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Engineering bone: challenges and obstacles

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Abstract

Repair of large bone defects is still a challenge for the orthopaedic, reconstructive and maxillo-facial surgeon. Availability of pluripotent stem cells from either autologous or allogenic sources and the potential of inducing the osteogenic phenotype is motivating exploration and development of custom-tailored materials known as "bioengineered bone constructs". In such cases, the clinical scenario involves either expansion of stem cells in monolayer and loading them into a porous scaffold prior to surgery or direct cell expansion within the scaffold, and implanting this novel construct back into the donor patient. In this review, we delineate, from an engineering perspective, the progress that has been made to date and the challenges remaining in successfully translating this promising (but not yet definitively established) approach from bench to the bedsite.

Keywords: tissue engineering - bone - biomaterials - stem cells

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Health relevance

Bone healing *in vivo* is generally considered to be biologically optimal since the vast majority of defects in this tissue heal spontaneously with minimal treatment. However, among the 6 millions fractures occurring every year in the United States, 5-10% will require further treatment for compromised healing because of either interposition of soft tissue, improper fracture fixation, loss of bone, metabolic disturbances, impairment of blood supply and infection [1]. In addition, in certain clinical settings, large pieces of bone must be resected to treat benign and malignant tumours, osteomyelitis as well as bone deficiencies and abnormal loss in the maxillofacial area.

In these challenging situations, autologous bone harvested from donor sites such as the iliac crest is the preferred treatment [2]. Grafts of this kind are osteoconductive (they provide a scaffold on which bone cells can proliferate), osteoinductive (they induce proliferation of undifferentiated cells and their differentiation into osteoblasts), and osteogenic (they provide a reservoir of skeletal stem and progenitor cells that can form new bone). Since the available autologous bone supplies are limited and harvesting autologous bone is painful and entails procedures with risk of infection, it has become necessary to develop alternative techniques to overcome these drawbacks. In the past, surgeons used banked bone and natural or synthetic substrates; such materials had limited success because they only provided a scaffold which had to be invaded by bone-forming bioactive cells [3, 4]. In fact, these materials gave good clinical results only when they were implanted in small defects or were placed in direct apposition to bone.

For these reasons, several novel approaches are currently being explored, including the use of growth factors and stem cells, as potential alternatives. In this review, we delineate, from an engineering perspective, the progress made and the challenges remaining in the development of bioengineered bone utilizing skeletal stem cells and synthetic scaffolds (Fig. 1). Since, such "biological composites" will not depend on local recruitment of the osteocompetent cells needed for new bone synthesis, they will be of particular interest and usefullness in clinical cases in which the bed of the wound cannot provide these cells. Such cases include patients with large bone defects and those with reduced number of osteocompetent cells because of aging, osteoporosis, metabolic disturbances and irradiation treatment.

Identification of a reliable cell source

Optimal cell source

Cell sourcing is the first issue to address in the development of bioengineered bone. The characteristics of an optimal stem cell source include: no immunorejection, no graft-versus-host disease, no tumorigenicity, immediate availability, availability in pertinent quantities, controlled cell proliferation rate, predictable and consistent osteogenic potential as well as controlled integration into the surrounding tissues.

Autologous sourcing

An autologous source of skeletal stem cells is most desirable as cells are collected from each patient, thereby eliminating complications associated with immune rejection of allogenic tissue.

Bone marrow, a natural repository of skeletal stem cells, has been used as the source of such cells. When plated at low cell densities, the cells form pluripotent fibroblastic colonies clonal in origin which were initially referred to as "colony-forming units-fibroblasts" (CFU-F) [5]. They represent a small (0.001-0.01%) fraction of the total population of the nucleated cells present in marrow and are now known as "mesenchymal stem cells" (MSCs) [6]. The progeny of MSCs are "bone marrow stromal fibroblasts" (BMSFs). Under appropriate experimental *in vitro* conditions, MSCs can differentiate into bone, cartilage, adipose tissue and hematopoietic-supportive stroma cells (for reviews see [6-9]. *In vivo*, the osteogenic potential of MSCs was initially reported in studies in which the cells were either placed into diffusion chambers or under renal capsules. Then in 1991, Goshima *et al*. showed that MSCs loaded into ceramic scaffolds accelerate bone formation [10]. From the bone tissue engineering perspective, MSCs have a number of advantages: (i) their isolation is easy as it relies

