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Biologically active substances in bone morphogenesis. Literature review

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SUMMARY

Objectives. Understanding the mechanisms of bone morphogenesis is essential in solving problems related to reconstruction of bone defects. In a field of bone morphogenesis enormous array of biologically active substances have been proved as having more or less influence on bone growth, but no ideal guiding path for a new bone formation has been established yet. Therefore, bone tissue engineering, based on the laws of osteogenesis, becomes a significant niche for investigation. The aim of this article is to review recent scientific information concerning the role of bio substances in osteogenesis, their potentials in and future prospects.

Material and methods. Medline library database was searched, focusing on stem cells, osteoblast differentiation, bone growth factors and bone tissue engineering. Limits were review, research support. A quality assessment was carried out.

Results. A total of 63 articles matching our criteria were found: 20 reviews summarizing topics of growth factors, stem cells, tissue engineering and 43 articles representing studies of growth factors impact in osteoblast differentiation, bone regeneration and bone engineering.

Conclusions. The interactions among growth factors in osteoblast differentiation cascade are not fully understood. Moreover, methods of conversion of laboratory processes into clinically effective, reproducible, safe, economically viable and competitive products need to be improved.

Key words: bone morphogenesis, bone tissue engineering, growth factors, mesenchymal stem cells.

INTRODUCTION

Bone defects occurring after trauma or as physiological and pathological bone resorption refer a major challenge for healthcare specialists and manifest as global health problem. Correction of bone defects worldwide annually comprise approximately 2,2 million bone grafting procedures applying autografts and allografts (1). The source of bone for the grafts is either patient's own body in case of an autograft, cadaverous in case of an allograft and synthetic materials in case of alloplasts. Unfortunately, all of these grafting methods have significant disadvantages. The incidence of medical complications arising after surgery involving an autograft from the iliac crest is nearly 30% (2). Allografts are subject to an immune response and may transmit disease, while alloplasts are not suitable for large defects (2). Since traditional methods for reconstruction of bone defects are related to numerous limitations, employment of tissue engineering for replacement of deficient bone demonstrates a promising alternative (2). Bone tissue engineering appoints seeding and growing of a cell source on a scaffold using various bioactive molecules, following with implantation of the cells-bearing scaffold into the affected site (3). The aim of this article is to summarize recent scientific information about the role of biologically active substances in bone morphogenesis and engineering, their potentials and future prospects.

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MATERIAL AND METHODS

Types of studies

This review was conducted following PRISMA statement for reporting systematic reviews (4). All researches investigating bone morphogenesis, bone growth factors, bone tissue engineering were included.

Types of Publications

The following were excluded: Letters, Reviews, Editorials and Postgraduate thesis.

Literature search methods for identification of studies

MEDLINE (1994-2013) was searched applying keywords "Mesenchymal stem cells", "Bone growth factors", "Osteoblast differentiation" combined with each other and/or certain growth factors. Also, search criteria as "Bone morphogenesis" and "Bone tissue engineering" were utilized.

Data extraction

The following information was extracted: First Author, Year of publication, Research type, Comparison group, Duration/Follow-up, Method of outcome measurement.

Inclusion criteria

Experimental animal studies, clinical researches, histologic, histomorphometric, radiologic analysis, research duration up to 21 day.

RESULTS

Articles were searched according to PRISMA statement. 63 articles were found matching our criterias. 20 articles were excluded because of not being an experimental research and exploring soft tissue engineering. Total of 43 articles were included in this review (Figure 1).

Most of the experimental research articles (36, comprising 83.7%) were investigating growth factors participating in bone regeneration and mesenchymal stem cell differentiation to osteoblasts (7, 10, 12-27, 31-48). 5 (11.6%) were exploring isolation of stem cells (5, 6, 8, 9, 11) and 2 (4.6%) - bone engineering scaffolds (28, 29).

