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Review articles

Tissue engineering for bone regeneration: stem cells and growth factors in biomaterial scaffolds

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Background: Bone tissue engineering requires a scaffold conducive to cell attachment and maintenance of cell function, together with a rich source of osteoprogenitor cells in combination with osteoinductive growth factors. Bone loss as a result of trauma or disease is an increasingly serious health problem. The requirement for new bone to replace or restore the function of injured, damaged, or lost bone is a major clinical and socioeconomic need. Bone defects still represent a major challenge for orthopaedic and reconstructive surgeons.
Objective: This review aims at outlining the role of stem cells and growth factors in scaffolds, focusing on the use of mesenchymal stem cells and bone morphogenetic proteins as applied to the research and practice of bone tissue engineering.
Results and conclusion: Bone tissue engineering has been emerging as a valid approach to the current therapies for bone regeneration. Therefore, tissue engineering offers a number of possible strategies to the generation of living prosthesis that could integrate with host tissue reducing the need for further surgery or possible implant failure.

Keywords: Bone regeneration, growth factors, scaffolds, stem cells, tissue engineering.

Bone defects represent a challenge for orthopaedic and reconstructive surgeons. Efforts have been made to develop osteoconductive, osteoinductive and osteogenic bone materials. The generation of bioartificial bone tissues may help to overcome the problems related to donor site morbidity and size limitations. Tissue engineering has been emerging as a valid approach to the current therapies for bone regeneration. Tissue engineering began with the use of bioactive materials that were designed to interact with the body to encourage tissue repair and regeneration. An early example was the design and fabrication of artificial skin from collagen and glycosaminoglycan that was successfully used in the treatment of extensive burn injury [1]. Firstly, the term "tissue engineering" was derived from an organization of an endothelium-like structure on the surface of polymethylmathacrylate prosthesis [2]. Currently, the term tissue engineering indicates combinations of cells, scaffold materials, and bioactive molecules used to guide tissue formation (**Fig. 1**). Other examples were the growth of chondrocytes on the bioresorbable polymers of polyglycolic acid mesh [3] and the culture of hepatocytes in hollow fiber liver assist devices [4]. Nowadays, tissue engineering is an interdisciplinary field that applies the principles of engineering and of life science towards the development of biological substitutes for restoring, maintaining, or improving tissue or organ function [5]. Tissue engineering implies the use of tissue or organ-specific cells for seeding an exogenous scaffold and keeps the promise of one day replacing living tissue with living tissue designed and fabricated to meet the individual defects.

The first generation of clinically applied tissue engineering concepts in the area of skin, cartilage, and bone regeneration was based on the isolation, expansion, and implantation of cells from the patient's own tissue. Bone tissue engineering needs to overcome major challenges to allow clinical applications with predictable outcomes. This includes the isolation and expansion of cells with the potential to form bone-like tissue and to direct and maintain the phenotypic differentiation of the cells while being cultured in the scaffold.

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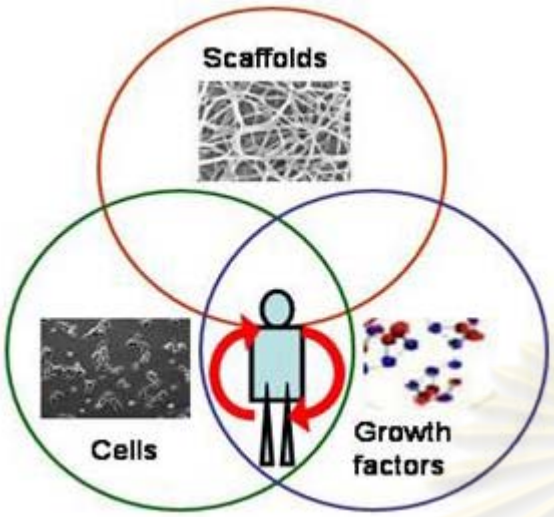

Fig. 1 Three major components in bone tissue engineering: cells, scaffolds, and growth factors.

