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New Research on BIOMATERIALS

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NEW RESEARCH ON BIOMATERIALS

PREFACE

Biomaterials serve as synthetic or natural materials used to replace parts of living systems or to function contact with living tissue. Biomaterials are intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body. A biomaterial is different from a biological material such as bone that is produced by a biological system. Artificial hips, vascular-stents, artificial peacemakers, and catheters are all made from different biomaterials and comprise different medical devices. This book presents new approaches to biomaterial development including multi-field bone remodeling, novel strategies for conferring antibacterial properties to bone cement, polyacrylonitrile-based biomaterials for enzyme immobilization and functionalized magnetic nanoparticles for tissue engineering.

Chapter 1 - The search of a non toxic material to replace bone remains a big challenge for scientists. This chapter summarizes the current research behind the use of hydroxymethylglutaryl coenzyme A reductase inhibitors (HMGRI) to induce bone formation. Bone morphogenetic proteins (BMPs) are important regulators in osteogenic differentiation. To discover small molecules that induce BMP-2, Mundy et al. examined more than 30 000 compounds from a collection of natural products and tested the effects of compounds on BMP-2 gene expression. They identified that statin, a common HMGRI, the rate-limiting enzyme in the mevalonate pathway, as the only natural product in that collection that specifically increased expression of the BMP-2 gene. In an in vivo study we showed that the amount of new bone formed by statin mixed with a collagen carrier was quantitatively assessed and results showed that 308% more new bone was formed in defects grafted with statin than those grafted with the carrier alone.

Further study showed that the statin-mediated activation of BMP-2 promoter was completely inhibited by the downstream metabolite of HMG-CoA reductase, mevalonate, indicating that the activation was a result of the inhibition of that enzyme. Therefore, it is possible that any HMGRI may have a similar effect of statin in the activation of the BMP-2 promotor. Naringin is a polymethoxylated flavonoid commonly found in citrus fruits and it is also a HMGRI. The authors compared the amount of new bone produced by naringin in collagen grafts to that produced by collagen grafts. A total of 490% more new bone was present in defects grafted with naringin in collagen than those grafted with collagen alone.

To study the mechanisms of the HMGRI-induced osteogenesis, using immunolocalization studies, we showed that on the early healing of the defects grafted with

statin, the VEGF, BMP-2, Cbfa1 expressed one day earlier than those grafted with the carrier alone.

To conclude, HMGRIs like statin and naringin in collagen matrix carriers have the effect of increasing new bone formation locally and can be used as bone graft materials.

Chapter 2 - Internal and surface bone remodelling of inhomogeneous long cylindrical bone is studied both theoretically and numerically in this chapter. Based on the theory of adaptive elasticity, two solutions for thermoelectroelastic problems of bone remodelling, are presented to study effects of mechanical, thermal and electric loads on bone remodelling process. The external loads may be of coupled axial force, external lateral pressure, electric, magnetic and thermal loads or the load as a result of a force-fitted medullary pin. The analytical solution is used for investigating bone remodelling process on the basis of assuming a homogeneous bone material. Though all bone is heterogeneous, assumption of homogeneity is made to entail a smoothing over features such as osteons, lamellae, fibres, and other structural elements, so that a continuum representation can be obtained. In contrast, the semi-analytical solution is developed for analysing bone materials that are assumed to be radially inhomogeneous. These two solutions are then extended to include magnetic effect. Numerical results are presented to verify the proposed formulation and to show the effects of mechanical, thermal and electric loads on bone remodelling process.

Chapter 3 - Bacterial infection remains a significant complication following total joint replacements, with infection rates ranging from 1% to 3% despite strict antiseptic operative procedures. The annual cost of treating joint prosthesis-associated infections in the United States alone is over US$300 million. Antibiotics such as gentamicin have been loaded into bone cement as one of the measures against prosthesis-related infection. Although antibiotic-loaded bone cements have been available for 30 years, a number of problems related to their use still persist. It is known that antibiotics are released from bone cement in a bi-phasic fashion with an initial peak release followed by slow release at decreasing levels which may continue for days to months. As a result, the protective effect of the antibiotic-loaded cement against bacterial infection is generally lost if bacterial contact with the implant occurs after a delay of several weeks, particularly as bacteria in biofilms on biomaterial surfaces have higher resistance to antibiotics than their planktonic counterparts. An increase in the antibiotic content may extend the period of protection against biofilm formation but the mechanical strength of the cement may be reduced, and higher levels of antibiotic release may lead to adverse effects such as kidney damage. Hence, it would be desirable to formulate new bone cements which can prevent bacterial infection and biofilm formation even after extended time without these accompanying problems.