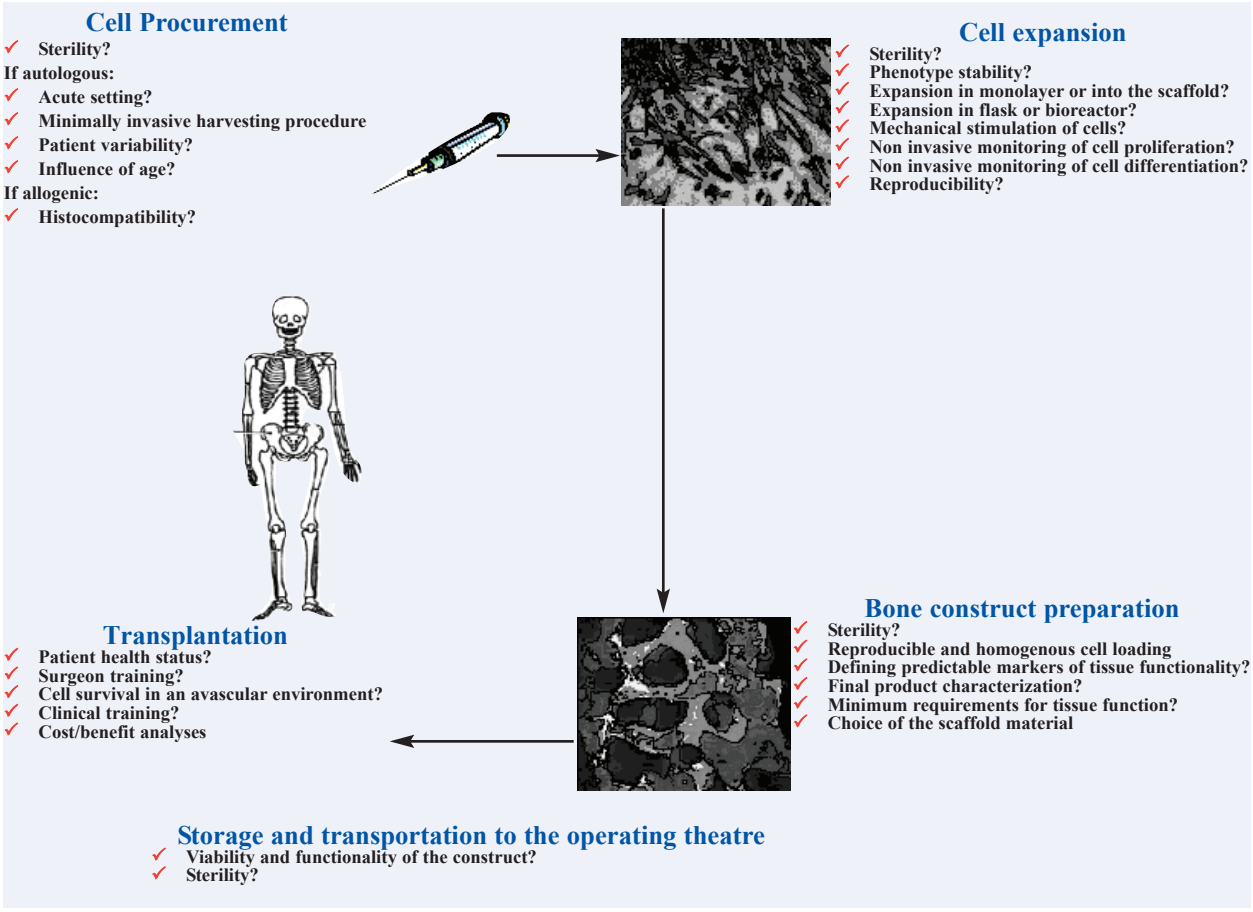

Fig. 1 Overview of the challenges and obstacles for engineering bone.

primarily on the ability of these cells to adhere to tissue-culture plastic [10]; (ii) they have a high proliferative potential [11-13]. In fact, it has been determined that almost half a billion cells could be obtained at passage 6 even when starting with 100-500 adherent MSCs [11]; (iii) the default pathway of MSCs is the osteogenic pathway; (iv) bone formation is not correlated to the number of cell passages as long as the human stem cells retain their proliferative potential [11, 14]; and (v) freezing conditions do not affect the osteogenic potential of MSCs, a condition that greatly facilitates their storage [10, 12].