Stem cells. The body houses several types of progenitor cells that are capable to divide many times, while also give a rise to daughter cells having more restricted developmental potentials. Examples of such stem cells include the totipotent zygote, as well as embryonic stem cells (ESCs), hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). The lineage of committed MSCs can fabricate a spectrum of specialized mesenchymal tissues, including bone, cartilage, muscle, marrow stroma, tendon, ligament, fat and a variety of other connective tissues. This class of progenitors, the MSCs, resides in bone marrow, around blood vessels (as pericytes), in fat, peripheral blood, fetal cord blood, skin, muscle, periodontal ligament, dental pulp. There are different methods and protocols to isolate MSC from particular tissue (5-11). Mesenchymal stem cell is involved in a myriad of biological signals when differentiating into osteoblast. In general, these biological signals, or bone growth signals, can be divided into the following groups:

a) Growth factors (IGF, FGF, BMP, TGF-β, PDGF etc.).

b) Signaling systems (Wnt signaling pathway, etc.).

Growth factors. They are small proteins that serve as signaling agents for cells. The main GFs are:

1) Fibroblast growth factors (FGF) are small polypeptide growth factors produced in the bone by various cells, including osteoblasts, macrophages, endothelial cells, and are stored in its active form in the extracellular bone matrix. When released or secreted, FGFs act in an autocrine and paracrine ways as a mitogen on many cell types. Among total of 25 FGFs, the best studied family member on bone regeneration is FGF-2 (also known as bFGF). Although FGF-2 alone is not capable of inducing ectopic bone formation, it plays an important role in the regulation of normal bone healing, as FGF-4 and -6 does (12). Moreover, it is suggested that FGF-18 functions in promotion of osteoblast differentiation. It is believed that FGF-18 accelerates osteogenesis by upregulation of BMP-2 as well as maintenance or upregulation of Fgfr1, -2 and -3 expression in osteoblasts (13).

2) Bone morphogenetic proteins (BMP) are growth factors secreted by osteoprogenitor cells, osteoblasts, bone extracellular matrix and have a variety of functions during development and cell differentiation. Over 20 members of the BMP family have been identified and at least 7 of them have documented osteoinductive capacities. When implanted ectopically, the osteoinductive BMPs can initiate the complete cascade of bone formation, including the migration of mesenchymal stem cells and their differentiation into osteoblasts. This bone induction occurs through endochondral as well as intramembranous ossification and results in the formation of normal woven and/or lamellar bone (14). These proteins have been subsequently cloned, sequenced and recombinant proteins manufactured. Among the BMP family, BMP-2, BMP-4 and BMP-7 have key roles during osteoblast commitment and differentiation. BMPs have primary effect on the pluripotent cells that are capable of differentiating into other mesenchymal cell types and BMP-2 can direct these cells to commit to an osteoblastic pathway, as BMP-4 and BMP-7 can (14, 15).

3) Platelet-derived growth factor (PDGF) is considered as one of the key regulators for general tissue repair (16). During the early phase of wound