In this article, two new strategies which the authors have developed to confer antibacterial properties to bone cement are described. The first strategy employs norfloxacin-containing monomer, while the second uses chitosan nanoparticles which may be further derivatized with quaternary ammonium groups as one of the components in the commercial poly(methyl methacrylate) bone cement. The norfloxacin moieties or chitosan nanoparticles can be used to confer antibacterial properties to plain bone cement or to further enhance the effects of antibiotic-loaded cement. These modified cements offered two distinct advantages: high mechanical strength even with an additive to bone cement powder loading of 15%, and long lasting antibacterial effect. Antibacterial assay using *Staphylococcus aureus* and

Staphylococcus epidermidis showed a 10^3 to 10^4 reduction in the number of viable bacterial cells upon contact with plain bone cement modified with chitosan or norfloxacin at an additive to bone cement weight ratio of 15%. The antibacterial effectiveness remained high even after the modified bone cement had been immersed for three weeks in an aqueous medium. No significant cytotoxicity effects were encountered with mammalian cells (mouse fibroblasts), as measured using the MTT assay. The results indicate that these strategies for modifying commercial bone cements are promising for combating joint implant infection.

Chapter 4 - As a kind of biomaterial for biotechnologies such as enzyme immobilization, polyacrylonitrile-based membranes have received much attention in the past years. However, as synthetic materials, the relatively poor biocompatibility of these membranes probably causes to some non-biospecific interactions between enzymes and the membrane surfaces, therefore, resulting in the partial denaturation of enzyme protein and the loss of enzyme activity. In order to depress these unfavorable interactions, the membrane surfaces should be modified to create biofriendly microenvironments for the immobilized enzymes. Following this idea, various copolymers were synthesized by the water phase precipitation copolymerization of acrylonitrile with maleic acid, acrylic acid, *N*-vinyl-2-pyrrolidone, 2-hydroxyethyl methacrylate (HEMA), 2-methacryloyloxy-ethyl phosphorylcholine (MPC), and sugar-containing vinyl monomers, respectively. They were fabricated into asymmetric membranes by phase inversion method or nanofibrous membranes by electrospinning process. Protein adsorption and cell adhesion measurements indicated that the surface biocompatibility of the polyacrylonitrile-based membranes could be improved to some extent through these copolymerizations. However, biomimetic surface modifications such as the incorporation of phospholipid moieties and the immobilization of biomacromolecules (chitosan and gelatin for examples) further enhanced the biocompatibility for the studied membranes. Lipase from *Candida rugosa* was chosen as a model enzyme to immobilize on some of the membranes through physical adsorption and/or covalent binding. Results demonstrated that, for enzyme immobilization, the biocompatibility of membrane was one important requirement, as the biocompatible surface could reduce non-biospecific enzyme-membrane interactions, created a specific microenvironment for the enzyme and thus benefited the enzyme activity. On the other hand, the enzyme loading and the activity retention of immobilized lipase on the nanofibrous membranes were much higher than those on the asymmetric membranes. Because the large specific area of nanofibrous membranes could greatly increase the catalyzing ability of immobilized enzymes due to the remarkable reduction of diffusional restriction for substrate and product transport, as well as provide more rooms for enzyme immobilization.

Chapter 5 - The adsorption of protein from blood plasma onto a biomaterial is the initial event that takes place in most *in vivo* settings, determining the interaction of inflammatory cells with the material surface. In this study, fibrinogen (Fg), was incubated overnight at 37°C on three ^{14}C-labelled polycarbonate-based polyurethanes (PCNUs) (synthesized with either 1,6 hexane diisocyanate (HDI) or 4,4'-methylene bis-phenyl diisocyanate (MDI), poly (1,6-hexyl carbonate) diol (PCN) and 1,4-butanediol (BD) in different stoichiometric ratios (HDI:PCN:BD 4:3:1 or 3:2:1 and MDI:PCN:BD 3:2:1) (referred to as HDI431, HDI321 and MDI321 respectively) and then incubated with either U937 macrophage-like cells or enzymes (cholesterol esterase (CE) or carboxyl esterase (CXE); resembles monocyte-specific esterase