Recently, stem cells with osteogenic potential have also been isolated from a number of other tissues including blood, adipose tissue, skeletal muscle, lung, deciduous teeth, synovium (for review see [15]). However, before these new sources could be considered as viable alternatives to bone-marrow-derived MSCs, further studies in clinically relevant animal models are needed to better characterize the relative (compared to bone marrow) osteogenic potential of stem cells isolated from these alternative sources.

To date, use of stem cells in the acute clinical setting has not been possible in autologous therapy because of pertinent logistics, specifically limitations associated with harvesting bone marrow from the patient and with expanding such cells *in vitro* in extremely short period of time. Additional major hurdles associated with the use of autologous stem cells are: (i) decreased numbers with age ([16-18] and for review see [19]); and (ii) variability of the amount and quality of biopsied bone tissue (the source of autologous cells) from patient to patient.

Development of "off-the-shelf cells"

Limitations of the autologous approach in obtaining stem cells and the desire to obtain "marketable products" which could benefit as many patients as

possible have provided incentives for the development of generic cell lines, which can be taken off the shelf as, and when, needed for patient treatment. These universal cells would have the following advantages: (i) availability through the development of large cell banks; (ii) consistency and efficacy because only cells with desirable characteristics and controlled critical parameters are selected and amplified; and (iii) sterility and assurance of compatibility through extensive safety testing. Until recently, it was difficult to envision utilization of allogenic generic cells in orthopedics as it was believed that their transplantation would require immunosuppressive drugs to reduce associated risks of rejection. However, this dogma has been recently shaken by data providing evidence that cultured MSCs exhibit a poorly immunogenic phenotype (*i.e*., evidenced by MHC class I^+, MHC Class II-, and low level of expression of costimulatory molecule)[20]. In fact, addition of MSCs to either autologous or allogenic T-lymphocytes stimulated by irradiated allogenic peripheral blood lymphocytes, dendritic cells or phytohaemaglutinin, resulted in a dramatic decrease in the proliferation of the T cells [20-22]. *In vivo*, a single intravenous administration of MSCs led to a modest, but significant, prolongation of skin graft survival [23]. These data have greatly enhanced the therapeutic appeal of MSCs because they raised the possibility of creating universal cell lines. However, MSCs have also been shown to prevent rejection of B16 melanoma cells in allogenic recipient mice sugggesting that they could favor tumor engraftment [24]. Further investigations are undoubtedly needed to better understand the exact mechanism by which MSCs suppress T-lymphocyte activation. In the meantime, patients that may be at risk for tumor growth should be excluded from clinical studies that aim at evaluating various applications of MSCs.

Obtaining the right material scaffold

Cellular challenges

Cell-induced osteogenesis and chondrogenesis is highly dependent upon the substrate carrier which provides a permissive environment into which bone cells would migrate, proliferate, differentiate and deposit bone matrix (*i.e*., osteoconduction) [25]. Such substratum should have specific biochemical (*i.e.*, molecules of the extracellular matrix), physicochemical (such as surface free energy, charge, hydrophobicity) as well as geometric aspects (for example, three dimensional (3D), interconnected porosity) [26-28].

Biomaterial challenges

Scaffolds for engineering bone should satisfy a number of criteria. Such matrices should be: (i) biocompatible, *i.e.*, non-immunogenic and non-toxic; (ii) absorbable (with rates of resorption commensurate to those of bone formation); (iii) preferably radiolucent (to allow the new bone to be distinguished radiographically from the implant); (iv) osteoconductive; (v) easy to manufacture and sterilize; and (vi) easy to handle in the surgery room, preferably without preparatory procedures (in order to limit the risk of infection). It has been often claimed that scaffolds for engineering bone should have good mechanical properties. It is the authors' opinion that such requirements are not necessary as the main function of the scaffold is to support bone ingrowth and not to provide support to mechanical loading. When needed, sufficient mechanical stability can often be achieved by appropriate orthopaedic devices (intramedullary rods, internal plates or external fixators).

The following macro- and micro-structural properties of biomaterial scaffolds are critical for optimal cell ingrowth.

Porosity

Three-dimensional scaffolds for bone tissue regeneration require internal microarchitecture, specifically highly porous interconnected structures and a large surface-to-volume ratios, to promote cell in-growth and cell distribution throughout the matrix. Pore sizes in the range of 200-900 μm have performed most satisfactorily in these applications because, in addition to osteoprogenitor cells, they also enable endothelial cells to

migrate into the matrix and develop the vascular beds necessary to nourish the newly formed tissue.

