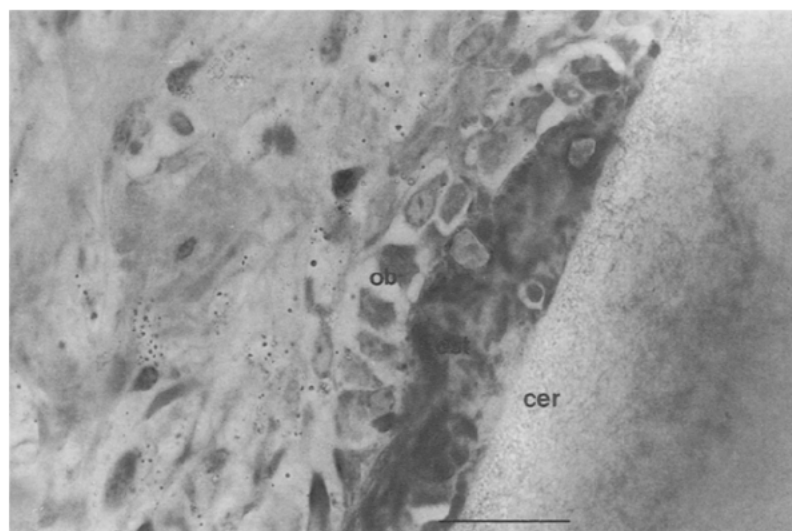


Fig. 2
Micrograph of osteoblasts (*ob*) differentiating at the surface of a calcium phosphate ceramic (*cer*) and forming an osteoid matrix (*ost*) on the material. The osteoblasts differentiate preferentially at the ceramic surface. Bar 80 μ m

cells that can erode the bone and which originate from the monocyte lineage.

Both types of cells are involved in the degradation-formation cycles responsible for the replacement of old by new bone.

The implantation of a bone substitute following surgical intervention always takes place at a bone healing site. The series of histologic reactions which characterise bone healing is well-known. Four stages may be observed when the site has become stabilised:

- 1) inflammatory stage;
- 2) fibrous callus stage;
- 3) bony callus stage;
- 4) remodeling stage.

The implantation of any material into bone tissue upsets the healing reaction at the periphery of the implant. An onslaught of monocytes and macrophages occurs from the first stage onwards, these becoming fixed to the material surface. Such cells will phagocytose the material or the debris released from it, depending on its characteristics. Some of these cells merge to form multinucleate cells known as giant cells. Particles less than 50 μ m in size are usually phagocytosed while larger particles are more often surrounded by macrophages and giant cells. Stephen Horowitz in Philadelphia [15] has shown that phagocytosis enhances the release

of mediators of inflammation, particularly $\text{TNF}\alpha$ which then stimulates the synthesis of Granulocyte Macrophage Colony stimulating Factor (GM-CSF) and interleukins (IL-6) by the osteoblasts. Each of these molecules recruits new macrophages, osteoclasts and other inflammatory cells at the interface with the material. $\text{TNF}\alpha$ also stimulates PGE2 production by osteoblasts which may then decrease $\text{TNF}\alpha$ production by macrophages and activate osteoclast activity (Fig. 1).

Bioactive materials do not induce these tissular, cellular and molecular reactions (Table 3). The healing process is characterized by the differentiation of osteoblasts from fibroblasts present in the connective tissue which has replaced the hematoma (Fig. 2). It is important to note that in the case of porous materials, osteogenic cells do not differentiate homogeneously within the entire volume delimited by the ceramic. Most of the osteoblasts differentiate in close proximity to the ceramic and are apparently immobilised at the material surface as they are at the surface of the bone trabeculae. These cells exhibit polar activity, which means that the extracellular matrix is synthesised by the cell pole in contact with the material (Figs. 2, 3). This leads to the development of an osteoid matrix at the material surface. Subsequent mineralisation of this matrix does not seem to be influenced by the nature of the material.