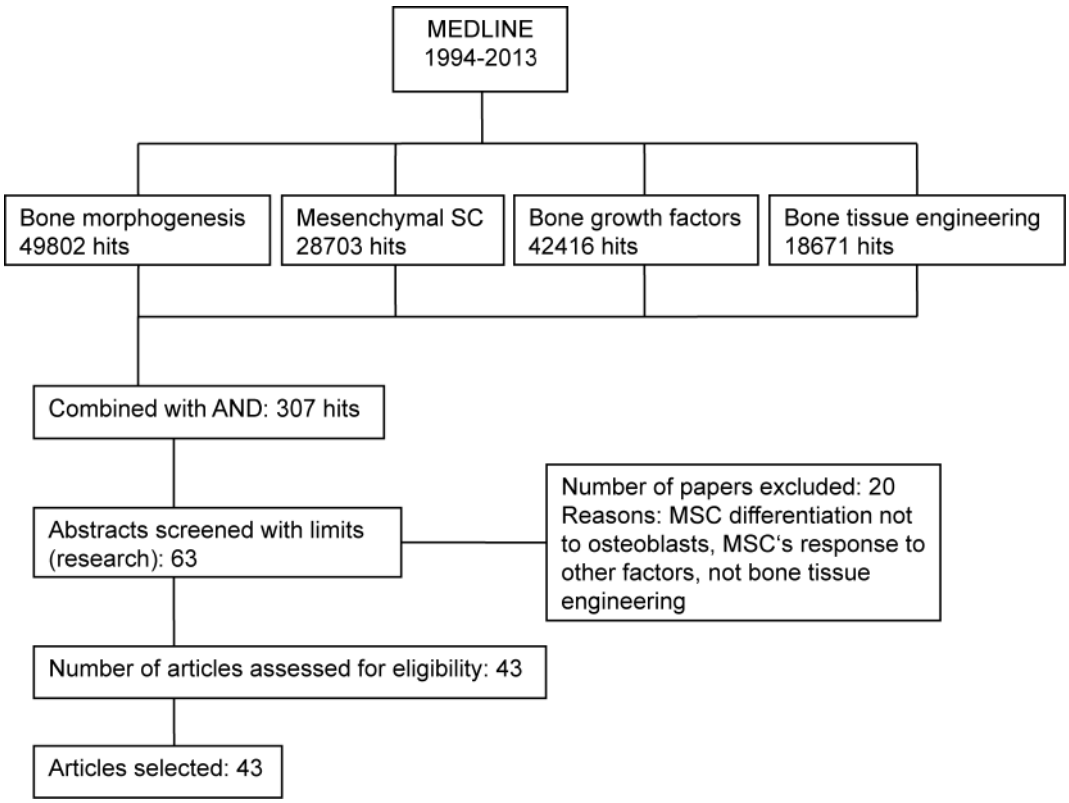

Fig. 1. MEDLINE search

healing, platelets are the major source of PDGF. After injury and hemorrhage, platelets aggregate and release cytokine-loaded granules containing various amounts of PDGFs. Upon release, PDGFs stimulate the recruitment of neutrophils, macrophages, and mesenchymal cells, which then serve as an ongoing source of PDGFs during the healing process. PDGF also enhances proliferation of various bone cell types and enhances angiogenesis. On the other hand, it does not have powerful bone-induction properties. Overall, platelet-derived growth factors physiologically mainly act as nonspecific mutagen, but their effects on cell chemotaxis and neovasculogenesis may be particularly important during would healing (16, 17).

4) Insulin-like growth factors (IGF). IGF-I and IGF-II are small single-chain polypeptides that play an important role in bone metabolism and are essential to skeletal growth and bone mass maintenance. They are synthesized by multiple tissues and elicit their effects in an endocrine, paracrine, and autocrine ways. IGFs are the most abundant growth factors produced by bone cells and are stored in the bone matrix at the highest concentration of all growth factors. Although there is still debate about its exact role in bone cell proliferation and differentiation, IGF has an antiapoptotic effect on (pre)osteoblasts and enhances bone matrix synthesis (17). The evidence suggests that the major effects of insulin-like growth factors are promotion of the late-stage differentiation and activity of osteoblasts (18).

5) Transforming growth factor β (TGF-β) is multifunctional growth factor with a broad range of biological activities on various cell types in many different tissues. Three isoforms of TGF-β have been found in humans (TGF-β1 through -β3). TGF-β is synthesized by many different cell types and is stored as an inactive complex with latency-associated peptide in the extracellular bone matrix. Another major source of this factor is platelets in the blood clot formed after a fracture. In general, TGF-β stimulates migration of osteoprogenitor cells and is a potent regulator of cell proliferation, cell differentiation, and extracellular matrix synthesis. Its use for bone regeneration has been evaluated in various experimental settings that show both stimulatory and inhibitory effects on bone formation. Overall, stimulatory effects on bone healing and bone formation predominate (33). TGF-β1 strongly enhances ectopic bone formation induced by BMP-2, resulting five-time greater bone volume than induced by BMP-2 alone, when implanted into mouse muscle tissue. This indicates a strong connection between TGF-β1 and BMP in signaling differentiation of osteoblasts (19).

6) Vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptors (VEGFRs) are primarily involved in angiogenesis. *In vivo*, VEGF induces angiogenesis as well as permeabilization of capillaries (20). During bone repair, these newly formed vessels and vascular changes are crucial for nutrient supply, transport of macromolecules, and invading cells. The important role of VEGF during bone regeneration has been shown in various experimental models, demonstrating stimulation or disruption of the normal bone regeneration process in response to VEGF administration or inhibition, respectively (21).