Topography and surface chemistry

Particle size, shape, and surface roughness affect cellular adhesion, proliferation, and phenotype. Specifically, cells are sensitive and responsive to the chemistry, topography and surface energy of the material substrates with which they interact. In this respect, the type, amount and conformation of specific proteins which adsorb onto materials surfaces, subsequently modulate cell functions [29]. Hydrophobic biomaterials support bone tissue healing by promoting adsorption and retention of proteins (such as fibrinogen and fibronectin initially derived from blood serum) that modulate cell adhesion [30]. More importantly, calcium-based ceramics undergo dissolution and precipitation at their surfaces. These events lead to formation of a carbonate-containing hydroxyapatite layer which promotes attachment of bone forming cell [31]. Such property is refered as osteoconductivity.

Clinical challenges

The size and anatomical shape of the bone defect in which the tissue-engineered construct will be placed, should be considered because different regions of the human body have different functional loads, type of bone (trabecular or compact) and degree of vascularity. All these parameters have to be taken into account when choosing material substrates for bone-tissue engineering applications. Such materials may be either injectable or in block shape, the latter requiring a surgical implantation procedure. On one hand, injectable materials (small particles or semi-liquid polymers that could crosslink in situ) would be preferentially used into irregular closed defects (for example, unopened fractures with bone loss, bone non-unions and cysts). On the other hand, block materials (in combination with either metal plates or other forms of fixation) are more appropriate for extensive, segmental, long-bone defects.

Scaffolds for bioengineered bone: a second life for bone fillers

To date, there are no Food and Drug Administration (FDA) approved orthopaedic devices that incorporate tissue-derived components such as cells. There are, however, bone fillers (chiefly calcium based ceramics) that have been assessed as scaffolds for delivering MSCs (for review see [19]).

Proof of concept was initially made in rodent models in which the regenerating ability of bioengineered constructs was always superior to the bone regenerating ability of the scaffold alone ([32, 33] and for review see [34]). However, the size of the defect was limited to maximum 6 mm. To show clinical applicability, studies were pursued in large animals in load bearing situations mimicking more closely the clinical setting. In the case of hydroxyapatite-tricalcium phosphate [35] or porous hydroxyapatite [36] ceramic scaffolds, MSC-loaded ceramics contained more bone when compared to scaffolds alone. However, no scaffold resorption was observed within the time frame of the experiments leaving the defect filled with "a composite tissue" of unknown mechanical properties. Similarly, in the case of natural coral exoskeleton, MSC-loaded scaffolds elicited more bone formation than that obtained with scaffolds alone. In contrast to MSC-loaded hydroxyapatite-tricalcium phosphate [35] or porous hydroxyapatite [36] ceramic scaffolds, however, complete resorption of the natural coral scaffold as well as corticalization and formation of a medullary canal with mature lamellar bone were observed in the most favorable cases (3/7 animals). Taken together, these results are promising as they suggest that MSC-loaded constructs lead to reconstruction of large-size bone defects, albeit with suboptimal results (for review see [34]). Nevertheless, in all the studies mentioned, the osteosynthetic material was not excised leaving undetermined the issue of the biomechanical properties of the newly formed bone.

Cell delivery in an avascular environment

One of the most limiting aspects in obtaining bioengineered bone suitable for repairing large (>10