(MSE) in specificity). Degradation was measured by counting the radiolabel release into the supernatant. There was no effect of Fg on U937 cell-mediated degradation of HDI431 and HDI321, whereas the degradation of MDI321 was inhibited, with less MSE protein in cell lysate from cells adherent to MDI321 ($p<0.05$). In contrast, the CE-mediated radiolabel release from MDI321 was not inhibited by Fg, whereas it was from HDI431 and HDI321. There was no effect of Fg on the CXE-mediated radiolabel release for any of the 3 PCNUs. Although the difference in the Fg effect appears paradoxical (cells with MDI321 vs enzyme data for MDI321), it must be considered that Fg could inhibit other cell-mediated degradative activities. However, this study suggests that the effects are specific, relating to material differences, enzyme substrate specificities, as well as cell processes.

Chapter 6 - An alloy intended for medical application is subject to preclinical and biological evaluation in order to ensure compliance with the specification regarding patients safety. The preclinical evaluation includes biological evaluations described in ISO 10993-1 (cytotoxicity, sensitization, irritation, toxicity, genotoxicity, implantation, haemo-compatibility) on one hand, as well as physico-chemical tests concerning localized corrosion and the realease of metallic cations on the other hand. In recent years, a comprehensive study was conducted on localized corrosion of and the release of cations, particularly nickel, from stainless steels commonly used for medical devices and consumer products in permanent contact with the human body. The authors present here selected results for stainless-steel grades used in applications such as medical instruments (AISI 431), hypotubes for angioplasty (AISI 304L), and implants (AISI 316L - ASTM F138-97, MP35N). The same grades are also used for watches and similar products that come into contact with the skin. In this context European Directives were decreed imposing a limit to the tolerable rate of nickel release concerning products that are into direct and prolonged contact with the skin.

The study reveals a significant anisotropy of localized corrosion and nickel release, depending on the orientation of the test surface with respect to the casting and rolling direction of the steel. Cross-sectional specimens (transversal cuts with respect to the rolling direction) show a substantially higher sensitivity to corrosion phenomena compared to longitudinal cuts, and they release nickel ions at rates 10 to 100 times higher.

These findings indicate that orientation needs to be taken into account when interpreting test results, in particular when comparing different grades of steel, as well as in product and production design.

Chapter 7 - Much more than a passive spectator, extracellular matrix (ECM) acts on the stage of tissue and organ development as main actor, playing both structural and functional characters. For this reason, the casting of tissue engineering has chosen native ECM as useful template for rebuilding tissues by themselves or associated with synthetic matrices. Apart from composition, also the distribution of molecular cues within the matrix may play important role in controlling and guide the morphogenetic processes. However, the role of spatial modulation of matrix cues is still poorly understood.

This article overviews the development of natural and synthetic extracellular matrices (ECMs) for use in tissue engineering that aim to mimic functions of the native ECM of developing and regenerating tissues. In addition to the potential therapeutic uses of these materials, they also provide model systems for basic studies that may shed light on developmental processes.

Chapter 8 - Magnetic nanoparticles for medical applications have been developed by many researchers. Since magnetic nanoparticles have unique magnetic features not present in other materials, they can be applied to special medical techniques. Magnetite cationic liposomes (MCLs), one of the groups of cationic magnetic particles, can be used as carriers to introduce magnetite nanoparticles into target cells since their positively charged surface interacts with the negatively charged cell surface. Magnetite nanoparticles conjugated with antibodies (antibody-conjugated magnetoliposomes, AMLs) are applicable to introduce magnetite nanoparticles into target cells specifically, even when target cells coexist with the other kinds of cells (e.g. mesenchymal stem cells in bone marrow aspirates). Since the cells labeled with magnetite nanoparticles could be manipulated using magnets, we applied this technique to tissue engineering and termed 'magnetic force-based tissue engineering (Mag-TE)'. Both of magnetic force and functionalized magnetite nanoparticles were used in various process of tissue engineering: Isolation and *in vitro* expansion of target cells; seeding of target cells into three dimensional (3D) porous scaffolds; or construction of multilayered cell sheet-like structures and tubular structures. Thus, the applications of these functionalized magnetite nanoparticles with their unique features will further improve tissue engineering techniques.