Sources of stem cells for bone tissue engineering

Stem cells and progenitor cells represent an important promise in the therapy of several pathological conditions. Stem cells are a key subset of cells in the body that function as ancestor cells to produce a variety of types of functionally specialized mature cells in a given tissue, while at the same time maintaining the capacity of self-renewal. Self-renewal of stem cells is a process of continuous division to reproduce themselves. The result of this process produces one cell that is exactly like the mother cell and one cell that takes on biological functions that are different from those of the mother cell. Without self-renewal, each activation event would lead to the progressive loss of the originating stem cell population. Stem cells are the source of all new tissues arising from repair and remodeling and are modulated by signals that regulate their activation, proliferation, migration, differentiation, and survival. Stem cells give rise to progenitor cells and are different from progenitor cells by their capacity for self-renewal, or self-regeneration. In contrast, progenitor cells have a limited capability for self-renewal and are committed to progess toward a different phenotype. Progenitor cells are derived from stem cells and retain the differentiation potential and high proliferation capability.

The current sources of stem cells and progenitor cells used in bone tissue engineering include bone marrow, periosteum, cartilage, muscle, fat, and vascular pericytes [6]. These cells can also be expanded in vitro for use in bone tissue engineering and other engineered-tissue applications. Culture-expanded and selected cell populations include connective tissue progenitors [7], bone marrow stromal cells [8], mesenchymal stem cells [9], and adult multipotential progenitor cells [10]. Adult stem cells derived from the bone marrow have been well characterized in relation to stem cells originating from other tissues. Mesenchymal stem cells reside in contact with the hematopoietic progenitors in the bone marrow cavity. Recently, mesenchymal stem cells have been isolated from the periosteum [11, 12], trabecular bone [13, 14], adipose tissue [15, 16], synovium [17], deciduous teeth [18], umbilical cord [19-22], periodontal ligament [23, 24], and dental pulp [25, 26].

Periosteum comprises osteochondral precursor cells that can be induced to differentiate into osteogenic and chondrogenic lineages under defined culture conditions. Periosteal cells are easily isolated from periosteum biopsy harvested under sterile conditions and expanded in culture through many generations. Cells are generally cultured in basal medium such as alpha-minimal essential medium in the presence of 10 % fetal bovine serum. Periosteal cells have a fibroblastic morphology in monolayer culture and adhere to the tissue culture substrate. By light or phase contrast microscopy, human periosteal cell cultures display a rather homogenous population of fibroblast-like cells as shown in **Fig. 2**. When cultured in a monolayer in the presence of demineralized bone matrix, these cells acquire an osteoblastic morphology with upregulation of alkaline phosphatase activity (**Fig. 3**).

An explant culture system can be established to isolate umbilical cord mesenchymal cells from the Wharton's jelly of human umbilical cord. Spontaneous outgrowth from small pieces of Wharton's jelly or explant culture technique allows the motile nature of mesenchymal cells, which migrate away from an explant in culture (**Fig. 4**). Following repeated trypsination of a primary culture, umbilical cord mesenchymal cells still possess outgrowth potential. However, the spontaneous cell outgrowth technique generally utilizes around two weeks to reach a confluent cell culture. We have demonstrated that the cells could be expand *in vitro* and induced to differentiate into osteoblasts as shown by expression of alkaline phosphatass [19, 20]. In addition, adipose tissue has been shown to contain multipotent stem cells, which have the capacity to differentiate into cells of