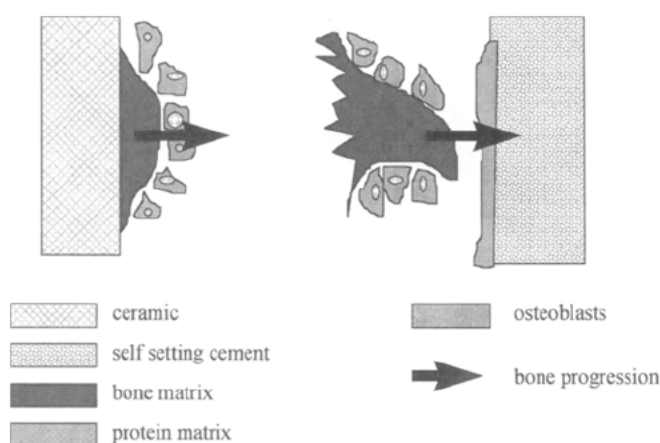


Fig. 3
Integration process of HA-ceramics and DCPD-based self-setting cement. The osteoblasts differentiate at the ceramic surface and synthesise an osteoid matrix and invade the rest of the pore. There is an ingrowth of bone toward the cement through a foreign body reaction

The bone formed in contact with the material initially exhibits an immature structure with randomly oriented collagen fibers and high cell density. The remodeling process which takes place in the bone formed at the material surface leads to the replacement of immature by layered bone. The implanted material is also subjected to remodeling and is degraded by osteoclasts and giant cells. As bone becomes synthesised in the degraded zone the material gradually becomes replaced by bone, this being the basis of osteointegration (Fig. 4).

After the release of calcium phosphate debris, a foreign body reaction identical to the histologic reaction induced by metal and polymers (Fig. 5) may occur in the bone marrow between the bone trabeculae. However, unlike the reaction to metal and polymers there is no activation of osteoclasts. This is of paramount importance as regards the bone-biomaterial relationship. It means that the osteolysis resulting from the presence of wear debris is dependent on the physico-chemical nature of the debris released by the materials. Harada et al. [13] demonstrated that the phagocytosis of calcium phosphate particles sintered at high temperature did not trigger the synthesis of inflammation molecules by the macrophages. High temperature sintering decreased the surface area and thus the exchange surface with the biological environment. The fragments of calcium phosphate materials are degraded in the low pH compartment of the cells. The solubility of such particles may be an important factor controlling non-activation of the macrophages involved in their phagocytosis.

Modification of the surface and bioactivity of osteoconductive materials

The observation of physico-chemical modifications occurring at the surface of osteoconductive materials may be of interest, and help to explain the biological properties of such materials. Identical reactions take place at the surface of amorphous materials such as silicate-phosphate glass or crystalline materials