Chapter 9 - Chitosan has been investigated as a non-viral vector because it has several advantages such as biocompatibility, biodegradability and low toxicity with high cationic potential. However, low specificity and low transfection efficiency of chitosan as a DNA carrier need to be overcome for clinical trials. In this review, chemical modification for enhancement of cell specificity and transfection efficiency was explained. Also, the chitosan derivative formulations in vivo were included.

Chapter 10 - An alloy intended for medical application is subject to preclinical and biological evaluation in order to ensure compliance with the specification regarding patients safety. The preclinical evaluation includes biological evaluations described in ISO 10993-1 (cytotoxicity, sensitization, irritation, toxicity, genotoxicity, implantation, haemocompatibility) on one hand, as well as physico-chemical tests concerning localized corrosion and the realease of metallic cations on the other hand. In recent years, a comprehensive study was conducted on localized corrosion of and the release of cations, particularly nickel, from stainless steels commonly used for medical devices and consumer products in permanent contact with the human body. We present here selected results for stainless-steel grades used in applications such as medical instruments (AISI 431), hypotubes for angioplasty (AISI 304L), and implants (AISI 316L - ASTM F138-97, MP35N). The same grades are also used for watches and similar products that come into contact with the skin. In this context European Directives were decreed imposing a limit to the tolerable rate of nickel release concerning products that are into direct and prolonged contact with the skin.

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These findings indicate that orientation needs to be taken into account when interpreting test results, in particular when comparing different grades of steel, as well as in product and production design.

Chapter 11 - The field of Tissue Engineering, now under the larger umbrella of Regenerative Medicine, has developed from the field of Biomaterials. In the past, biomaterials used for the repair and replacement of tissues were designed to have minimal interaction and impact on the human body and have the potential to last the lifetime of the patient. Today, implant materials are being designed to elicit desired and specific responses in cells that promote the degradation of the material and its replacement with organized living tissue. Although degradable synthetic materials are available, they currently lack many physical and biological properties necessary to ensure the proper structure and function of regenerated tissues. An alternative approach involves using decellularized tissues as templates for regeneration. By doing so, the structure and function of the tissue may be retained, while allowing for repopulation by the recipient's cells. In this review, the development of decellularization techniques and their application in Tissue Engineering as scaffolds for the replacement and regeneration of tissues will be discussed.

CONTENTS

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Chapter 1

THE USE OF HMG-COA REDUCTASE INHIBITORS AS BONE INDUCTION MATERIALS

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ABSTRACT

The search of a non toxic material to replace bone remains a big challenge for scientists. This chapter summarizes the current research behind the use of hydroxymethylglutaryl coenzyme A reductase inhibitors (HMGRI) to induce bone formation. Bone morphogenetic proteins (BMPs) are important regulators in osteogenic differentiation. To discover small molecules that induce BMP-2, Mundy et al. examined more than 30 000 compounds from a collection of natural products and tested the effects of compounds on BMP-2 gene expression. They identified that statin, a common HMGRI, the rate-limiting enzyme in the mevalonate pathway, as the only natural product in that collection that specifically increased expression of the BMP-2 gene. In an in vivo study we showed that the amount of new bone formed by statin mixed with a collagen carrier was quantitatively assessed and results showed that 308% more new bone was formed in defects grafted with statin than those grafted with the carrier alone.

Further study showed that the statin-mediated activation of BMP-2 promoter was completely inhibited by the downstream metabolite of HMG-CoA reductase, mevalonate, indicating that the activation was a result of the inhibition of that enzyme. Therefore, it is possible that any HMGRI may have a similar effect of statin in the activation of the BMP-2 promotor. Naringin is a polymethoxylated flavonoid commonly found in citrus fruits and it is also a HMGRI. We compared the amount of new bone produced by naringin in collagen grafts to that produced by collagen grafts. A total of 490% more new bone was present in defects grafted with naringin in collagen than those grafted with collagen alone.

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To study the mechanisms of the HMGRI-induced osteogenesis, using immunolocalization studies, we showed that on the early healing of the defects grafted with statin, the VEGF, BMP-2, Cbfa1 expressed one day earlier than those grafted with the carrier alone.

