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Bone morphogenetic proteins and tissue engineering: future directions

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ABSTRACT

As long as bone repair and regeneration is considered as a complex clinical condition, the administration of more than one factor involved in fracture healing might be necessary. The effectiveness or not of bone morphogenetic proteins (BMPs) in association with other growth factors and with mesenchymal stem cells in bone regeneration for fracture healing and bone allograft integration is of great interest to the scientific community. In this study we point out possible future developments in BMPs, concerning research and clinical applications.

Introduction

In a bone regeneration setting such as delayed fracture, aseptic bone necrosis or other critical defect, bone morphogenetic proteins (BMPs) have proved key in enhancing the natural ability of the surrounding tissues to produce bone healing. If the mechanical conditions are fulfilled, these molecules are able to address progenitor cells in the bone-forming cascade to allow the repair of the damaged tissue. This action seems efficient when a considerable number of mesenchymal stem cells are available in the local environment.

In the complex clinical conditions associated with bone repair and regeneration, the involvement of more than one healing factor is needed. The more difficulty in healing expected, the availability of more factors, such as an adequate osteosynthesis device and application of growth factors and progenitor cells, are required. On the other hand when local damage is limited, mechanical stabilisation is unnecessary and the site is rich in progenitor cells, correct healing will require at most only the application of growth factors.

In this chapter we point out possible future developments in the application of BMPs. The rationale of the use of protein, starting from the relationships among the different type of factors applied in the system, is considered. The first issue is to improve the protein carrier, and the use of bovine collagen is discussed as well as the possible application of different carriers in different preparations. The usual preparation time, which includes mixing the protein with the carrier through an aqueous system, may be inappropriate in some circumstances. An initial rapid efflux ('dumping') of the protein was suggested upon the observation of heterotypic ossification. Thus we discuss various preparations and methods of application. An injectable material is now foreseen as the best product to obtain early application of the protein in difficult clinical conditions.

Finally, we explore the possibility of coupling the protein with other growth factors and/or with mesenchymal stem cells to obtain a more reliable biological therapeutic product. We conclude by looking at gene delivery of the BMP in allograft healing and delayed union.

BMP carriers and local delivery systems

Despite the significant evidence for stimulation by BMPs of bone healing that has been demonstrated in animal models, future clinical investigations will need to better elucidate some open questions, i.e. the ideal delivery system for human BMPs, the determination of suitable dosage and the real concentration of BMPs at the graft site, and future developments and applications.

To exert their biological effect, BMPs need to be combined with carriers for controlled release.[88] Carriers act as delivery systems for BMPs by retaining these growth factors at the site of injury for a prolonged period and by providing initial support for the attachment of cells and formation of regenerated tissue.[138] Controlled delivery systems are necessary in order to avoid uncontrolled ectopic bone formation in non-bony tissues.[110,154,161]

Essential requirements of a suitable carrier are the ability to provoke the best possible inflammatory responses, the formation of an interface with the surrounding biological tissue, and ideal porosity in order to allow first the infiltration of cells and then vascular ingrowth. In addition, carriers should be biodegradable but allow protection to BMPs from degradation for a period sufficient to induce a specific amount of bone mass at the treatment site. Finally, carriers should be sterile, immunologically inert, non-toxic and user-friendly.[7,134] The incorporated factors should be continuously released and controlled because of the very short half-life of most growth factors *in vivo*.[93]

Various formulations of delivery system may be designed to meet different mechanical requirements according to the type of tissue to be regenerated. Vascular ingrowth is essential in bone formation whereas, in cartilage, carriers should deal with compressive and

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shear stresses. For these reasons scaffolds have increased in complexity, mimicking the properties of the extracellular matrix in some cases, or responding to physiological modification of pH in others. The manufacture of the carrier also defines its ability to successfully deliver BMPs at the injured site.[66]

At present, the clinically available delivery devices for rhBMPs are far from ideal, because large doses of BMP are required for the desired osteogenic result.[45] In fact there is more BMP in a single dose than in 1000 humans, and objections can be made as to cost and safety.[59,88] Finding suitable carriers for BMPs is a great challenge for researchers. To date, despite the ready availability of rhBMPs for clinical use, the dilemma facing clinicians and the biotechnology industry is how to achieve optimal delivery systems that can decrease the dose of BMP, maintain a more sustained release pattern and be effective for osteoconduction.[102] Taking all these factors into consideration, workers have put their efforts into searching for efficient, simple and cheap delivery systems for drug targeting. Delivery systems can be divided into four major categories.[134]

Of natural carriers such as collagen, hyaluronans, fibrin, alginate, silk and agarose, the most commonly used is bovine collagen for delivery of rhBMPs.[59] The advantage of these materials is good biocompatibility; drawbacks are the natural source, processing, possible disease transmission and immunogenicity.[138]

Inorganic materials, such as calcium phosphates, calcium sulphates and bioglass, can mimic the natural bony structure and are, for example, produced as injectable paste, granules and blocks.[138]