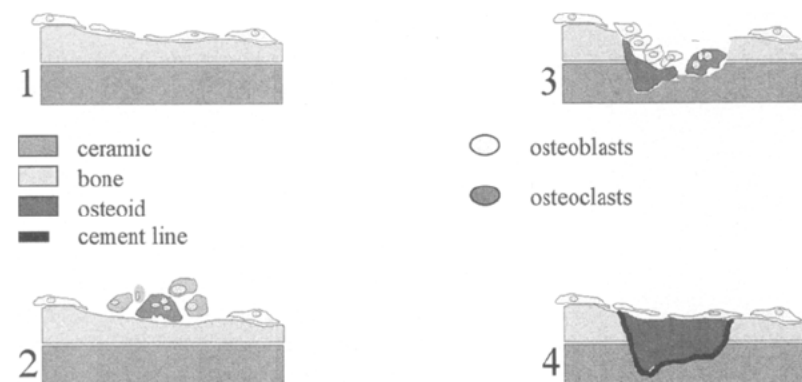


Fig. 4
Remodeling of calcium phosphate ceramics

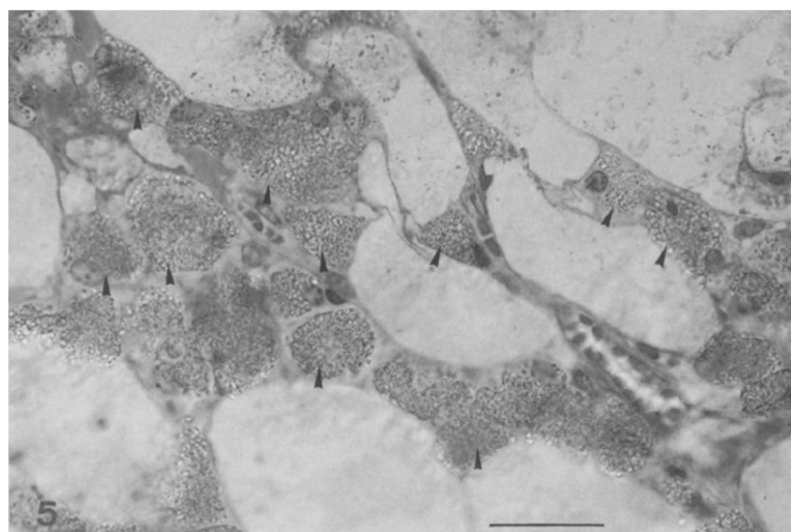


Fig. 5
Micrograph of bone stroma containing numerous macrophages which had phagocytosed calcium phosphate particles (arrowheads). No abnormal bone resorption could be evidenced in the proximity of these cells. Bar: 80 μ m

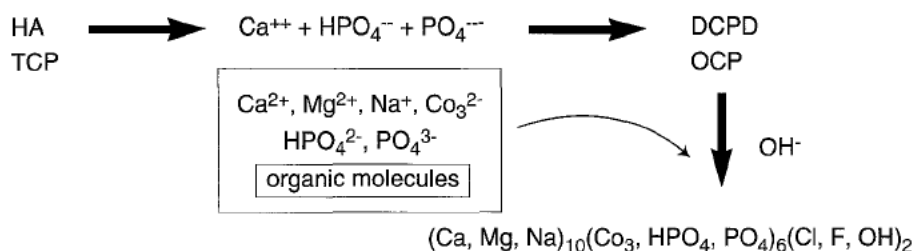


Fig. 6
Formation of carbonate apatite at the surface of HA or TCP ceramics. Ions released from the surface of the ceramic are precipitated at the material surface in the form of an apatite containing different ions or even small organic molecules from the biologic environment. Intermediary components (DCPD: dicalcium phosphate dihydrate, OCP: octocalcium phosphate) are possibly formed

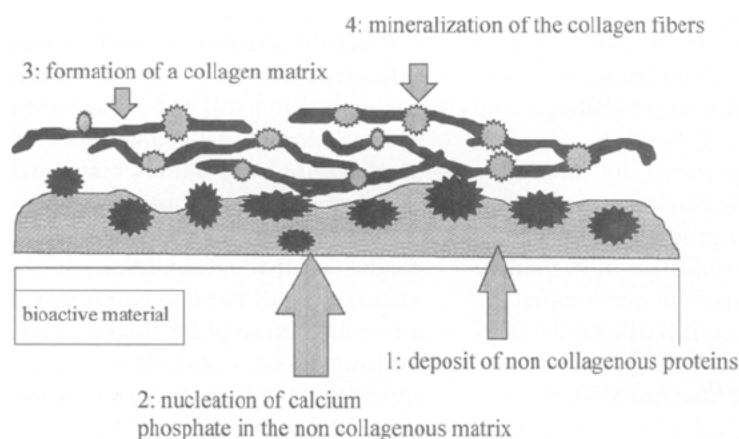


Fig. 7
Sequence of events occurring at the surface of bioactive material in rodent cell culture model