7) Parathormone (PTH) is a major systemic regulator of bone metabolism. It is secreted from the parathyroid glands and travels through the bloodstream to act

upon bone (22). Whereas continuous PTH infusion causes *osteitis fibrosa*, its intermittent subcutaneous administration enhances bone formation. It has been suggested that PTH-induced stimulation of bone formation is due to an increase in osteoblast number. This increase in osteoblast amount is not dependent on osteoblastogenesis, rather is the result of activation of existing bone-lining cells that undergo hypertrophy and resume matrix synthesis. Another proposed theory for its effect is the inhibition of osteoblast apoptosis. Overall, PTH may preferentially stimulate osteoblast differentiation to immature osteoblasts but inhibit further maturation of cells (23-25).

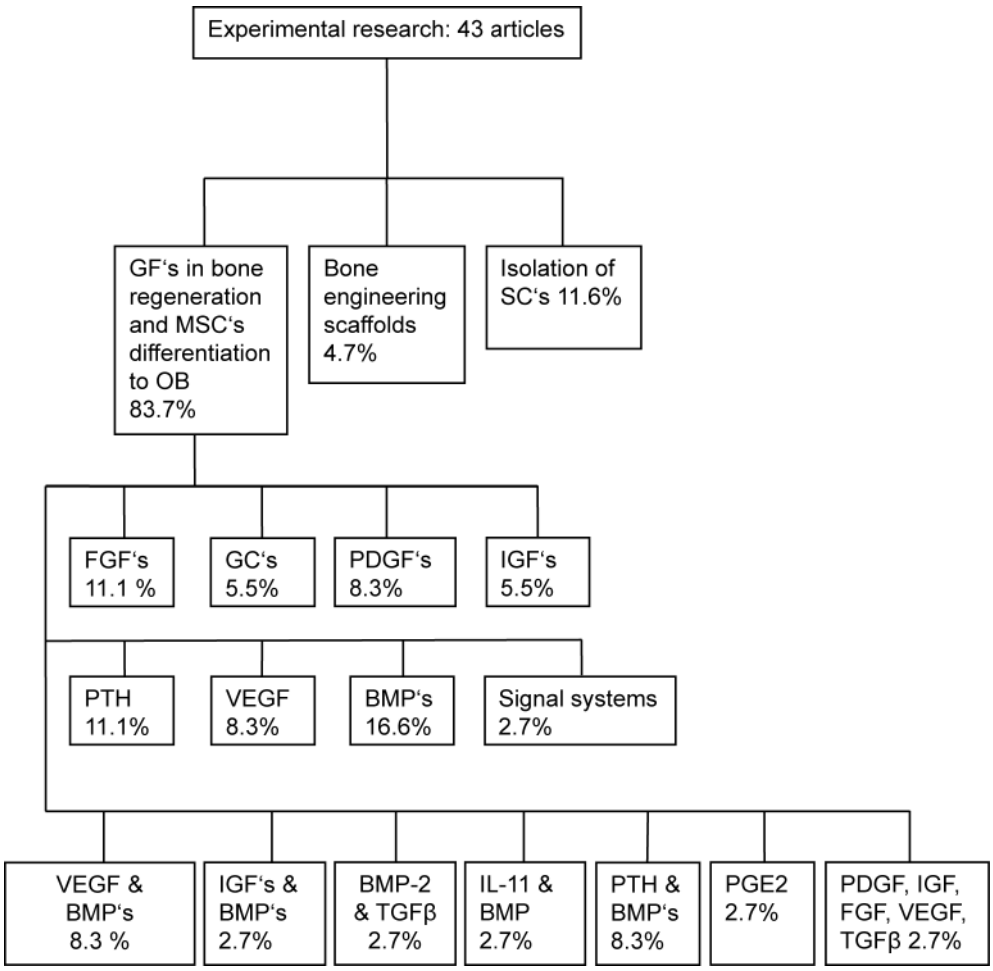

Fig. 2. Review summary

8) Glucocorticoids (GC) direct osteoblasts to differentiate and mineralize. Moreover, osteoblasts need to be triggered by GC's in a specific time-window during the early stages of development. Once the cells have entered the osteoblast differentiation pathway the mineralization process seems to be independent of GCs. (26)

Signaling systems

Signaling systems are composed of various transmembranous proteins and their ligands and are divided into Wnt and Notch pathways.