Synthetic materials such as polylactic acid (PLA) and polyglycolic acid (PGA) or their copolymers such as polylactic-co-glycolic acid (PLGA) are widely used as biodegradable implants for orthopaedic application.[84]

Composites consist of a combination of the materials mentioned above, the advantages of individual materials optimising those of another material class. The ideal composite material should combine osteogenic (cells), osteoinductive (growth factors) and osteoconductive (structural) properties to promote tissue regeneration.[52]

Recently great attention has been paid to the subject of nanoparticles and microparticles for drug delivery. Most common materials in the design of nanodevices as delivery carriers are synthetic materials, natural polymers and hydroxyapatite-based particles.[73] PLA, PGA and their composite PLGA have been used in animal models as carriers for nanoparticle-based delivery systems for BMPs.[71,86,128,129,136,156] In a study by Ruhe et al., rhBMP-2 release was observed to depend on composite composition and nanostructure, as well as on the pH of the release medium.[128]

Microspheres based on collagen–hydroxyapatite have also been evaluated as rhBMP-4 carriers in rabbits.[155] Bone regeneration was observed in the animal group treated with BMP-4 particles whereas, in the group receiving the carrier alone, the defects were filled with fibrous tissue and inflammatory cells. Chitosan-sodium alginate microspheres have been studied *in vitro* in bone-marrow-derived cells.[121] Chen et al. evaluated dextran-based microspheres and nanospheres for BMP delivery[20–24] and demonstrated how much rhBMP release was influenced by changing the ratios of the components.[23]

Nanoparticle technology applied to BMP delivery appears the most promising approach in the future of bone tissue engineering, and further investigations must focus on this field in order to find ideal carriers for growth factors.

Dosage and concentration of BMPs

In clinical practice, BMPs are used for acute fracture treatment and healing of bony defects, delayed unions and non-unions. Two growth factors of the TGF-β superfamily, BMP-2 and BMP-7, have received approval for restricted clinical administration;[14,44,57] rhBMP-7 (Osigraft®, Stryker-Biotec, Hopkinton, MA) is available as 1 g lyophilised powder containing 3.5 mg eptotermin-α with bovine collagen 1 and can be applied as a suspension. According to the manufacturer, not more than 2 g (7.0 mg eptotermin-α) should be administered to any one individual.[38] BMP-2 (InductOs®, Wyeth, Gosport, UK) is available as a kit containing 12 mg dibotermin-α (1.5 mg/ml), to be applied in a bovine collagen 1 matrix. According to the manufacturer, not more than 24 mg dibotermin-α should be administered to any one individual.[38]

Both growth factors have also been applied ‘off label’ in delayed healing with promising results,[35] although only BMP-7 is approved for the treatment of non-unions. Pharmacokinetic studies showed that BMP release is characterised by an initial burst effect, followed by a more gradual release; in the initial phase the carrier can lose up to 30% of its BMP.[45,50] In addition, the high dose resulting from this initial rapid release determines a supraphysiological concentration of BMPs, which can be related to severe complications such as ectopic bone formation within the spinal canal, generalised haematomas in soft tissue and bone resorption around implants.[18,49,53,59,125] Therefore the effective dosage of BMP required in humans are fairly high. One pack of Osigraft® (rhBMP-7) contains 3.5 mg of eptotermin-α and, since 1 kg of human bone yields &1 mg of BMP, the application of one vial is equivalent to the total amount of BMP-7 in the skeleton of two people.[9] As a result, the high local and consequently low systemic concentrations of incorporated growth factors may reduce the overall dosage per application. Furthermore, because of the very short half-life of growth factors (60–240 min), direct and continuous application of the factors at the required site is necessary.[163,167]

Preclinical and clinical studies have revealed little evidence of toxic effects and few adverse events have been reported. A low rate of antibody formation following administration of BMPs has been observed in some cases, without clinical consequences.[60] In another study, antibody responses to rhBMP-2 were detected in less than 1% of people treated for spinal problems. For rhBMP-7, low immune responses have been observed in 38% of cases without adverse clinical effects.[119] Long-term effects are yet to be demonstrated.

To date the effective dosage of BMPs related to the size of the gap to be filled has not been established, i.e. the use of one 3.5 mg vial of Osigraft® (OP-1) in recurrent non-union without osseous gap and two vials for non-union with bone loss has not yet been validated. In addition, we do not know the retention rate of the OP-1 in the application site. Retention of the growth factor depends on BMP immobilisation in the delivery system,[7] and much effort is currently being put into finding and producing delivery carriers for BMPs that do not cause loss of their activity. Immobilisation of the BMPs in a delivery system may be achieved by adsorption, entrapment, immobilisation or covalent binding.[99]