such as calcium phosphate ceramics. The heterogeneous nucleation of carbonated apatite [6, 14] which occurs (Fig. 6) at the material surface, may be a prerequisite for bioactivity. John Davies [5] from the Centre for Biomaterials of the University of Toronto, investigating the interface between bone and bioactive implants developed an *in vitro* model based on the culture of rat bone marrow cells at the material surface. These studies showed that a protein matrix was rapidly deposited at the material surface and acted as a nucleation site for calcium phosphate crystals. Collagen fibers which assembled at the surface of this mineralised matrix during growth of the calcium phosphate crystals were then

subjected to autonomous mineralization (Fig. 7). This mode of integration, observed under TEM, creates a partition between the material and bone apparent as a layer of mineralised tissue (0.5 μm) composed of non-collagenous proteins from the bone extracellular matrix and constituting a cement line identical to that present between the osteons.

Ducheyne et al. [8] and El-Ghannam et al. [9] reported that modification of the bioglass surface markedly influenced osteoblast activity. They suggest that the calcium phosphate-rich layer promoted adsorption and concentration of proteins from the culture medium and was utilised by the osteoblasts to form the mineralised extracellular matrix.

Differences in the biological properties of solids and pastes

Solid osteoconductive materials are brittle and fragile and very difficult to shape during surgical intervention. This is why calcium phosphate hydraulic cements have been developed, either for direct injection as pastes into the implantation site or for modelling to the shape of this latter. Setting results from precipitation of a different phase to the one in suspension in the paste or liquid. The resulting mechanical strength is due to interconnexion of the resulting crystals. The compressive strength may vary from a few MPa at the beginning of setting to 20-50 MPa a few weeks later. In contrast to ceramics, the different crystals remain separate from each other so that the resulting surface area is very high. Numerous formulations may be used to obtain the setting of this calcium phosphate phase and Driessens et al. [7] have listed more than one hundred; 14 set within 10 mn and their compressive strength exceeds 2 MPa. We studied the integration of a DCPD-based self-setting cement [11]. The sequence of biological events leading to integration of such materials into the bone differs from that occurring at the contact of ceramic or glass (Fig. 3). The surface of the former materials is generally surrounded by numerous macrophages and giant cells indicating a foreign body reaction. Osteoblasts then differentiate at some distance from the material. Trabeculae progress towards the material surface across the foreign body reaction while the surface recedes due to the rapid rate of degradation resulting from the high surface area. The presence of a proteinaceous deposit between the crystals at the material surface is often noted. This differs from the osteoid matrix, is acellular and appears rapidly after implantation, remaining between the bone and the material. The resulting ossification which integrates this material is thus centripetal and not centrifugal as in the case of ceramics or glasses.

Extending the limits of use with biotechnology

For two main reasons, the indications for all bone substitutes are relatively narrow. Firstly, their mechanical properties are poor. Secondly, their integration is linked to the ability of the implanted sites to furnish osteogenic cells which will then grow into the substitute.

This latter condition is often impossible to satisfy in human and the material must therefore carry all the necessary factors to allow the surrounding tissue to synthesise a bone matrix.

Bone Morphogenetic Proteins are members of a protein group in the TGF β family. They were first described by Urist from the UCLA in 1965 [25], who showed that this protein group induced enchondral ossification after implantation into the mesenchymal tissue. It was later demonstrated that bone induction by these extracts resulted from a multistep cascade of events comprising chemotaxis, mitosis and differentiation. Such proteins may be obtained by extraction from a demineralised bone matrix but only in very low amounts. Purification of the different protein fractions within the extracted protein soup is in addition very difficult. Recombinant human morphogenetic bone proteins (rhBMP2) have been obtained by genetic engineering and are produced by the Genetic Institute in Boston. These recombinant proteins have been shown to induce bone formation when implanted in bone defects in large mammals or apes, bringing about a sequence of events which leads to the endochondral formation of bone tissue. In order to be active such molecules have to be associated with a carrier. Osteoconductive materials are ideal for this role: they do not denature the molecules which become fixed to their surface, release occurs during carrier degradation, and no foreign body reaction is induced which would impair osteogenesis. Numerous studies [23] have demonstrated the interest of hybrid materials consisting of calcium phosphate-bone morphogenetic proteins for the induction of ectopic bone formation.