Wnt and Notch pathways may serve as switch-type regulators in bone remodeling since they are involved in the stabilization of SC recruitment to the different cellular species as well as in the acquisition of precise phenotypic features.

Wnt. Canonical Wnt signaling system encourages mesenchymal progenitor cells to differentiate into osteoblasts. It is also related to bone remodeling and/or modeling. Wnt proteins, secreted as cysteine-rich glycoproteins, bind to G protein-coupled receptor proteins and activate at least three distinct pathways: canonical (b-catenin-dependent), Ca2 and planar polarity. Of these three, the canonical pathway is best understood. Wnts are composed of 19 extracellular signaling molecules, but which of these exactly are involved in bone metabolism has not been fully elucidated yet (27).

Notch. The Notch signaling mechanism leads to suppressed osteoblast differentiation. Direct interactions between Notch proteins and their ligands, Delta or Jagged, leads to the cleavage, release and nuclear translocation of the Notch intracellular domain (NICD). This complex (or the product of the NICD target gene, Hey1) binds to Runx2 (a key transcription factor associated with osteoblast differentiation.) and inhibits osteoblastogenesis. NICD also inhibits Wnt signaling mediated by β-catenin and, either directly or through its interactions with other transcription factors may inhibit osteoblastogenesis (27).

Extracellular matrix. The last component of the *in vitro* bone model is the scaffold, which provides a structural and logistic template for the developing tissue and can markedly affect cell behavior. Several types of porous scaffolds have been shown to support *in vitro* bone formation by human cells, including those made of ceramics (HA, TCP), native (bovine skin atelocollagen) and synthetic polymers and composite materials (28, 29).

In overall, articles studying growth factors, involved FGF's in 11.1% of papers (12, 13, 31, 32), GC's in 5.5% (10, 26), PDGF in 8.3% (16,40,42), IGF's in 5.5% (17, 18), PTH in 11.1% (21, 23-25), VEGF in 8.3% (20, 22, 36), signal systems in 2.7% (27), BMP's in 16.6% (7, 14, 15, 33-35), combination of VEGF and BMP's in 8.3% (37-39), IGF's and BMP's – in 2.7% (40), BMP-2 and TGF beta in 2.7% (19), Il-11 and BMP's in 2.7% (44), PTH and BMP's in 8.3% (45,46,48), PGE2 in 2.7% (47) , PDGF, IGF's, FGF's, VEGF, TGF beta - in 2.7% (41) (Figure 2).

DISCUSSION

The sequential phases of osteoblast commitment and differentiation are regulated by a variety of complex activities, including hormones, GF, mechanical stimuli, cell–cell and cell–matrix interaction, controlled by interconnected signaling networks and leading to activation of specific transcription factors and, in turn, their target genes (30).

A number of growth factors have been shown to be expressed during different phases of experimental fracture healing. Basing on these findings, it is thought that growth factors, particularly TGF-β, BMP's, FGF's, PDGF's and insulin-like growth factors (IGF's) may serve as potential therapeutic agents enhancing repair of the bone (30).

FGFs act in an autocrine and paracrine ways as a mitogen on many cell types. FGF plays an important role in cell recruitment and expansion during the early phase of bone regeneration. Various studies showed FGF exposure and dose dependence on bone regeneration enhancement when FGF-2 or FGF-4 was combined with BMP's. Administration of FGF at early stage (2-4 days) following implantation into mice muscle increased the amount of newly formed bone, whereas its administration at later time points (days 6-8 or 9-11) had no effect. Low FGF dosages in combination with a single BMP dose synergistically enhanced BMP-induced bone formation; however, bone formation was inhibited by coadministration of higher FGF dosages. In rats, intramuscular implantation of FGF-2 and BMP-2 at a ratio of 1:10 or higher, had more stimulatory effect on the bone differentiation markers when compared to BMP alone. These mentioned studies suggest a ratio-dependent FGF/BMP effect, where mitogenic stimulus of FGF at high concentrations overrules the BMP-induced osteogenic differentiation (31, 32).