In the case of adsorption, conformational changes may occur and the release of the protein may be less sustained. With entrapment, hydrophobic polymeric matrices are known to release bioactive agents over extended periods of time;[83] however, during carrier material processing, pH and temperature conditions can lead to denaturation of the protein. Covalent binding to the carrier may be performed by production of a fusion BMP protein with a domain of specific binding to a biomaterial.[145] Anyway, covalent immobilization may negatively affect the binding of the growth factors to their receptors as it could lead to subsequent dimerization of the receptors in the plane of the membrane.[99]

Animal models have been studied to evaluate systemic distribution and pharmacokinetics and the retention of BMP at the site of orthopaedic injury, through specific BMP targeting using radiolabelled [125]I OP-1 associated with different carriers.[6,97] Human studies are difficult because of legal problems in combining

OP-1 with a radioactive tracer; it would be hard to gain the approval of an ethical committee or the recipient for the application of a radioactive isotope, as radiation exposure would be prolonged and repeated. In animal studies, rabbits have been exposed to radiation for periods ranging from 6 h to 35 days. The most investigated tracer [99]TC is inappropriate because of its short half-life, so longer-lasting isotopes are required (e.g. [125]I).

Regarding effective dosage, retention at the injury site and osteoinduction, major studies conducted *in vivo*[12,17,35,44,54,67,101,127,140,166] have been based on therapeutic efficacy corresponding to healing of three quarters of the cortical bone involved. Such studies show osteoinduction after local delivery of OP-1, but the *in-vivo* activation of local cells by growth factors, already studied *in vitro*,[88,122,159] has not yet been elucidated. Although expensive, further studies should focus on local osteogenetic induction processes using OP-1 and nuclear medicine (PET or PET-CT).

Injection of BMPs

In a number of procedures involving BMPs, a scaffold is used to enhance the local bone growth. The disadvantages of the use of autogenous material, such as additional surgical procedures, donor site morbidity and complications, are well known.[56,58,162] This has led to increased interest in scaffold material derived either from bone graft or from substitutes, e.g. allogeneic grafts, xenografts, demineralised bone matrix or synthetic materials.[52] However, the application of BMPs with morcellised material requires open surgery to create a comparatively large approach, and some of the BMPs may be diluted in the surrounding tissues, thus losing their effect.

Minimally invasive local application methods reduce the risk of ectopic bone formation due to high concentrations of circulating BMP or an incorrectly placed BMP carrier. Injectable products have been developed, directly applying growth factors into the fracture gap with a syringe without exposing the fracture zone. BMP-2 injected with a calcium phosphate paste (α-BSM) accelerated osteotomy healing in a rabbit model.[95] Injectable applications are currently under clinical investigation and not yet approved. Further clinical trials are required, which may enable future developments in tissue engineering to be applied to bone defect healing.

Use of BMPs in association with other growth factors

Proliferating growth factors

The bone forming process is a cascade of events which include the involvement of different types of cells and growth factors (Table 1). Peptide growth factors stimulate the activity of osteoprogenitor cells and osteoblasts and may enhance osteogenesis. Fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are strongly expressed during fracture repair. They are both necessary to bone healing, but are not osteoinductive. Other growth factors, particularly present in platelet-rich plasma (PRP), have been proposed for clinical administration, e.g. platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β1) or prostaglandin agonist.[10] Callus formation can be improved by the physical application of an osteosynthesis device and, on observing the release profile of cytokines during femoral nailing, all the main growth factors were found to have increased, particularly VEGF and PDGF and in lesser quantities insulin growth factor 1 (IGF-1) and TGF-β1.[51]

It is important to underline also the action of single BMPs as well as in association with others of the BMP family group. It has been stated that BMPs produce bone by a complex series of events involving a subset of proteins all capable of inducing bone formation by themselves, including BMPs -2, -3, -4 and -6. Concurrently other cytokines that are not BMPs may facilitate bone formation in other ways, e.g. FGF, which has an angiogenic effect that promotes neovascularisation, and PDGF and IGF-1 acting as local modulators.[160]

Since the lack of bone formation is often due to the limited ability of the surrounding tissue to induce a vascular supply at the site of regeneration, VEGF is considered by many authors to be of pivotal importance because of its ability to enhance local angiogenesis. Vessel formation is the earliest process in the bone regeneration; through the fine capillaries, progenitor cells can be recruited to start the process of osteoblastic differentiation. *In-vivo* experiments have proved the synergistic effect of dual delivery.[72,116,157]

The association of BMP and VEGF has been explored, and it was demonstrated that the beneficial effect of VEGF on bone healing elicited by BMPs was dependent on the ratio of the two proteins. However, the mechanism of action (including the timing of the protein release) has not been clarified yet.[118]

The same results can be achieved with gene transfer on different cells to produce BMP and VEGF, but the level of VEGF production should be low and constant over time. Another element influencing the result is related to the type of cells involved, which is an important consideration for cell-based gene therapy and tissue engineering.[94] In particular, the combination of BMP-2 and VEGF induced significantly early bone formation, and VEGF transfection produced more blood vessels relative to the conditions without VEGF. Thus, VEGF might enhance BMP-2-induced bone formation through modulation of angiogenesis on periosteal derived cells.[131]

