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Molecular therapy for bone regeneration in horses

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Summary

Complicated and catastrophic fractures in horses have high failure rates. Horses often succumb to bone nonunion or contralateral limb third phalanx rotation as a consequence of long-term lameness of the fractured limb. Molecular therapy has proven useful for the acceleration of bone repair and regeneration. Even a transient local production of osteoinductive growth factor may induce a clinically relevant degree of bone formation. Molecular, or gene, therapy locally delivers a nucleotide sequence that encodes for an effective growth factor to the site of fracture healing to increase the local concentration of this growth factor. Direct gene therapy has shown to be effective for in vitro bone formation and in vivo healing acceleration by using various viral and nonviral gene delivery vectors. Cell-mediated gene therapy has also been successfully performed by using wide range of carrier cells. These cell-mediated and direct gene therapies have provided numerous promising results in recent years and may become as practical alternative solutions for the potential treatments of equine bone repair and regeneration.

Keywords: gene therapy, fracture healing, osteoinduction, local drug delivery, delivery vectors

Molekulartherapie in der Knochenregeneration beim Pferd

Komplizierte und katastrophale Frakturen beim Pferd zeigen hohe Komplikationsraten, welche oft in Pseudarthrosen (non-unions) resultieren oder in der contralateralen Extremität durch mechanische Überbelastung zu Hufrehe und daraus folgender Rotation des Hufbeines führen. Modernere, molekulare Therapien versprechen eine Beschleunigung der Frakturheilung und Knochenregeneration. Sogar vorübergehende, lokale Produktion von osteoinduktiven Wachstumsfaktoren können zu einer klinisch signifikanten Beschleunigung der neuen Knochenbildung führen. Die molekulare oder auch Gentherapie bringt eine Nukleotid-Sequenz vor Ort, welche lokal das Gewebe kodiert an der Frakturlinie effektive Wachstumsfaktoren zur Beschleunigung der Frakturheilung zu produzieren. Dass die direkte Gentherapie erfolgreich neue Knochenbildung anregt, wurde bereits in vitro und in vivo nachgewiesen, wobei die Nukleotid-Sequenzen mit viralen und nichtviralen Vektoren appliziert wurden. Auch die Zell-mediierte Gentherapie wurde mit verschiedenen Zelltypen bereits erfolgreich angewendet. Diese Therapiearten sind vielversprechend und werden in Zukunft eine praktische Alternative zur herkömmlichen Frakturbehandlung darstellen, auch für Pferde.

Schlüsselwörter: Gentherapie, Frakturheilung, Osteoinduktion, Lokale Drug Delivery, Vektoren, Orthopädie

Introduction

Musculoskeletal disorders are the most important health problems and the most common cause of performance morbidity in horses (*Jeffcott* et al. 1982). The risk of catastrophic injuries in Thoroughbreds racehorses, most of which involve fractures, has been reported as 1.4 horses per 1,000 starts in Kentucky (*Peloso* et al. 1994), 1.7 per 1,000 starts in California (*Estberg* et al. 1996), and 0.8 per 1,000 starts in the United Kingdom (*McKee* 1995). Reportedly, the catastrophic fractures have resulted in the 45% career ending and 40% mortality rate in horses (*Ducharme* et al. 1996). Equine bone, in general, has small windows of opportunities to restore natural integrity and this characteristic makes horses particularly vulnerable to bone failure. Surgical techniques of internal and external fixations have improved over the past decades in both implant size and in established techniques of repair (*Auer* 1996, *Levine* et al. 2007). Even with these improvements, however, equine bone could particularly benefit from molecular therapeutic technologies for an acceleration and enhancement of fracture healing.

Osteoinductive growth factors

Use of osteoinductive growth factors for the purpose of improving bone healing has been established over decades of experimental use and has been applied in animals and human clinical patients. Growth factors are defined as certain types of signaling molecules that induce cell differentiation and tissue regeneration. Increased expressions of endogenous growth factors associated with osteogenesis or bone formation have been identified and their functions have been studied. This includes bone morphogenetic protein (BMP), LIM mineralization protein (LMP), transforming growth factor-beta (TGF-β), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (*Zachos* and *Bertone* 2005). Of these, an induction of bone formation or enhancement of bone healing in experimental models has been demonstrated with application of exogenous recombinant human (rh) proteins such as rhBMP2 (*Murnaghan* et al. 200%), rhBMP4 (*Han* et al. 2005), rhBMP7 [osteogenic protein-1 (OP-1)] (*Friedlaender* et al. 2001), rhTGF-β (*Nielsen* et al. 1994), and rhPDGF (*Nash* et al. 1992). Use of rhIGF has also improved bone healing when it was combined with rhTGF-β (*Schmidmaier* et al. 2002)