Presently, BMP-2, 4, and 7 are known as playing a crucial role in bone healing by means of their ability to stimulate differentiation of mesenchymal cells to osteochondroblastic lineage. A number of preclinical studies have assessed the efficacy of recombinant human BMPs (rhBMPs) in the healing of critical-sized bone defects and in acceleration of the fracture healing. Recombinant human BMP-2 has demonstrated efficacy in the healing of critical-sized bone defects in rat, rabbit, sheep, and dog models (33). Sciadini and Johnson evaluated effectiveness of rhBMP-2, delivered in a collagen sponge, for the healing of a critical-sized radial defect on dog model. All dogs were treated by either autogenous bone graft or a collagen implant containing 0, 150, 600, or 2400 μg of rhBMP-2, while control group was treated applying collagen sponge alone. All defects that had been treated using either autogenous bone graft or either collagen implant, containing various doses of rhBMP-2, after 24 weeks radiographically and histologically were confirmed as having similar bone union. None of the eight defects that had been treated applying collagen carrier alone have healed. However, the biomechanical properties of the defects that have been treated using the lowest dose of rhBMP (150 μg) were superior to that of the defects that have been treated with the higher doses. This finding was attributed to the lack of cyst-like voids occurring when administrating higher doses of rhBMP (33, 34). In many other preclinical trials, BMP-2 and 7 showed synergistic effect.

Several experimental studies reported attempts to enhance bone regeneration by combining TGF-b and BMPs. Despite the differences in experimental settings, isoforms used and delivery vehicles, most studies showed an additive or synergistic effect of the combination. Studies of ectopic bone formation after implantation of these factors to the mice muscle showed that TGF-β enhanced BMP-7-induced bone formation in a dose-responsive manner. This enhancement is believed to be indirect (stimulating osteoprogenitor cell recruitment, synergistic effect on angiogenesis and mitogenic effects) and direct (enhancing osteoblast differentiation process). Despite the conflicting results of numerous *in vitro* experiments, it seems TGF-β1 may have stimulatory effects on osteoblast differentiation during the early stage and an inhibitory effect on differentiation and mineralization at later stages. Although TGF-β1 almost certainly inhibits matrix mineralization at later stages, the *in vivo* consequences of these inhibitory effects might be limited since expression of TGF-β receptors is downregulated when cell differentiation progresses. This results a decreased responsiveness to TGF-β1 at later differentiation stages (35).

Several *in vitro* studies imply a BMP/VEGF-regulated coupling between osteogenesis and angiogenesis through reciprocal signaling. Cocultures demonstrated capability of osteoblast-like cells to stimulate proliferation of endothelial cells by producing VEGF,

whereas endothelial cells stimulated the differentiation of osteoprogenitor cells by producing BMP-2 (36). Further, BMP-induced differentiation of preosteoblast- like cells enhanced endogenous production of VEGF (37). So far, VEGF has been combined with BMPs attempting to enhance bone regeneration. The combined implantation of VEGF-expressing cells with BMP-2 or BMP-4- expressing cells synergistically enhanced bone formation at an ectopic implantation site. As VEGF is primary involved in angiogenesis, some results suggest that exogenous VEGF stimulates the osteoblastic differentiation of cultured human periosteal-derived cells and might act as an autocrine growth factor (38, 39).

In animal models and clinical trials for osteoporosis systemic infusion of IGF increased bone formation, bone volume, and/or bone turnover. A limited number of studies have investigated the effect of IGF-I and BMP combination on osteogenesis *in vitro* and *in vivo*. *In vitro*, sequential exposure to BMP-2 followed by IGF-I led to synergistically enhanced mesenchymal cell proliferation and alkaline phosphatase (one of the main genetic markers expressed by osteoblasts) activity (40). Similar effects were seen after exogenous BMP-7 and IGF-I administration. Further, exogenously administered combinations of IGF-I and TGF-b synergistically enhanced cell proliferation and matrix synthesis in osteoblast cultures. A similar synergistic effect of IGF-I and FGF on cell proliferation and matrix synthesis in osteoblast cultures was detected (41).