BMPs and PRP

The same importance can be attributed to the association of BMP and PRP. It is well known that the growth factors present in this natural product include FGF, TGF-β1, PDGF, VEGF and IGF1. Although the use of the peripheral blood as a reservoir of growth factors is apparently simple, the efficacy of PRP activity is donor- and method- dependent because of the varying presence of growth factors. Nonetheless, for 15 years PRP has been investigated *in vitro* and *in vivo* and in clinical practice, in several fields of application. The use of a combination of PRP and BMP has been shown to result in improved vascular perfusion around bone defects and enhanced bone healing and density.[115]

The soluble fraction of PRP is considered to have strong proliferative activity on stem cells in the regeneration environment, stimulating recruitment and proliferation. Although the presence of osteoinductive cytokines in PRP is not proven, it has been hypothesised that PRP may contain a novel potentiator for BMP dependent osteoblastic differentiation.[148]

Finally, the problem of controlled delivery of the proteins after implantation remains a complex concern. There are several aspects to this topic, including manufacture of the drugs, coupling of different material (BMPs, other growth factors, PRP), carriers, stem cells and good manufacturing practice. A number of studies

Table 1
Application of growth factors and other bioactive peptides in musculoskeletal repair

Growth factors	Articular cartilage	Meniscus	Ligament/tendon	Bone	Muscle
IGF-1	√		√	√	√
FGF-2	√	√	√	√	√
PDGF			√		√
EGF	√	√	√		
TGF-β	√	√	√	√	
BMP-2	√	√		√	
BMP-7	√			√	
GDF-5	√		√		
VEGF			√	√	

challenging the pharmacokinetic problem address the subject of the protein carrier; the choices of both optimal growth factor and optimal application system are important in this. One option, for example, is material coating; this process allows the incorporation of growth factors and the controlled release of these factors during the healing process without the need for further devices.[133] In conclusion, growth factors are of paramount importance in the generation of new functional tissue. Their action can be applied to the cell during *in-vitro* differentiation or directly *in vivo*; the latter seems to be more effective in conditioning the site of bone formation. The issues of type of stimulus (one or more growth factors), their quantity and, most of all, time of release are still under consideration. We believe this topic is of great importance for the future of the BMP application, and more resources should be devoted to exploring in greater depth this field of modern regenerative medicine.

BMPs in association with mesenchymal stem cells

The clinical effectiveness of BMP application has been widely proven in a variety of situations, but a number of studies have pointed out the need to improve the pharmaceutical formulation.

Termaat et al. concluded a wide-ranging review by pointing out that the first clinical studies reporting on BMP-2 and -7 had demonstrated that bone formation was not always consistent. Possible explanations would be the relative osteoinductivity of the applied BMPs in presence of responding cells, or the inappropriate time point at which the BMPs were presented locally by their carrier.[147] Kloen et al. questioned the relative paucity of target cells and whether we can rely on chemotaxis to recruit osteoprogenitor cells or whether exogenous cells are required.[76] Hence, the need for a number of local, responding, undifferentiated cells has been addressed. The efficacy of adding precursor cells to BMPs *in vivo* has been shown using bone-marrow concentrates,[146] but the contribution of circulating cells is still undefined.[48,120,135]

There are controversies about the real effect of adding mesenchymal stem cells to the BMP application, first of all concerning their grade of differentiation. It has been proved that human bone-marrow-derived mesenchymal stem cells (BMMSCs), osteogenically differentiated before implantation, regenerate less bone than undifferentiated human BMMSCs *in vivo* once exogenous BMP-2 has been delivered to the implantation site.[69] This is consistent with *in-vitro* experiments which do not always show a significant increase of alkaline phosphatase after exposure to BMP.[34]

A recent study in goats of microsphere/poly(propylene fumarate) composite in a subcutaneous pouch concluded that bone formation induced by autologous cryopreserved MSC and BMP did not enhance ectopic bone formation compared with BMP alone.[70] The osteogenic potential of autologous MSCs in goats was clearly shown using ceramic scaffolds,[79,80] but the efficiency of cell seeding and their behaviour *in vivo* on the scaffolds applied in this study are unknown. The absence of bone formation in the cell-seeded composites without BMP-2 was as expected since, in contrast to ceramics, these synthetic polymers do not possess osteoinductive characteristics. In the absence of such an osteoinductive stimulus, MSCs could have differentiated into fibrous tissue in the microsphere/scaffold composite. However, the limits of the study are clearly the absence of a proper scaffold and the type of *in-vivo* model.