However, the action of the BMP family is not limited to bone tissue. Hogan [17] proposed that depending on the concentration, low levels of BMP would stimulate cell proliferation whereas high levels would inhibit it.

BMPs have been observed in nearly all developing visceral and somatic organs, i.e. brain and sympathetic neurons, heart, liver, lung, skin, hair follicles, craniofacial structures, branchial arches, placenta, and skeletal elements [26, 28]. BMP-2 and 4 are mammalian homologs of dpp and 60 A proteins associated with dorsoventral patterning in the fruit fly embryo [26], and of activin in the amphibian *Xenopus* [29]. BMP or BMP antecedent molecules pervade the earliest stages of development of all animal life from sea urchins, silkworms and earthworms to baboons and human beings.

It should be noted that BMPs are active at very low doses: only 0.1-100 ng/l are required to produce embryo axial skeleton abnormalities in the chick.

Recent advances in our comprehension of the effect of BMPs on development were presented at the Second International Conference on BMPs held in Sacramento [27]. Zhang and Bradley reported that knockout mice deficient in BMP-2 exhibited extensive aberrations in development of the heart. Mutation of BMP-14 resulted in a marked inhibition of bone size in mice. Recent work on the inactivation of BMP-12 and 13 would indicate a role in joint morphogenesis. BMP-7 has been shown to play a part in kidney and eye development.

Other growth factors, without being inductive, have shown an *in vitro* effect on isolated bone cells and certain have shown a similar effect *in vivo*. bFGF is mitogenic for osteogenic cells under certain culture conditions and several studies have revealed activation of the synthesis of different extracellular matrix molecules [21]. This effect was observed in laboratory animals in which bFGF had progressively been administered into a fracture site during the early healing phase [1]. Most hormones and growth factors affect ossification process. This effect, to be optimum, is very often synergistic, taking place after that

of one group of molecules and before that of another. The complexity of these sequences is little understood, but explains the difficulties encountered in the therapeutic use of such molecules. The very low active dose of BMPs or other growth factors and their action at the cross-roads between development, patterning, cell multiplication and differentiation constitutes a further difficulty and must incite caution in selecting a suitable indication for such molecules.

Next generation materials

Proteins are not the only factors, in the field of hybrid materials which can enhance the activity of mineral carriers; osteogenic stem cells may also be used to this end (Fig. 8). The nature of the synthetic carrier material and the techniques of recovery and cell culture will require further development.

The mechanical properties of osteoconductive materials which can be used as carriers will need to be improved, especially with regard to their mechanical properties. Composite ceramics in which the pores of the macroporous ceramic framework are filled with a DCPD based self-setting cement have been developed in which the mechanical properties (compressive strength and Young's modulus) are very similar to those of cancellous bone [12]. This ceramic can be shaped during surgical intervention or even screwed. The bone colonisation capacity is conserved as the material filling the pores is highly soluble and can be degraded as the bone grows into them. The growth of bone within the material compensates any mechanical weakening due to material degradation. The inclusion of whiskers in the calcium phosphate matrix can also confer the ceramic with the properties of a composite material.