To conclude, HMGRIs like statin and naringin in collagen matrix carriers have the effect of increasing new bone formation locally and can be used as bone graft materials.

Keywords: HMGCo-A reductase inhibitors, statin, naringin, bone induction

1. Bone Induction Techniques

1.1. Introduction

Bone induction is needed to augment fracture healing and to fill osseous defects. Over the years autogenous cancellous bone grafting has been considered the gold standard in this form of treatment (Lane et al. 1999). The major limitations of using autogenous grafting are the inadequacy of supply and surgical morbidity; including donor site pain, paraesthesia, and infection, which can approach 8% to 10% (Younger and Chapman 1989). Moreover, graft resorption poses a severe problem. In an experimental study, endochondral bone grafts showed 65% volume loss (Zins and Whitaker 1983). Allografts, an alternative to autogenous grafting, seem to be biologically inferior and are associated with infection and inflammation (Strong et al. 1996).

To overcome this problem, investigators have attempted to develop synthetic composite grafts that are intended to mimic the natural components required for bone healing. Three general approaches have been developed.

The first approach, matrix based therapies simply introduce biocompatible implants to replace the missing bone, and they consequently depend on the recruitment of endogenous osteoprogenitors to repair the osseous tissue. Some are based on titanium fibre metals and ceramics composed of hydroxyapatite, tricalcium phosphate, or both (Bucholz et al. 1987, Hollinger et al. 1996, Johnson et al. 1996, Moore et al. 1987, Urist et al. 1984, Wolff et al. 1994). Optimally, they have a porous nature that facilitates bony ingrowth and allow osteoconduction. They are not bone inductive but can be used as carriers for other bone inductive techniques.

The second approach, factor based therapies directly provide osteoinductive stimuli. Growth and differentiation molecules have been introduced to bone defects such as using demineralized bone matrix (Einhorn et al. 1984, Feighan et al. 1995) or bone morphogenetic proteins (Cook et al. 1994b, Cook et al. 1995, Gerhart et al. 1993, Heckman et al. 1991, Mayer et al. 1996, Stevenson et al. 1994, Yasko et al. 1992).

In the third approach, the cell based therapies, cells with osteogenic potential are transferred directly to the site requiring augmentation. They do not depend on local osteoprogenitors for the synthesis of new bone at the site of the defect and are particularly attractive for patients in whom the host tissue bed has been compromised. (Egrise et al. 1992, Inoue et al. 1997, Quarto et al. 1995, Tabuchi et al. 1986). The second and the third approaches, which involve bone induction, will be discussed in more detail.

1.2. Factor Based Approaches

Numerous mediators have been implicated as the predominant growth factors in bone healing with members of the transforming growth factor-β (TGF-β) superfamily of polypeptide growth factors being the most notable. Its members include TGF-β1 through TGF-β5 and other peptides, including the bone morphogenetic proteins (BMPs), and growth differentiation factors (GDFs). This group of growth factors appears to regulate the proliferation and expression of differentiated phenotypes for many cell populations, including chondrocyte, osteoblast, and osteoclast precursors. The extracellular matrix of bone is the largest source of TGF-βs in the body (Centrella et al. 1994). TGF-βs are also present in the graft haematoma after release by platelets and are synthesized additionally by mesenchymal cells.

Other growth factors present in the callus during the bone healing process include fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and insulin-like growth factors (IGFs) (Bourque et al. 1993, Einhorn 1996, Einhorn and Trippel 1997). Fibroblast growth factors are well known mitogenic and angiogenic factors that are important in neovascularization and wound healing (Radomsky et al. 1998). FGFs were used with demineralized intramembranous bone matrix to promote allogeneic bone graft healing (Lu and Rabie 2002). Platelet-derived growth factors act as local tissue growth regulators and initially were isolated in blood platelets, underscoring one of the important roles of the clot in fracture healing. Insulin-like growth factors are other examples of matrix synthesizing growth factors that are important in bone healing (Sandberg et al. 1993, Simmons 1985, Trippel 1998). D'Ippolito et al. (2002) showed that hepatocyte growth factor in concert with vitamin D may promote growth and differentiation of human bone marrow stromal cells into osteogenic cells.