Indeed an osteoconductive scaffold, as well as appropriate test conditions, are necessary to demonstrate the cooperative effect of BMPs and MSCs. It is known that the addition of osteogenic protein-1 further enhances the weak osteoinductive properties of hydroxyapatite when loaded with human MSCs. Alkaline phosphatase activity measurements and scanning electron microphotography have demonstrated increased cellular attachment and proliferation into hyaluronic acid (HA) pores in the loaded samples.[151] It has been hypothesised that the HA can interact with the cells and generate potent inductive substance release into the medium, inducing uncommitted cells to differentiate into the osteolineage.[92] However, the observation of a great amount of calcified tissue around a femoral critical defect in nude rats may not be as predictive of the real contribution of undifferentiated or differentiated cell in bone healing.[74] *In-vitro* experiments, looking at the Affymetrix (Santa Clara, CA) gene profile of MSC stimulated by BMP-7, show the ability of the protein to activate gene expression of differentiation and also down-regulation of the cell cycle, thus promoting bone induction. Meanwhile, cytokine osteoblast down-regulation promotes osteoclastogenesis and osteoclastic bone resorption.[85] This probably means that swift activation of the mineralisation in the surroundings of the fracture callus does not indicate permanent mechanical stability.

Finally it is important to underline different responses to the BMP stimulus among different species; MSCs mediated by transcription factor(s) behave differently in rodents compared with humans.[113]

BMPs and bone allograft application

Allograft contains osteoinductive growth factors and other non-collagenous proteins present in the matrix, that support new bone formation.[124] However, the osteoinductive capacity of massive allografts is very frail.[150] Several studies pointed out the need for newly formed vessels creeping inside the natural bone canals to achieve allograft incorporation. The high rate of fractures observed in clinical practice in structural bone allograft is the result of the accumulation of micro-cracks that cannot be repaired by the necrotic bone because there is no vascular supply.[16,29,39]

Recently our group demonstrated that blood marrow-derived laboratory-expanded MSCs contained in a collagen- and PRP-based carrier can improve structural allograft integration in a 16-weeks metatarsal sheep model.[98] We were able to show significantly greater new bone formation inside the treated allograft in comparison with the control group (graft alone), and also increased presence of newly formed vessels and better mechanical properties of the new bone inside the graft.

The same result has been channeled to using BMPs added to the allograft to improve incorporation and mechanical stability. Numerous animal studies demonstrated increased ability bone–allograft integration when rhBMPs, mixed with a collagen type I carrier, were added to the site of interest.[27,28,130] Hence, BMPs do have a role in this type of application, but the efficacy in different indications has to be definitively proved. As well as a quick response in the formation of new bone around the implant, longer-term observation studies found evidence of bone lysis. Some authors reported that BMPs were able to up-regulate osteoclast-like activity *in vitro*,[55,64,68,78] leading to greater allograft porosity when BMPs were added to a massive bone allograft *in vivo*, stimulated graft remodelling and enhanced resorption of bone.[13,31]

In our experiment performed with the same model of sheep metatarsal bone, adding BMP-7 to the allograft, we observed an early bone callus formation on the radiographs at 1 month, increased at 8 weeks and followed by callus remodeling and graft resorption at 16 weeks.[36]

This is consistent with the findings of Cullinane et al.,[31] who demonstrated a significant resorption rate with a massive bone allograft in a canine model treated with rh-OP-1 at 12 weeks postoperatively. The results obtained in this further study were very similar to those of their previous study, confirming the high stimulation of graft resorption when rh-BMP-7 was used.[91] This is also confirmed by other authors dealing with experimental model of impaction grafting using morcellised allograft and BMP.[13]

In our study[36] we could confirm the different allograft integration pattern that occurs in the presence of a clinical dose of BMP-7, in comparison with observations of the same animal model when using MSCs and PRP, when prompt direct integration at the host–graft junction occurred without the need of a bulky external callus. The histological findings were consistent with a high presence of newly formed vessels without increasing allograft porosity. In contrast, the use of BMP produced an abundant presence of external callus followed by resorption with high vascularity in the histology section at 16 weeks.

More recently (unpublished data) we used a new group of animals and mixed the protein with MSCs, to test whether these could better induce host–allograft integration. A sequence of plain radiographs revealed results much more similar to those achieved by the use of MSCs and PRP. The final histology confirmed increasing presence of new bone around and inside the graft, better healing patterns at the junctions and better mechanical performance in relation to the group treated with BMP alone. The same result was not achieved with MSCs alone. This study thus confirmed the need for a mechanical stable scaffold (allograft) associated with MSCs and growth factor to achieve the best possible result in tissue regeneration of critical defects. The differentiation of readily available precursors can enhance the bone appositional phase from an early stage in remodelling, without the marked resorption evident when using growth factor only.

There are several issues involved in the use of BMP in experimental surgery related to allograft integration such as dosage, type of carrier, local conditions (inflammation, graft mechanical stability) and, most importantly, the presence of available precursor cells. In the vast majority of allograft replacements for clinical indications, the local environment of healing has a poor presence of stem cells. This is due to repetitive surgery (repeated ostheosynthesis), failure of prosthetic devices (bone resorption and scar tissue formation), muscle excision and antiblastic activity (tumour surgery). In these conditions the action of the protein may be unbalanced and the final result can be impaired by the prevalence of osteoclastic activity. The concurrent use of MSCs seems to optimise the activity of the protein as a bone regenerative product.