Ogushi et al. [20], in Cleveland, showed in the mid eighties that porous ceramics soaked in a cell suspension were able to induce bone formation in subcutaneous sites in rats. At the same time, Bab et al. [2] demonstrated that bone formation in ectopic sites could be obtained from a very limited number of

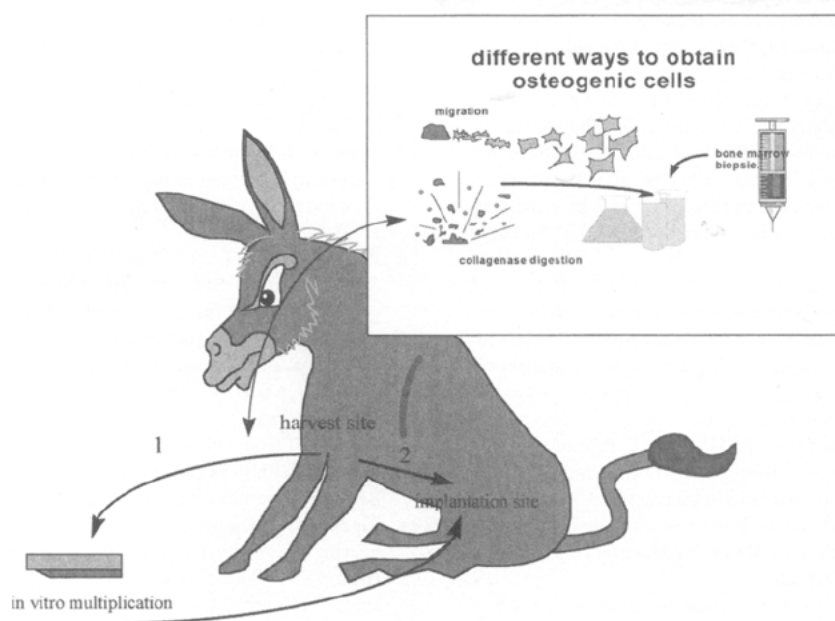


Fig. 8

Autologous osteogenic cells can be harvested and different ways to obtain them can be used. They can be re-implanted either after a period of *in vitro* multiplication or directly without any growth in culture

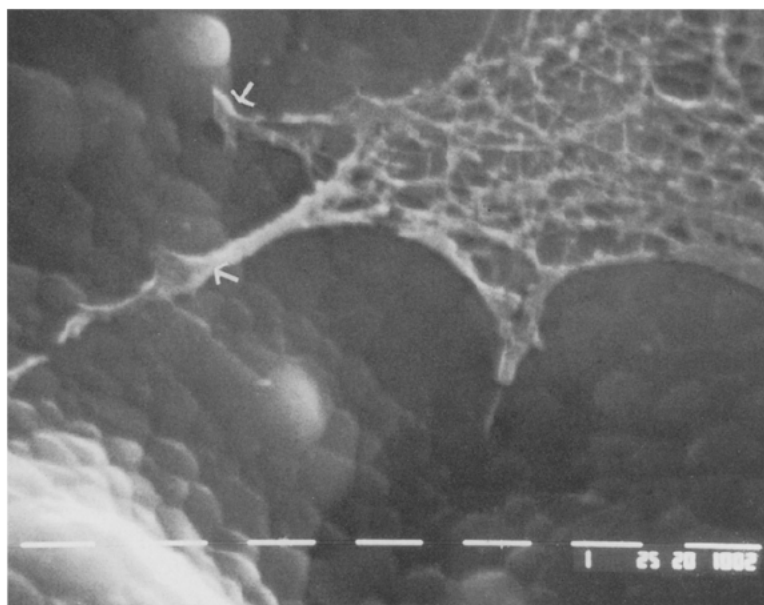


Fig. 9

SEM of fibroblasts grown at the surface of a calcium phosphate ceramic. The cell membrane has been removed with detergent showing the actin cytoskeleton fixed at the ceramic surface

stem cells. During the late eighties, we in the histologic laboratory of Toulouse-Rangueil University, immobilised osteogenic cells grown *in vitro* at very high density on the surface of calcium phosphate ceramics (Fig. 9). We were thereby able to harvest osteogenic cells and to

multiply them *in vitro* before implanting the cell line in association with a carrier. This kind of experiment produced encouraging results in the dog, although highly variable [10].