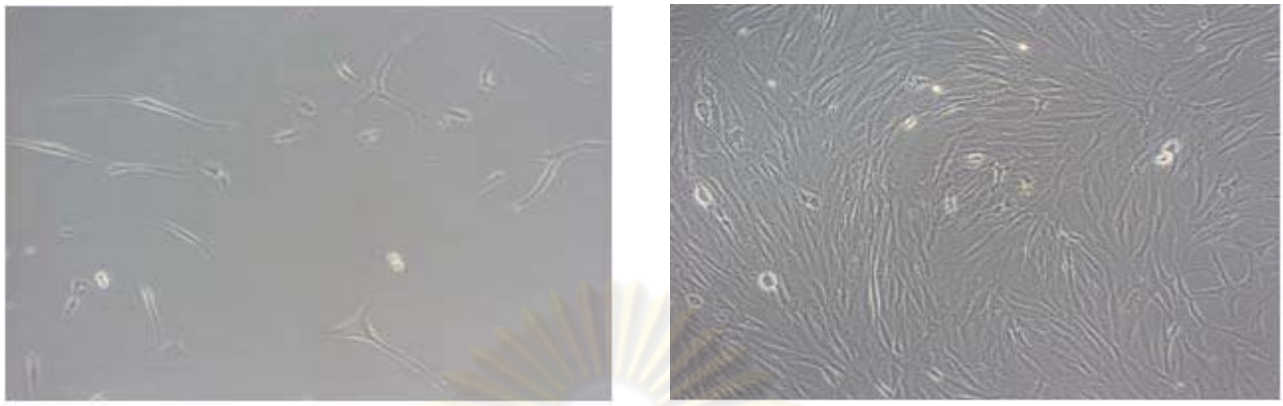

Fig. 2 Human periosteal cell cultures from periosteum growing in vitro on the floor of a plastic dish about 5 days (left) and 10 days (right) after the initial seeding (x 10).

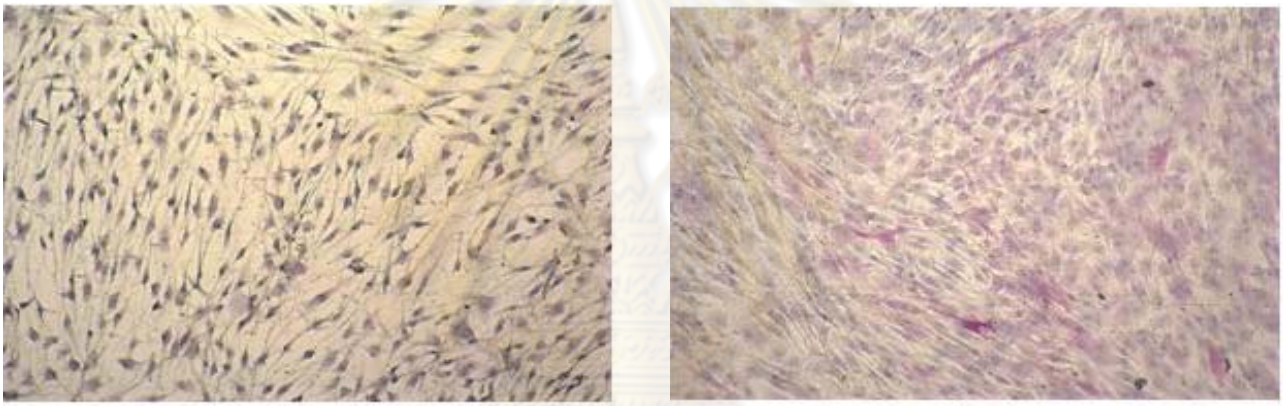

Fig. 3 Osteoblastic differentiation of human periosteal cells. Untreated cells are negative for alkaline phosphatase (left). Cells treated with demineralized bone matrix show positive staining of alkaline phosphatase (right) (x 10).