BMPs and gene therapy

Gene therapy is a technique whereby new genes are introduced into cells in order to treat disease by restoring or adding gene expression. Theoretically, it may be useful for a wide spectrum of diseases, including the treatment of bone and joint disorders.[152] Some diseases of the locomotive system cannot be cured successfully because of the limited healing capacity of most of the tissues constituting the musculoskeletal system. Thus, ligaments, tendons, menisci and articular cartilage all have low blood supply and reduced cell turnover.[43,63] Even bone, which is normally capable of regeneration, can be problematic, particularly in degenerative disorders such as osteoporosis or in situations such as delayed union.[46,126]

Numerous growth factors and other proteins capable of promoting regeneration of these tissues have been identified, such as BMPs. These molecules have been demonstrated to have great potential in stimulating bone growth, but their clinical application is complicated by delivery problems.[62,88,96,123] The main issue is the provision of a sustained, biologically appropriate concentration of the osteogenic factor at the site of the defect. These factors have exceedingly short biological half-lives, usually in the order of minutes or hours rather than days or weeks. Delivery also needs to be concentrated locally to avoid ectopic ossification and other unwanted side effects.[8] Because systemic delivery by intravenous, intramuscular or subcutaneous routes fails to satisfy these demands, there has been much interest in developing implantable slow-release devices. However, release is still not uniform over time. In most cases, there is an initial rapid efflux ('dumping') of the protein, which spikes the surrounding tissue with wildly supraphysiological concentrations of growth factor. Clearly such systems, although capable of increasing osteogenesis, are clumsy and inefficient.[8,37,104,112,153]

Research into genetic manipulation of bone healing is based on the hypothesis that gene transfer could achieve more satisfactory osteogenic promotion.[4,90,109] The advantages of gene delivery include the ability to establish a local, endogenous synthesis of authentically processed therapeutic proteins at the site of deterioration or injury, whereby therapeutic substances are persistently produced directly by local cells.[152] The concept is to transfer genes encoding osteogenic factors to cells in the location of osseous lesions. Unlike the recombinant protein, the growth factor synthesised *in situ* as a result of gene transfer undergoes authentic post-translational processing and is presented to the surrounding tissues in a natural, cell-based manner.

Unfortunately cells do not spontaneously take up and express exogenous genes. Moreover, delivery of foreign genes to recipient cells is limited by normal extracellular and intracellular protective mechanisms. As the peptide structure is dissimilar to that of the recipient species, the foreign proteins are recognised and removed by phagocytes, T-cell responses, opsonins, limited movement through pericellular matrices and collaboration of other degradative enzymes.[42] For this reason successful gene transfer requires vectors, which can be viral or non-viral. Gene transfer with viral vectors is known as transduction, whereas gene transfer with non-viral vectors is known as transfection.[8,108,114]

Gene vectors

The ideal gene delivery vector is non-toxic, non-immunogenic, easy to produce in large quantities, efficient in protecting and delivering DNA into cells (preferably with specificity for the target cell) and capable of regulating and controlling the levels of transgene protein expression in the transduced cells. This ideal vector remains to be discovered.[152] Various techniques have been deployed for introducing new genes into mammalian cells for the purpose of gene expression. Based on vector genesis and their cellular approach, these systems are divided into three major categories: viral vectors, synthetic vectors and physical methods.

Viral vectors used in orthopaedics are retrovirus (oncoretrovirus or lentivirus), adenovirus or adeno-associated virus (AAV), and herpes simplex virus (Table 2). With the exception of lentivirus, all of these have been used in human clinical trials. The only such clinical trials yet initiated in the orthopaedic area involve gene transfer to joints.[33,40,65] Retroviral vectors have the ability to integrate their genetic material into the chromosomal DNA of the cells they infect.[5,117] Retroviruses offer the potential advantage of integrating genes into host chromosomes for long-term stability in dividing cells. However, the insertion site is random and for this reason there are some huge concerns about the safety of these vectors, because they may activate or inactivate genes critical to normal host cell functioning and they could recombine with cellular or viral DNA or RNA producing new oncogenic viruses or replication-competent retroviruses.[61,111,141] Moreover, they cannot transduce non-dividing cells (this may, however, be overcome by using lentiviral vectors).[103,164]

Adenoviruses and AAVs are DNA viruses that deliver genes episomally to the nuclei of the cells they infect. Adenovirus is a non-enveloped, medium-sized (80 nm), linear, double-stranded (36 kb of nucleotides) DNA virus.[139] Adenoviruses have highly evolved mechanisms for delivery of DNA to cells and, unlike retroviruses, are not dependent on cell replication for infection. Following

Table 2
Viral vectors used in musculoskeletal gene therapy[108]