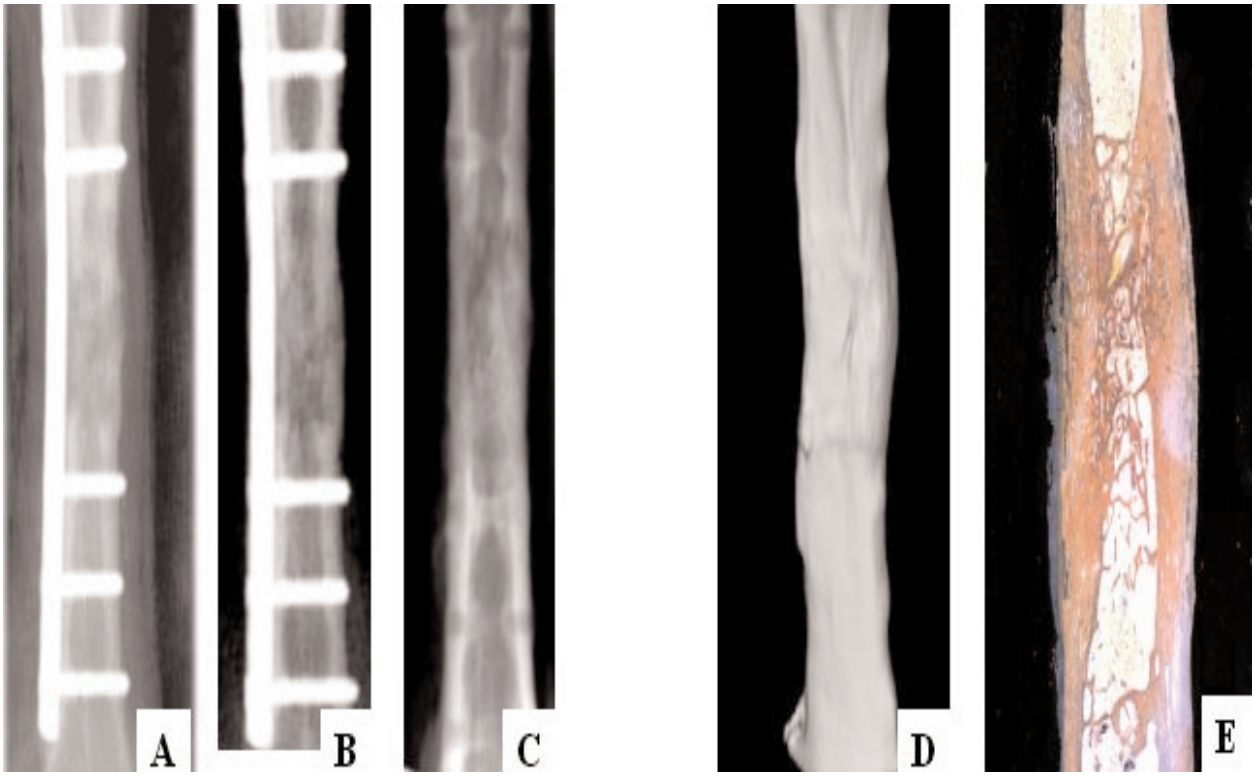

Fig. 2 Autologous MSCs from adult sheep were expanded *in vitro* onto coralline hydroxyapatite scaffolds (CHA). This method of culture induced formation of a "periosteal-like layer" which surrounded the implant. X-rays were taken (A) 6, (B) 10, and (C) 14 months after a 25-mm resection of the metatarsus of adult sheep and transplantation of the bone construct. Gradual remodelling of the implant occured. (D) Representative three-dimensional reconstruction of computer-assisted tomography scans of the metatarsus taken at 14 months. Note that at this time, the animal had undergone removal of the osteosynthetic material for 2 months. The hydroxyapatite mineral content of the newly formed bone and of the normal adjacent bone was 1,015 and 1,474 mg/ml, respectively. (E) Longitudinal histological sections of defects filled with bone constructs. Although some residual bone tissue was still present in the medullar cavity, remodelling of the tissue-engineered bone led to formation of new cortical bone matching the architecture of the adjacent metatarsus. By that time, the scaffold had completely disappeared. Reprinted with permission from "De Novo Reconstruction of a Functional bone by Tissue Engineering in the Metatarsal Sheep Model " published in Tissue Engineering (in press, 2005).

mm) bone defects is the ability to orchestrate early initiation and development of a functional vascular network. Unlike organ transplants in which a preexisting vascular network supplies oxygen and nutrients, synthetic bone constructs (the ultimate efficacy of which depends on the presence of viable MSCs [37]) are devoid of preexisting vascularization. Upon implantation, survival of such bone constructs will, therefore, depend on surrounding diffusive nutrient supply and waste removal processes until the engineered tissue become vascularized. This aspect is critical when the respective bone defects are large, as nutrient diffusion is effective within 150-200 µm from the blood supply source [38-40]. In addition, *in vitro* studies have shown that, under hypoxic conditions, massive death of MSC occurs within 3 days [41]. All in all, these data strongly suggest that the ultimate efficacy of engineered bone will depend on timely delivery and exchange of oxygen and nutrients from surrounding blood vessels to MSCs. To meet this challenge successfully, acceleration of the vascular invasion has been attempted by improving the design of scaffolds and supplementing them with angiogenic molecules. Delivering MSCs mixed with a cocktail of molecules that reduce MSCs sensitivity to hypoxia might be an alternative strategy of

untapped potential. Alternatively, MSC delivery as a "periosteal layer" (rather than within the core of the scaffold) might favor direct contact of MSCs with surrounding, normoxic healthy tissues, which will provide ready access to the blood supply and allow survival of greater numbers of cells in the tissue engineered construct. In fact, successful healing of large bone defects has been achieved by delivering MSCs as a "periosteal layer" (Fig. 2) [42].