1.2.1. Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is essential for bone formation. VEGF is an angiogenic cytokine; it is a secreted mitogen, a powerful regulator for angiogenesis, and is vasodilatory (Ferrara and Davis-Smyth 1997, Bates et al. 1999). There are at least six VEGF isoforms termed VEGF-A, -B, -C, -D, -E, and placental growth factor. Furthermore, the exon splice variants for VEGF-A generate four molecular species, having 121, 165, 189, and 205 amino acids, and are designated $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{205}$, respectively. VEGF has a direct and singular action on endothelial cells (Yancopoulos et al. 2000). In addition, VEGF stimulates permeabilization of blood vessels and plays a central role in vasculogenesis regulation (Neufeld et al. 1999). VEGF also promotes new collateral vessel growth and undoubtedly is a major therapeutic benefit for disorders characterized by inadequate tissue perfusion (Takeshita et al. 1997). VEGF and FGF-2 serve complementary roles, and thus act synergistically in the production of new blood vessels (Nehls et al. 1998). VEGF may also induce proliferation and differentiation of osteoblasts by stimulating endothelial cells to produce osteoanabolic factors (Deckers et al. 2000, Wang et al. 1997). A close correlation exists between expression of VEGF, vascularization and bone formation in a glenoid fossa model with forward mandibular positioning (Rabie et al. 2002, Shum et al. 2004).

1.2.2. Bone Morphogenetic Proteins

Among the growth factors tested in heterotopic and orthotopic locations, BMPs, either in native or recombinant forms (rhBMPs), appear to be the most promising (Cook et al. 1994a, Cook et al. 1994b, Yasko et al. 1992). Urist made the key discovery that demineralized bone segments and extracts of demineralized bone induce bone formation when implanted subcutaneously or intramuscularly in animals (Urist 1965). Subsequent purification studies of these bone inductive proteins resulted in the identification of many members of BMPs (Özkaynak et al. 1990).

BMPs are low molecular weight glycoproteins which have a pleomorphic function that ranges from extracellular and skeletal organogenesis to bone generation and regeneration. Bone induced by BMP in postfetal life recapitulates the process of embryonic and endochondral ossification (Wozney et al. 1988, Wozney and Rosen 1998). BMPs are important regulators in osteogenic differentiation during fracture repair (Nakase et al. 1994, Onishi et al. 1998, Sakou 1998). Wang et al. (1993) showed that BMP-2 caused commitment and differentiation of a multipotential stem cell line into osteoblastlike cells. Upon stimulation with BMP-2, the cytoplasmic signaling molecules Smad1 and Smad5 are activated and form a complex with the effector, Smad4 (Heldin et al. 1997). Subsequently, a complex of Smad1/Smad4 or Smad1/Smad5 translocates into the nucleus with or without establishing physical interactions with other transcription factors (Heldin et al. 1997). Clinically, native human BMP has been used successfully for the treatment of established nonunions and spinal fusions (Johnson et al. 1988a, Johnson et al. 1988b, Johnson et al. 1992).

BMP-4, BMP-6, and to a lesser extent BMP-5, have also been shown to induce new bone formation (Wozney and Rosen 1998).

Through recombinant gene technology, BMPs are available in sufficient amounts for basic research and clinical trials. rhBMP-2 and rhBMP-7 induce structurally sound orthotopic bone in various experimental systems. These BMPs have the capability of healing critical size defects in rodents, dogs, sheep, and primate models when combined with collagen, demineralized bone matrix, or biodegradable polymers (Aspenberg et al. 1992, Bostrom et al. 1996, Cook et al. 1994a, Cook et al. 1994b, Heckman et al. 1991, Kirker-Head et al. 1995, Kirker-Head et al. 1998, Yasko et al. 1992). Zegzula et al. (1997) successfully induced bone formation with restoration of cortices and marrow elements with the use of rhBMP-2 delivered in porous poly(DL-lactic acid) implants in rabbits. Studies reported to date with rhBMPs have been related largely to animals, although clinical trials are underway in the United States and in Europe (Lane et al. 1999).