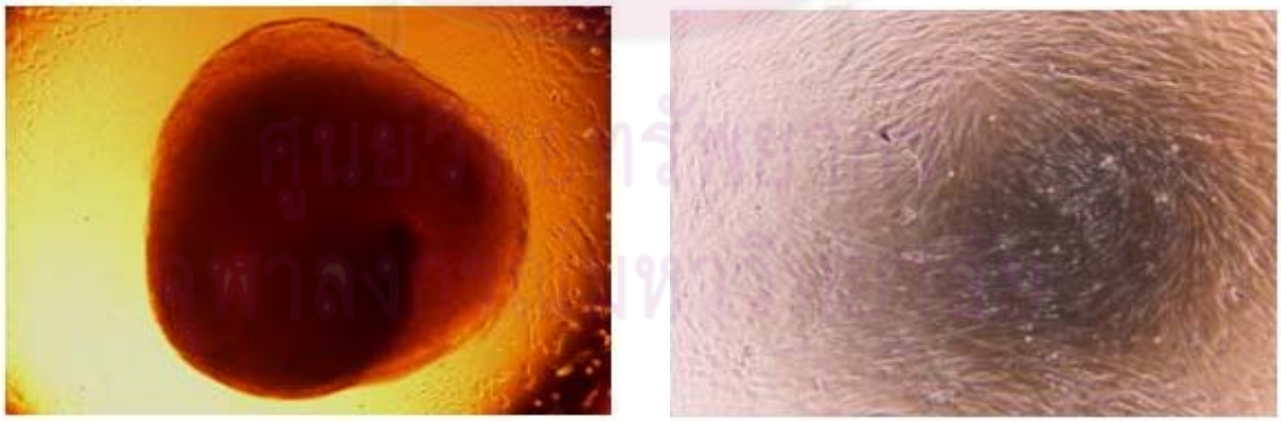

Fig. 4 Primary explant culture for isolating mesenchymal cells from Wharton's jelly of human umbilical cord. Explant and early stage outgrowth approximately 7 days after explantation (left) Confluent cells in monolayer culture about 14 days after explantation (right) (x 10).

connective tissue lineages including bone, fat, cartilage and muscle, in the presence of lineage specific growth factors [27, 28]. Osteoprogenitor cells have also been isolated from skeletal muscle in mice and humans [29]. Muscle biopsy and liposuction are attractive alternatives in cell-based tissue engineering strategies.

The induction of bone formation was first described when the bone marrow derived cells were transferred to ectopic sites delineating the osteogenic properties of bone marrow-derived mesenchymal stem cells [30]. It has been proposed that all highly specialized types of hard tissues, including cortical and trabecular bone, tendons, ligaments and cartilage as well as stromal microenvironment supporting and regulating hematopoiesis, originate from a common type of early mesenchymal progenitor cell [31-34]. The osteogenic precursor cells are presumed to originate from the stromal stem cells possessing self-renewing potential. The developmental pathway that the mesenchymal stem cell pursues to differentiate into osteoblast is still under intense investigation.

Table 1. Examples of cells, matrices, and growth factors used in bone tissue engineering.

Cells
Bone marrow stromal stem cells
Mesenchymal stem cells
Matrices
Absorbable
Synthetic polymers
Polylactic acid
Polyglycolic acid
Natural polymers
Collagen
Chitosan
Nonresorbable
Synthetic polymer
Polytetrafluoroethylene
Synthetic ceramics
Calcium phosphate
Growth factors
Bone morphogenetic proteins
Polypeptide mitogens

Matrices and biomaterial scaffolds

Osteoconductive materials enhance the attachment, migration, and distribution of cells responsible for the bone-healing response. This is a three-dimensional process that depends on the chemical surface properties of the implant, its three-dimensional structure and porosity, and its mechanism of degradation. When porous osteoconductive matrices are implanted into bone, cells from surrounding tissues migrate into available void volume of the matrices. The process is characterized by an initial ingrowth of fibrovascular tissue and new blood vessels. This tissue invades the void volume of the scaffold and is followed by new bone formation. The efficacy of osteoconductive matrices is a function of the chemical surface of the implant. This surface may have a direct effect on the cells that come in contact with it. The surface may also serve as a site on which various bioactive molecules from the wound site become concentrated, including growth factors and adhesion molecules [35-37].