Virus	Type	Chromosomal integration	Duration of expression	Maximum insert size	Disadvantages
Adenovirus	DNA	Episomal	Short	37 kb	Cytotoxic; Immunogenic
AAV	Single-stranded DNA	Episomal	Medium/long	5 kb	Immunogenic; Difficult propagation
Retrovirus	RNA	Random insertion	Long	7 kb	Mutagenic
Lentivirus	RNA	Positive	Long	7 kb	HIV-related safety issues
Herpes simplex	Double-stranded DNA	Episomal	Short	30 kb	Cytotoxic

AAV, adeno-associated virus.

internalisation of the adenovirus, its genome is translocated into the cell nucleus, but it remains extra-chromosomal which minimises the risks of insertional mutagenesis and of non-transmission to the progeny of dividing cells.[111,139] Furthermore, adenoviruses are easy to manipulate using standard cloning techniques and can be produced in high titres, allowing them to be used *in vivo*.[30,32] The important drawback of adenovirus vectors is the high antigenicity of both the virion itself and cells infected with first-generation adenoviruses, because such cells secrete viral proteins and elicit an immune response that eventually results in their clearance from the body. The combined effects of episomal localisation and immunogenicity cause transgene expression from first-generation adenoviruses to be quite brief.[42] As an example, Zhao and colleagues recently determined that the *in-vivo* duration of BMP expression from fibroblasts transduced with Ad-BMP2 was less than 2 weeks.[165] The immunogenicity can be eliminated by using a third-generation virus, so-called gutted adenovirus vector, that contains no viral coding sequences. However, third-generation adenoviruses are difficult to manufacture and they can be propagated only in the presence of helper viruses that contain the missing viral genes necessary to form a viable capsid.[132] In spite of these limitations, first-generation adenoviruses continue to be extremely useful for defining which regenerative factors or groups of factors can best stimulate bone regeneration.[152]

AAV is a non-pathogenic, non-enveloped, small (20 nm), single-stranded DNA (5 kb of nucleotides) parvovirus that has the characteristic of being far less antigenic than adenovirus, and is considered very safe. Because of its ability to interact with both dividing and non-dividing cells and its nearly ubiquitous tropism, AAV is considered one of the most promising vectors and can be produced in high titres. However, AAVs are difficult to construct and induce an innate host immune response that does not necessarily require viral transcription. This fact has impaired progress in their utility for gene therapy in human clinical trials, and at the moment research is not focused on their use.[42,111]

Finally, vectors derived from herpes simplex viruses are difficult to manufacture and are considered potentially dangerous because of the large size of their genome, which includes many wild-type genes with unknown functions. For these reasons their use for gene transfer is limited.[15,82,143] Non-viral gene transfer using synthetic vectors may be an alternative method for gene delivery, providing higher safety. Non-viral vectors (naked DNA, DNA–protein complexes, DNA–polymer complexes, plasmid DNA) are usually cheaper and safer than viruses. Their main problem lies in their low efficiency particularly *in vivo*, compared with viral vectors, but the future is expected to see more sophisticated systems. However, at the moment none of the non-viral delivery systems provide the highly efficient transduction rates of viruses.[75,81]

Delivery strategies

Both viral and non-viral vectors can direct the constitutive expression of individual factors to sites targeted for regeneration. Two basic gene therapy strategies can be followed: vectors are either directly delivered to *in-vivo* sites (*in-vivo* gene therapy) or used to transduce, in tissue culture, cells that are subsequently implanted into animals (*ex-vivo* gene therapy).

The advantages of the first approach are that it involves only one step and it should be an off-the-shelf technology, thus more popular with surgeons. The disadvantages are that it is more difficult to achieve standardized, high transduction efficiencies, and targeting specific cells only is extremely problematic in clinical practice.

The advantages of the second approach are that standardised and high transduction efficiencies can be achieved when gene transfer is performed in an *in-vitro* setting. However, this technique is more complex and therefore may not be cost effective and may confer increased risk of bacterial contamination. Furthermore, the anatomy and topography of some organs may not allow the homing of genetically modified cells. Both approaches are under investigation and have been attempted with respect to a wide range of conditions.[152]

Orthopaedic applications in bone regeneration

Although addition of BMPs to cancellous allograft bone has proved successful for cavitary bone defects, fracture healing and spinal fusion, the same is not true for large segmental defects that require exogenous BMP activity for at least 1 week.[107] Freeze-dried rAAV-coated structural allografts have emerged to engender revitalised cortical bone with host factors that will persist for weeks following surgery and facilitate revascularisation, osteointegration and remodelling. In view of the empirical advantages of rAAV vectors for orthopaedic gene therapy[137] and the clinical potential of this vector, Koefoed and colleagues evaluated the osteogenic and remodelling properties of rAAV-caAlk2-coated allografts in a murine femur model.[25,77] They found that the efficacy of this coating may be derived from four effects that were never observed in uncoated or AAVLacZ-coated allografts: osteogenesis, inhibition of the foreign-body reaction, angiogenesis and osteoclastic resorption of the allograft.