1.2.3. Core-Binding Factor Alpha (Cbfa1)

The core-binding factor alpha (Cbfa1) gene, also referred to as polyoma enhancer-binding protein, runt-related transcription factor-2 (Runx2), and acute myelogenous leukemia factor (AML-3), a downstream effector of BMPs, plays a key role in osteoblastic differentiation (Ducy et al. 1997, Komori et al. 1997). The role of Cbfa1 in bone formation has been demonstrated in Cbfa1-deficient mice that manifest a complete absence of osteogenesis (Komori et al. 1997, Otto et al. 1997), and genetic mutations in the Cbfa1 gene have been associated with the multiple skeletal abnormalities in patients with cleidocranial

dysplasia syndrome (Lee et al. 1997, Mundlos et al. 1997). There are three isoforms of the Cbfa1 gene product that differ in their N-terminal sequences but which have identical 3' ends: Cbfa1/org, Cbfa1/iso and Cbfa1/Osf2 (Shui et al. 2003). The Cbfa1 protein has been found to bind the promoter of several genes that are expressed predominantly in osteoblasts (Ducy et al. 1997). All the three isoforms of Cbfa1 have been shown to increase the expression of osteocalcin, osteopontin, and collagen type I genes, and isoforms I and II, but not III, induce alkaline phosphatase (ALP) expression (Harada et al. 1999). Furthermore, forced expression of Cbfa1 in non-osteoblastic mesenchymal stem cells leads to the acquisition of an osteoblastic phenotype by these cells (Ducy et al. 1997). A variety of bone anabolic hormones and skeletal growth factors that affect osteoblast differentiation and function have been shown to regulate Cbfa1 gene expression. BMP-2 increases expression of the Cbfa1 type II transcript during osteogenic differentiation of primary rat osteoblasts, MC3T3-E1 osteoprogenitor cells, and C2C12 premyoblasts (Banerjee et al. 2001), and transiently (within 1 hour) increases the level of Cbfa1 mRNA in human marrow stromal cells (Gori et al. 1999). The Cbfa1 mRNA has also been shown to be transiently upregulated by BMP-4/-7, TGF-β1, or ascorbic acid in C3H10T1/2, C2C12, or MC3T3-E1 cells (Ducy et al. 1997, Lee et al. 1999, Tsuji et al. 1998, Xiao et al. 1998). Nishimura et al. (2002) showed that Cbfa1 expression required the activation of the cytoplasmic signaling molecules, Smad1 and Smad5 by BMP-2. Moreover, Cbfa1 is essential and also sufficient to induce osteoblastic differentiation in C2C12 undifferentiated mesenchymal cells. Shui et al. (2003) recently found that in contrast to rodent systems where osteoblast differentiation is associated primarily with increases in Cbfa1, in human bone marrow stromal cells, it is associated with increases in Cbfa1 activity, without a change in mRNA or protein levels. Yang et al. (2003) showed that Cbfa1 and BMP-2 have distinct, but complementary, roles in osteogenesis and that their combined actions may be necessary for optimal bone formation.

1.2.4. Demineralized Bone Matrix (DBM)

DBM is one osteoinductive material that has been developed to a stage that it can be used routinely in clinical situations. It was considered an acceptable alternative to autogenous grafting in various craniofacial, oral surgical, periodontal, and hand reconstructive procedures (Glowacki et al. 1981, Kaban et al. 1982, Sonis et al. 1983, Upton and Glowacki 1984). Implantation of DBM in segmental defects in rats resulted in complete regeneration in less than 8 weeks (Narang et al. 1973). At 12 weeks after the operation, the mechanical properties of the new tissues were comparable to bone formed in the early stages of normal fracture repair (Einhorn et al. 1984). The capacity of the rat to regenerate bone in response to DBM is extraordinary; such implants induced repair of segmental fibular defects that were too large to be healed even by autogenic bone grafts (Oikarinen and Korhonen 1979). Clinically, the use of DBM in the form of pulverized bone has been shown to induce osteogenesis predictably and thus bone healing in craniofacial defects (Mulliken and Glowacki 1980, Mulliken et al. 1981, Mulliken 1982, Mulliken et al. 1984). The bone induction capacity of DBM was attributed to its content and diffusibility of BMPs and other cytokines that interact with the undifferentiated osteogenic precursor cells in the host bed and cause them to differentiate into functionally active osteogenic elements (Wozney et al. 1988, Tuli and Singh 1978). The BMP was found to diffuse to significant distances along surfaces