New trends

Polymers (either naturally-derived or synthetic ones) are another promising category of potential materials for bone tissue engineering applications.

Natural polymers are extracted from animal and vegetable sources. These compounds are of major interest in tissue engineering since they are biocompatible, biodegradable and natural substrates onto which cells can adhere, proliferate, and function [43]. Additionally, such material substrates can be prepared in various forms including strips, sheets, sponges and beads.

Synthetic polymeric materials, free of potential contamination, have proven versatile and are used to develop biocompatible substrates tailored for specific medical applications [44]. One of the polymers that has been considered for potential use in skeletal repair is the poly (lactic-co-glycolic acid) (PLAGA) copolymer and its homopolymer derivatives. Various chemical techniques were investigated and were successful in developing three-dimensional porous structures that can be custom-designed to the size and shape of bone defects in individual patients. The most widely used processing method for polymeric scaffolds involves solution-cast/particulate-leaching to tailor three-dimensional porous structures [45]. Recent advances in computer technology enabled development of new fabrication, such as rapid prototyping (RP) techniques, for use in the tissue-engineering field. RP techniques utilized layered manufacturing approaches, whereby 3D objects are fabricated layer by layer via processing of solid sheet, liquid or powder material stocks [46, 47]. These computer-controlled fabrications theoretically allow reproducible fabrication of polymer scaffolds, which may be combined in a customized way with highly uniform pore morphology and pore interconnectivity. These new technologies, however, have limitations similar to those associated with conventional methodologies because they also use organic solvents, operate at high processing temperatures and result in limited pore size that require use of porogen particles to enlarge pore size.

Use of polymers as scaffolds for tissue-engineering applications has several advantages. These materials are amenable to chemical modification needed to promote and/or improve cell adhesion and subsequent cell functions pertinent to new tissue formation (described in the sections that follow). Additionally, polymers have been studied extensively for applications in tissues such as cartilage, blood vessel, nerve conduits, liver.

Despite a plethora of polymeric materials developed for bone-tissue engineering purposes, few constructs have been evaluated in *in-vivo* (even in small) animal models [48, 49]. This limitation of polymer-based scaffolds is likely due to lack of bioactivity, which prevents or limits either cell adhesion to biomaterials or subsequent cell functions pertinent to new tissue formation and further tissue integration at the implant-host- tissue interface. To overcome these limitations, some researchers used composites combining osteoconductive materials with polymer-based materials such as HAP-coated PLGA scaffolds [50].

Material scaffolds intended as engineered matrices should both assist and stimulate tissue regeneration by promoting cell functions pertinent to new tissue formation. In an attempt to address this issue, chemical modifications of either the surface or bulk of materials that endow scaffold with biological cues (which *in vivo* regulate attachment, proliferation and differentiation of various cells) have been developed.

A way to improve cell adhesion and migration onto a scaffold whose material surface originally favours weak cell-matrix interactions, is to tether cell-binding peptides that mimic and induce cell-extracellular matrix (ECM) protein interactions to the biomaterial surface through physical or chemical modification methods [51]. Many investiga-

tions focused on the interactions of bone-forming cells with various integrin-binding domains such as the arginine-glycine-aspartic acid (RGD) oligopeptide [52-54]. Since the RGD peptides modulate attachment of many cell types (including osteoprogenitor, osteoblasts, and endothelial cells) chemical modifications of material substrates to induce select functions of specific cells (such as osteoblasts) requires design, synthesis and evaluation of novel peptides that have such unique properties[55].

Incorporation of growth factors (which, upon release, promote tissue regeneration during different phases of bone-fracture healing) on the scaffold material could modulate proliferation and differentiation of implanted cells. In this respect, use of bone morphogenetic proteins (BMPs), which induce differentiation of uncommitted mesenchymal stem cells into osteoblasts, has been of particular interest [56]. Angiogenic factors, such as vascular endothelial growth factor (VEGF) may also be incorporated into the synthetic material scaffolds to promote *in vivo* angiogenesis [57].

Theoritically, three strategies could be attempted to immobilize bioactive molecules onto synthetic material: (i) covalent linkage *via* a chemical process that does not alter the biological activity of the immobilized protein [58]; (ii) non covalent binding of growth factors *via* specific molecules (i.e., heparin-like molecules) immobilized at the material surface. Such strategies enabled preservation of the bioactivity of growth factors and their sustained release from material substrates [59-61]; and (iii) physical entrapment of growth factors into delivery-vehicles and release during degradation of the carrier material [62-64].