Very few osteogenic stem cells and many contaminant cells are obtained

during biopsies, whether of the bone marrow tissue or of cancellous bone. Bruder et al. [4], in the biology department of the Case Western University in Cleveland, produced monoclonal antibodies to segregate human osteogenic stem cells and thereby to increase the cell recovery yields.

Differentiated cell lines lose their characteristics when grown *in vitro*. Osteogenic cells were shown to lose their ability to produce a mineralised matrix and bone-specific proteins as growth progressed. However, an osteogenic medium containing dexamethasone and β -glycerophosphate was developed so that synthesis of the mineralised matrix by the cell line could be maintained for longer [3]. A further difficulty became apparent when such cells were grown *in vitro* at a high rate of multiplication. An aneuploidy occurred, after several days or weeks of culture, which could make this type of graft particularly hazardous after a long period of growth and requiring new research to overcome this problem.

Conclusions

The bone substitutes used during the last decade were based on a biomimetic concept which meant that the materials were developed to exhibit chemical or structural analogy with part of the bone matrix. Most of these materials were not engineered and a specific design process was bypassed in favor of trial-and-error optimisation [22]. Material sciences, nanotechnology and molecular biology would contribute to the synthesis of the ideal surfaced-biomaterials.

Trial and error optimisation has already provided appreciable results in orthopaedic biomaterial technology. Bioactive glass and calcium phosphate ceramics especially developed for the orthopaedic field were obtained in this way. It was then discovered that the epitaxial growth of mineral crystals modified the surface, thus conferring such materials with specific biological properties.

Could the epitaxial organization of small molecules on a crystalline surface lead to the development of new biomaterials exhibiting the required functional properties? Such surface optimisation could determine whether the cells consider the material surface: as a self component of the implanted tissue or as a foreign body.

The adjunction of cells or active molecules to such surfaces might compensate the absence of a cellular component and the observed failure of the healing process. Whatever the nature of the active molecules fixed to or assembled at the surface, the indications for the use of such materials will be considerably different from those of today.

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Biotechnologie, substituts osseux et réparation osseuse

Résumé : Pour des raisons de sécurité infectieuse et réglementaires, les substituts osseux de synthèse pourraient voir leur usage augmenter dans les prochaines années. Ces matériaux généralement ostéoconducteurs et bioactifs, sont des composés inorganiques qui montrent des relations complexes avec le tissu osseux de cicatrisation dans lequel ils sont implantés. Ils diffèrent de plus des matériaux métalliques ou polymériques utilisés en chirurgie orthopédique par leur capacité à ne pas induire de réaction ostéolytique par leur produit de dégradation. Cette dégradation est une même étape essentielle du processus d'ostéointégration. Leurs propriétés biologiques semblent liées à une modification de surface par croissance épitaxiale de cristaux de phosphates de calcium. Des modèles biologiques ont été réalisés *in vitro*. Différentes mises en forme de matériaux peuvent être obtenues, mais quelles qu'elles soient, les limites d'utilisation de ces matériaux sont liées aux capacités de cicatrisation du tissu osseux dans lequel ils sont implantés. L'ingénierie des protéines pourra éventuellement reculer ces limites. Diverses protéines recombinantes dont les protéines morphogéniques osseuses sont candidates pour conférer aux matériaux substitutifs du tissu osseux des propriétés d'ostéoinduction qu'ils ne possèdent pas intrinsèquement. A moyen terme, le couplage de cellules souches osseuses à des matériaux ostéoconducteurs devrait permettre d'obtenir des matériaux ostéogéniques se rapprochant du tissu autologue frais. Les matériaux de la prochaine génération combineront les techniques de la biologie moléculaire et une optimisation des propriétés de surface des vecteurs des facteurs ostéoinductifs ou ostéogènes.

Mots-clés : Réparation osseuse – Biotechnologie – Biomatériaux – Substituts osseux – Ostéoconduction – Ostéoinduction