Cells and material scaffolds play an essential role in the development of new tissue. The cellular component is required for the generation of new tissue through production and maintenance of extracellular matrix. The primary purposes of the scaffold materials are to provide mechanical stability to the construct and to provide a framework for three-dimensional organization of the developing tissue (**Table 1**) [38]. The coordination of cellular and scaffold components is important for the success of an engineered tissue construct. Three-dimension porous scaffolds play a crucial role in both cell targeting and cell transplantation strategies. Scaffold matrices serve as space-holders to impede intrusion of surrounding tissues. They provide surfaces that facilitate the attachment, survival, migration, proliferation, and differentiation of stem cells and progenitors. They also provide space in which vascularization, new tissue formation and remodeling can take place (**Fig. 5**) [39]. In addition, scaffolds can deliver cells into a graft site, facilitating their retention and distribution into the new tissue area.

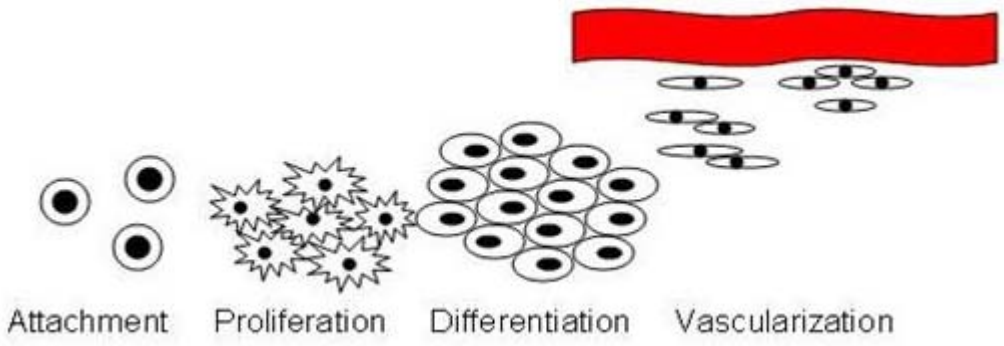

Fig. 5 The sequential stages in the new tissue formation include attachment, proliferation, differentiation, and vascularization.

A number of biodegradable materials have been utilized for tissue scaffolds, including ceramics and polymers. The primary application of ceramics has been in bone tissue engineering, where porous formulation of hydroxyapatite have been used to carry osteoprogenitors derived from bone marrow or periosteum. In general, ceramics have long degradation times, often on the order of years. While polymers have been extensively used as scaffold materials, their degradation times have a wide range from days to several months. Normally, polymer scaffolds are in the form of fibrous meshes, porous sponges or foams, or hydrogels. The more common polymers used in fibrous meshes and foams include the linear polyesters, including polyglycolic acid (PGA), polylactic acid (PLA), polycarpolactone (PCL), polyethylene glycol (PEG), and natural polymers, such as collagen and hyaluronic acid (HA). Polymeric hydrogels have the unique advantage of being injectable, permitting less invasive delivery of the construct, and minimizing surgical risks. Common hydrogel substrates comprise the copolymers of polyethylene oxide and polypropylene oxide known as pluronics and natural polymers including alginate and agarose.

Scaffold materials play a crucial role in providing mechanical stability to constructs and holding cells in place. These bioactive matrices are designed to support cell attachment to the polymer through cell surface adhesion proteins. Typically, polymers have been synthesized that have been a polypeptide sequence of arginine-glycine-aspartate (RGD) for integrin receptor binding (**Fig. 6**) [40].This allows the scaffold to effectively mimic the extracellular matrix and induce attachment of cells directly to the material.