Another approach to promote bone cell functions pertinent to neotissue formation uses the gene therapy principle. In this case, the scaffold matrix is the vehicle for delivery of genes encoding for a growth factor. Implanted cells in contact with such a material will integrate the DNA and will act as local in vivo bioreactors, secreting plasmid-encoded proteins that augment tissue repair and regeneration [65] in a transitory way since the transfected DNA will not be inserted into the genome. "Gene activated matrices" have certain advantages over "growth factor coated matrices", specifically stability of the immobilized molecule, low manufacturing cost as well as capability to modulate the pattern of expression of growth factors by judicious choice of the promoter on which the gene of interest is fused.

The use of such technologies for bone tissue engineering applications is just emerging and requires further studies including *in vivo* animal models.

Producing bioengineered bone of clinical grade

A major challenge to the tissue engineering field is to produce bone of clinical grade at an acceptable cost. This endeavor entails a true paradigm shift for bone biologists who are usually initiators of such projects. Products of such endeavors should comply to policy issued by healthcare regulatory authorities. Such requirements impact various aspects of product development including processing, in vitro testing, pertinent animal studies, and clinical trials. To address this challenge, multidisciplinary teams (involving clinicians, engineers, biologists and regulatory agency personnel as well as industrial representatives) must be formed and work together to achieve the desirable goals.

Different regulations exist in different countries [34], but the USA policy is regarded as the most comprehensive regulatory approach and relies on regulating tissue-engineered-products through a risk-based regulatory scheme. In February 1997, the Food and Drug Administration (FDA) published two documents entitled "A Proposed Approach to the Regulation of Cellular and Tissue-Based Products" and "Reinventing the Regulation of Human Tissues" that established the current framework for the regulation of cell- and tissue-engineered products. The intention of this FDA act is to: (i) control the spread of infectious disease; (ii) prevent handling that may damage tissue products; and (iii) ensure safety and efficacy of such products.

This aforementioned regulation was described in and disseminated by the publication of three documents by the Federal Register entitled:

- "Human Cells, Tissues, and Cellular and Tissue-Based Products; Establishment

Registration and Listing- Final Rule " published on January 19, 2001, which requires that establishments that manufacture tissue-engineered products, as well as all tissue engineered products, to be registered with the FDA.

- "Eligibility Determination for donors of Human Cells, Tissues, and Cellular and Tissu-based Products- Final Rule" issued on May 25, 2004, which established regulations regarding donor screening and testing for communicable diseases.
- "Current Good Tissue Practice ,for Human Cell, Tissue, and Cellular and Tissue Based Product Establishments; Inspection and Enforcement - Final Rule" published on November 24, 2004, which requires establishments which manufacture tissue- engineered products to follow current Good Tissue Practices.

Current FDA regulations address the extent of acceptable cell manipulation. Thus "minimally manipulated" tissue-engineered products (*i.e.*, cryopreserved cells which are not otherwise processed, positive selected CD34+ cells, etc.) are regulated solely by §361 of the Public Health Services Act (PHSA) regarding control of communicable diseases whereas "more than minimally manipulated" tissue-enginnered products (*i.e.*, preparations that involve either *ex vivo* expansion or genetic manipulations) must use either established Investigational New Drug (IND) or Investigational Device Exemption (IDE) mechanisms, and comply with both Good Tissue Practices (GTPs) and Good Manufacturing Practices (GMPs). Any cell product that contains *in vitro* expanded stem cells will, therefore, be considered as a "more than minimally manipulated" product and will require either IND or IDE clearance. Compliance with these FDA regulations implies the following:

(i) Record keeping. Recording all components utilized during manufacturing of the tissue-engineered product as well as description of all appropriate procedures used during preparation and sterilisation of the cell-containing product is required by the FDA. Moreover, appropriate informative product labelling should be used and efficient tracking of the product should be in place.