The effect of various doses of PDGF-BB in combination with BMPs in a collagen matrix was studied on craniotomy defects. Whereas BMPs caused a dose-dependent increase of bone volume and radiopacity in the defect site, BMP induced bone formation was inhibited when adding higher PDGF-BB doses and, in turn, enhanced by its low doses (42). The properties of PDGF for active stimulation of bone resorption could also be attributed to a reduction of available surface for bone formation resulted by increased number of osteoclasts (42). The effect of the PDGF/IGF combination has been studied on partial-thickness tibial defects in pigs. Comparison of growth factor-treated defects with empty intraanimal control defects showed increased callus area and thickness in PDGF/IGF-I combination group, whereas neither of the growth factors alone enhanced regeneration. Moreover, several *in vivo* studies showed formation of new bone, cementum and periodontal ligament in substantial amounts when combining PDGF-BB and IGF-I. In a clinical trial, the local application of a high dose (150 mg/mL) of rhPDGF/rhIGF-I significantly increased alveolar bone formation if comparing to controls without growth factor (43).

In vitro, IL-11 enhances osteoclast formation and bone resorption, but also stimulates expression of osteoblastic markers in mesenchymal progenitor cells. During osteogenesis, IL-11 synergizes with BMP-2 in a dose-dependent fashion, thus enhancing the *in vitro* osteoblastic differentiation of progenitor cells, resulting higher bone volume (44).

In some experiments PTH, a major systemic regulator of bone metabolism, has been combined with osteoinductive BMP's (45). Ectopically, systemic treatment applying PTH increased the local BMP-2-induced bone formation and reversed the age related decrease of osteoinductive potential of BMP-2 (58). Although systemic treatment with PTH enhanced healing of a partial-thickness tibial defect, no significant effects of BMP-7 or synergistic effect of the PTH/BMP-7 combination were found (46).

In a combination with BMP-2, a low dose of prostaglandin E2 significantly increased alkaline phosphatase activity and calcium content at ectopic implants in rats (47). Moreover, systemic administration of vitamin D3 significantly increased alkaline phosphatase activity and calcium content in ectopic BMP-2-loaded implants. So far, the mechanism behind this effect is not known and further studies are required to characterize this vitamin D3–BMP-2 interaction (48).

Current models of bone formation *in vitro* are based on the paradigm that cellular differentiation and function can be modulated by the same factors known to play a role during embryonic development. In order to engineer an environment supporting bone formation, combinations of biochemical and biophysical signals need to be presented at the cells in a three-dimensional setting in a way that allows cellular interactions with the surrounding cells and extracellular matrix. The complexity of signaling – with sequential and spatial gradients of molecular and physical factors affecting bone morphogenesis – raises considerable challenge for engineering of fully viable, functional bone. To date, these approaches have been evaluated in both animal experiments and clinical studies involving low numbers of patients, with inconclusive outcome and low bone regeneration efficacy. Overall, the clinical outcomes have been disappointing, especially with respect to restoration of large bone defects. The limited success of recent clinical studies may be attributed to a number of issues, primarily related to transformation of laboratory procedures to clinical application. Transmission of laboratory processes into clinically effective, reproducible, safe, economically viable and competitive products is generally acknowledged as complicated and challenging phase in the development of new clinical techniques. On the other hand, it has to be admitted that clinical results when using autologous bone transplants enriched by

various blood components (PRP and others) are quite satisfactory for a now, yet having a major disadvantage – morbidity of the donor site. In the context of bone tissue engineering, the difficulties reflect the multidisciplinary nature of the concept (49).

CONCLUSION

Each of the sources of osteogenic human cells - primary cells, MSCs, ESCs and induced pluripotent stem cells – has an ability to differentiate to osteoblasts. MSC's are the most frequently used exploring bone morphogenesis and engineering these days.

Each of biosubstances that participates in bone morphogenesis plays a distinct role in this process, and has a different impact on gradual stages of osteoblast differentiation and strogly interacts with each other. Isolated effects of particular GF's are quite well understood, nevertheless various combinations with each other have different effect on bone formation and needs for further experimental studies.

The difficulties of bone tissue engineering reflect the multidisciplinary nature of concept and requires further studies on biologically active molecules, scaffolds and conversion methods from laboratory procedures to clinical practice.

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