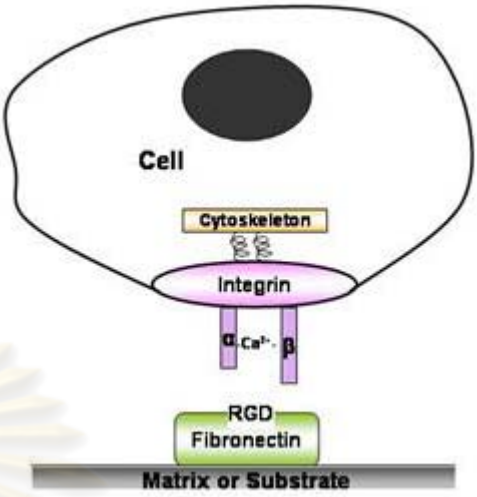

Fig. 6 Specific amino acid sequence of arginine-glycine-aspartate (RGD) for integrin receptor binding is responsible for cell recognition of polymer surface.

Growth factors and bone morphogenetic proteins

Growth factors exert their biological function by binding exclusively to cell-surface transmembrane receptors on the target cell. When binding to the extracellular domain of the receptor, growth factors stimulate the intracellular domain, leading to the activation of a specific signaling pathway. The effects of the signaling cascade culminate in the activation of transcription of a gene into mRNA, which is then translated into proteins for use intracellularly or extracellularly [41].

Bone matrix contains a number of growth factors, including bone morphogenetic proteins (BMPs), osteogenic protein-1 (OP-1), transforming growth factor- , insulin-like growth factors, and platelet-derived growth factor. Various osteoblastic culture models as well as *in vivo* experimental and clinical models have revealed that these growth factors influence cellular proliferation, differentiation, chemotaxis, and protein synthesis [42-46]. BMP-2, BMP-4, and BMP-7 have shown bone morphogenetic activity in animals [47] and could be quantitatively measured in human demineralized bone matrix using sandwich enzyme-linked immunosorbent assay [48-51]. There was a trend of decreasing extractable BMP-4 levels with increasing donor age. Demineralized bone samples with high osteoinductivity contain greater extractable BMP-4 levels than those with low osteoinductivity [50, 51].

BMPs initiate endochondral bone formation presumably by recruiting and stimulating progenitor cells of osteoblast lineage and by enhancing bone collagen synthesis. Evidence suggests that BMPs may play a significant role in the formation and maturation of skeletal tissues. BMPs and their receptors have also been demonstrated to be essential in osseous regeneration in fracture repair [52] .Difficulties in bone regeneration after injury can be related to abnormal or insufficient endogenous BMPs, their receptors, or a combination of both. Recombinant human BMP-2 and BMP-7/OP-1 (rhBMP-2 and rhBMP-7/OP-1) induce orthotopic bone in various experimental models. These recombinant BMPs have the capability of healing critical size defects in rodents, dogs, sheep, and primate models when combined with collagen,

guanidine hydrochloride-extracted demineralized bone matrix, or biodegradable polymers [53-57].

Recombinant human BMPs are now available more readily and can be isolated by molecular cloning technology. The encoding human genes were subsequently introduced into a Chinese hamster ovary cells and to *Escherichia coli* cells [58, 59]. Among the recombinant proteins, rhBMP-2 and rhBMP-7 have been tested in a number of orthopaedic indications as well as for application in the dental/maxillofacial surgery [60-64]. BMPs are water soluble and diffuse very easily in body fluids, so in order for it to maintain adequate concentration at the surgical or fracture site it is necessary to contain the BMPs in a carrier [65]. The major categories of carriers include inorganic materials, synthetic polymers, natural polymers, and allograft bone. Bovine type 1 collagen is currently used in the clinical setting. rhBMP-2 carried on a type I collagen sponge was approved for use in conjunction with a tapered, threaded intervertebral cage for the treatment of anterior interbody spinal fusion [66]. The composite device consisting of rhBMP-2 carried by the absorbable collagen sponge was trademarked as InFUSE (Medtronic Sofamor Danek) [67] and InductOs (Wyeth Europa) [68]. Most of the clinical trials to date that have used rhBMP-2 in spinal fusion have used the InFUSE bone graft system [69]. Clinical results have recently led to regulatory approval of rhBMP-7/OP-1 (Osigraft, Howmedica International S. de R.L.) [70]. Osigraft and NeOsteo bovine BMPs mixture (Sulzer Orthopaedics Biolodics) [71] have also utilized collagen based carriers.