(ii) Ensuring Biosafety of bioengineered bone (sterility, absence of mycoplasma, adventitious viral agent and endotoxins) necessitates systematic monitoring of both the manufacturing process and the final product. In this respect, an allogenic approach will be a less demanding strategy in terms of ensuring biosafety as one biopsy will permit to expand enough cells to prepare many "units" of bioengineered bone that will benefit many patients. Consequently, one set of analyses will suffice for the evaluation and approval of a batch of bioengineered bone; in contrast, an autologous appoach (one biopsy per patient) will benefit only one patient. Moreover, an allogenic approach allows sufficient lag of time to complete appropriate compatibility and sterility analyses before product release. In addition, experimental studies are primarily performed at a small-scale with cells cultured as monolayers and in open systems. This practice is not an optimal approach for obtaining the large quantities of cells generally needed for medical, health-care applications. Robust processes that protect both product and patient are necessary and require the use of clinical grade products, validated disposable devices, and development of high throughput closed-systems for obtaining safe and cost-effective products. In this respect, development of bioreactors, which allow automation and scale-up in a GTP/GMP environment is most desirable (for review see [66]). Finally, since the risk/benefit ratio is never a life/death ratio in orthopaedics as in other medical specialties (for example, cardiology), regulatory authorities will most probably emphasize biosafety and institute strict regulations for bioengineered bone.

(iii) Assessment of product purity. Assaying for residual growth factors and serum proteins is critical to ensure that no toxic products are administered to the patient along with the cell-therapy product. In this respect, it is noteworthy that proteins derived from fetal bovine serum have been shown to conbine with MHC class I cells and to trigger T-lymphocyte proliferation in mixed lymphocyte

reactions [67]. Antibodies against bovine 2 microglobulin have been found in mice to which syngeneic lymphoblasts cultured in medium containg fetal bovine serum were administrated [68]. These data substantiate the need of well-designed compatibility procedures that assure complete elimination of fetal bovine serum in the final cell-containing tissue engineered product.

(iv) Assurance of the final product characterization, consistency and bioactivity. Finding a molecular signature for bioengineered bone is a difficult issue because: (i) protocols currently used for MSCs isolation, at least MSCs derived from bone-marrow, rely primarily on cell adherence to tissue-culture plastic and give heterogenous populations; (ii) no clear-cut markers that allow identification of MSCs with predictable bioactivity exist yet. In this respect, autologous bioengineered bone with cells isolated on an individual patient basis (and, therefore, of very limited availability) might not be the ideal bone substitute. Even if robust protocols are in place, and because of the inherent variability of biologics, it will be difficult to manufacture tissue-engineered bone in a consistent manner and to achieve predictable clinical results. Finally, ensuring potency and consistency entails development of a set of predictable markers of osteogenic potential such as cbfa1, alkaline phosphatase, osteocalcin, etc. However contributions by molecular biologists (who would carry out multiphaseted analyses to assess gene expression in MSCs) with clinicians (who would assess the osteogenic potential of bioengineered bone in a clinical setting) are needed to better define and validate a set of markers predictive of the clinical efficiency of bioengineered bone. Such studies will establish the definition of acceptance criteria that must be met successfully before product release to the healthcaremarket. Last but not least, maintining and demonstrating the viability of bioengineered bone following long-term storage and transportation to end-users is "the Achille's heel" of this type of products. Cryopreservation could be envisioned for storing if the end product is a cell suspension but is less than an ideal condition as it is technically challenging, requires skilled manipulations and has high cost. In addition, cryopreservation cannot be extrapolated to store more complex tissue engineered products such as MSC-loaded-scaffolds, which presently need further development of new modalities of storage practice. These parameters must be taken into account early in the development process of bioengineered bone because they could have a dramatic impact on the formulation of the end product. Finally, pertinent cell physiology (for example, during product storage and transportation) is essential and must be preserved until use of bioengineered implants by healthcare personnel.

Conclusions

Undoubtedly, appropriately randomized clinical trials which clearly demonstrate benefits, if not superiority, of bioengineered bone compared to established treatments (such as autologous bone grafts) are needed. We envision that bioengineered bone will be primarily used for the treatment of large bone defects in which the supply of autologous bone is insufficient. For small-size bone defects alternative treatments such as transplantation of autologous bone (which is painful, entails risk of infection but, all in all, is efficient) exist or are curently under clinical evaluation. In order for bioengineered bone to find its place in the armentarium of the orthopaedic surgeon, cost/benefit analysis of this new appoach is needed if it is to be funded and fully exploited within the current, tough constraints of healthcare budgets. Transplanting bioengineered bone instead of an autograft represents a shift of paradigm for surgeons, who will need education and training in using this next generation of bone substitutes. Nevertheless, development of bioengineered bone has the potential to impact daily clinical practice and may be beneficial to patients with reduced bone healing potential because of age, osteoporosis, metabolic disturbances and irradiation treatment.

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