Gene therapy has been used to heal critically-sized defects in animal models and, although impressive amounts of new bone were deposited in response, these were insufficient to heal the defect.[2,11] An effective *in-vivo* gene therapy approach to healing a large bony defect without the addition of exogenous cells has yet to be demonstrated. Recently, rAAVs expressing BMP have been combined with cultured MSCs for *ex-vivo* and *in-vivo* models of bone healing.[2,26,47,100] Lieberman et al.[89] showed that bone marrow cells transduced with human BMP-2 produced sufficient protein to heal a segmental femoral defect in rats. However, before applications of this in limited human clinical trials, several proof-of-concept issues must be addressed using marker genes to verify the kinetics of the transgene expression and the biodistribution and localisation of transduction. In addition, safety issues for people with cancer undergoing tumour resection must also be clarified by demonstrating that the rAAV does not increase residual tumour growth or metastasis.[1]

Other important issues concern the demonstration that human gene targets have similar effects to their homologue mouse and rat genes used in preclinical studies, and that the transfer of multiple human genes is feasible and effective. There is also the necessity to demonstrate that the rAAV-coated bone allograft can be used in grafting critically-sized defects in large animal models with graft fixation techniques identical to those used in clinical practice. Finally, a major limitation is the non-porous cortical surface that prohibits uniform distribution of the rAAV coating before freeze-drying. Recently some authors have proposed surface demineralisation of the cortical bone allograft to increase surface adsorbance while retaining the structural integrity of the allograft,[158] but these studies need to be confirmed in larger animal models which can demonstrate the ability of the rAA-coated demineralised allograft to withstand biomechanical stresses and decreased vascularity.

Fracture healing

Great promise has been shown by gene therapy in the field of fracture healing.[19,149] Baltzer et al.[3]demonstrated enhanced fracture healing in rabbits using *in-vivo* adenoviral transfer of the BMP-2 gene. Fractures treated with Ad-BMP-2 had radiographic evidence of healing at 7 weeks, and complete ossification (histological) across the defect at 8 weeks. Control rabbits treated with Luciferase-Adenovirus (negative control) had radiographic and histological non-union at 12 weeks. Fractures treated with Ad-BMP-2 were also stronger and stiffer compared with controls. Southwood and colleagues[142] evaluated the use of Ad-BMP-2 for enhancing healing of infected fracture defects in rabbits; earlier bridging callus, increasing external callus formation (radiographic evidence) and earlier new bone formation (histological evidence) were seen in Ad-BMP-2-treated rabbits compared with Ad-LUC-treated controls.

Preclinical studies have demonstrated that gene therapy has great potential to promote fracture healing, but most of these studies were performed in small animal models; the next step would be a comparison of delivery methods in large animal models. Moreover, despite tremendous promise, the clinical application of gene therapy raises concerns about safety. Although extreme caution has been applied in gene transfer, any substantial morbidity will not be accepted in the treatment of non-fatal musculoskeletal conditions. Viral vectors are the most controversial aspect that needs to be addressed before gene therapy can constitute an effective option in clinical practice. There is need for a deeper understanding of the biological aspects of genetically modified cells; more studies should better define the risk of a permanent alteration which could potentially lead to neoplastic transformation.

BMPs: new indication for application

Recently new applications of BMPs have been found for the treatment of post-traumatic osteonecrosis of the femoral head. Some authors suggested combining core decompression with BMP-7, with osteoinductive potential to enhance bone repair in the femoral head.[105]

In Mont's preclinical study, defects were treated with bone graft and rhBMP-7 and moderate or excellent ratings were obtained. Defects that were left untreated did not heal. When treated with either bone graft and rhBMP-7 or bone graft alone, the trapdoor cartilage appeared to be essentially normal on visual inspection whereas depression was noted in untreated femoral heads.[106] Lieberman conducted a retrospective evaluation of 15 cases (17 hips) with osteonecrosis of the femoral head treated with core decompression and human BMP, following up for 53 (26–94) months. The procedures were a clinical success in 14 of 15 hips (93% 13 cases) with Ficat stage II disease;[41] of 17 patients, 3 had radiographic progression (Ficat stages IIA, IIB, and III) of the femoral head and were converted to total hip arthroplasty.[87]

In our unpublished experience we used a core decompression procedure in association with tantalum rod and rhBMP-7 for the treatment of osteonecrosis of the femoral head; 15 hips (13 cases) with symptomatic osteonecrosis of the femoral head were treated with an average follow-up of 24 (12–42) months. Good clinical results were obtained with 12 hips (80% success rate). Of four hips with stage IIIc disease (Steinberg classification),[144] three had radiographic progression and only one was converted to total hip arthroplasty at 11 months.

Preliminary results with the use of these methods are encouraging, but further randomised trials and additional extensive follow-up are required to demonstrate the safety and effectiveness of these procedures.

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