Studies reported to date with rhBMPs have been related largely to animals. Yasko et al. achieved more than 80 % unions using rhBMP-2 combined with type I collagen in femoral defect of rat model [72].Long-term healing of bone using rhBMP-2 in sheep was investigated by Kirker-Head et al. [73]. At twelve months' follow-up, all the defects were intact and woven and lamellar bone bridged the defect site. Cook et al used BMP-7/OP-1 to heal large segmental defects in rabbits, dogs, and primates [74-76]. In primate studies involving African green monkeys, ulnae and tibiae treated with BMP-7/OP1 achieved complete bridging of the osseous defect and new bone formation within six weeks. Although a variety of BMPs are being investigated for their therapeutic potential, a combination of different BMPs and other growth factors may prove to be more effective in bone formation. The use of BMP-2/BMP-7 and BMP-4/BMP-7 heterodimers has shown increased bone formation compared with their homodimer counterparts alone [77]. Boden et al. [78] have discovered an osteogenic protein named latent membrane protein-1 (LMP-1) that appears to work through different mechanisms. BMPs function as secreted ligands, whereas LMP-1 functions intracellulary by up-regulating the expression of several other osteogenic growth factors. Boden et al. revealed that this protein stimulated bone formation in several different experimental models [78-80]. Although the development of these growth factors for clinical use has been slowly ongoing, these growth factors remain a promisingly powerful means of enhancing bone healing and stimulating bone growth.

The delivery systems currently available for recombinant BMPs include demineralized bone matrix, synthetic polymers, type I collagen, hyaluronic acid gels and a variety of bone graft substitutes, including hydroxyapatite and coralline hydroxyapatite. The ideal BMPs delivery system may be dependent on multiple factors, including anatomic location, local soft tissue envelope and the mechanical strain environment. Other conditions that should be considered include the possible timed release of BMPs from the delivery system, the ability of the delivery system to control and maintain a proper dose of BMPs and the presence of the proper substrate that will enhance cell recruitment or attachment. It is also essential for the delivery system to refrain from generating some type of immune or inflammatory response that may inhibit the regenerative process.

Conclusion

Tissue engineering is an attractive field of research and is bound to drastically change clinical practice in orthopaedic and reconstructive surgery. Osteogenic cells for bioactive implants are easily available following harvesting and expansion. Osteoinductive factor may further enhance bone formation within engineered tissues. In addition to understanding the process of tissue generation, it is crucial to increase the sophisticated level of techniques used to characterize engineered tissues. The simply used tool for investigation of tissue is histology. This method allows scientists to evaluate the extent to which the morphology of the generated tissue resembles native tissue. Furthermore, immunohistochemistry provides for the detection of tissue-specific proteins. Additionally, it is useful to examine the presence of

matrix proteins, but also to quantify their levels in generated tissues. A variety of basic biochemical assays are available for quantification of total collagen, proteoglycan, and elastin in engineered tissues. Similarly, enzyme-linked immunosorbent assay (ELISA) techniques using antibodies to tissue-specific proteins would also allow quantitation of these components in body fluid and generated tissue. Thus, the collaboration of biochemical scientists, physicians, and tissue engineers to translate these approaches to comprehend generated tissues is of great potential interest to the field of tissue engineering.

List of abbreviations

BMPs=bone morphogenetic proteins,
HA=hyaluronic acid,
ELISA=enzyme-linked immunosorbent assay,
LMP-1=latent membrane protein-1 (LMP-1),
PCL=polycarpolactone,
PEG=polyethylene glycol (PEG),
PGA=polyglycolic acid,
PLA=polylactic acid,
RGD=arginine-glycine-aspartate,
OP-1=osteogenic protein-1.

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