Clinical application of rhBMPs has been reported in human and veterinary hospitals for the treatment of various osseous disorders. In human clinical trials, the rhBMP2 and rhBMP7 have been applied for tibial or femoral nonunion with favorable outcomes (*Friedlaender* et al. 2001). In veterinary fields, rhBMP2 has been clinically used for small animal patients (*Faria* et al. 2007, *Jones* et al. 2008) and horses (*Perrier* et al. 2008).

Direct gene therapy

Application of recombinant protein of osteoinductive growth factors can be challenging in adult horses. Large doses of rhBMPs may be required not only due to the size of the bones but also the fact that human proteins have appeared to be more effective in laboratory animals (rat, rabbit) than large animals (sheep, goat, and dog) (*Heckman* et al. 1991) and may not be cost-effective for clinical use in large animal species. For these reasons, alternative strategies of growth factor delivery should be considered in horses.

Gene therapy has a great potential as a method for application of growth factors in the acceleration and enhancement of bone healing. This is because gene therapy can induce a higher local concentration of therapeutic molecules for a longer time period compared to protein delivery, and manufacture of gene therapy products is likely much less expensive than recombinant protein (*Goldstein* et al. 1998). Also, gene therapy can produce a more biologically active form of the growth factor since the molecules are synthesized by autologous cells in situ (*Chen* 2001). Genes of growth factors can be delivered to bone by viral vectors (adenovirus [Ad], retrovirus [Retro], lentivirus [Lenti], adeno-associated virus [AAV]) or nonviral vectors (electroporation, gene activated matrix, liposomal vectors, and sonoporation). Currently, viral vectors are preferred for gene therapy due to the high transduction efficiency, although nonviral methods may be more economical and have less immunogenecity (*Oligino* et al. 2000).

Viral gene delivery

Acceleration and enhancement of bone healing by direct injection of the Ad vectors encoding BMP genes has been demonstrated in numerous animal experimental models (*Zachos* and *Bertone* 2005). Adenovirus has a number of advantages including the ability to generate high viral titers, its high transduction efficiency, and its ability to transduce both dividing and non-dividing cells (*Evans* et al. 2009). In contrast to Ad-vector, recombinant AAV has gained much scientific attention as having a superior safety profile, because it is non-pathogenic, immunologically inert virus, and can induce sustained transgene expression (*Evans* et al. 2009) The ectopic and orthotopic bone formations have been induced by AAV-BMP2 (*Chen* et al. 2004, *Gafni* et al. 2004). Use of nonviral gene delivery methods, such as electroporation and use of lipid based transfection reagents can also effectively deliver molecular therapies to sites of bone formation, however will not be discussed here because the application in horses has not been yet reduced to practice.

Cell gene delivery (Indirect or ex vivo Gene Therapy)

The use of living cells as a vector for molecular therapy, however, offers additional benefits over viral vector delivery, including the option for molecular engineering to release paracrine and autocrine bioactive factors, direct integration of the cell into the regenerative process, and a broad diversity of medical applications. Stem cells can be engineered for induction of cell differentiation toward various tissue types and can provide the machinery to release an array of local endogenous growth factors (*Zachos* and *Bertone* 2005). Additionally, direct delivery of viral or nonviral vectors to treat fractures and bony disorders require large volumes of purified agents and the potential risks of failure of vector containment, systemic immune reaction to viral vector, and seeding of nontarget tissues with vectors raise safety concerns that are much reduced with the cell serving as an intermediary vector. Various types of carrier cells can serve as a source of osteoprogenitor cells. Pluripotent stem cells, isolated from various tissues, have shown promising results; however, several types of non-stem cells have also been used as gene delivery cells for bone disorders.

Bone marrow-derived stem/stromal cells

BMD-MSC have shown to differentiate into various mesenchymal lineages including bone, cartilage, adipose, muscle, and tendon, including equine BMD-MSC (*Zachos* et al. 2006). Bone formation and regeneration have been demonstrated by gene augmented BMD-MSC and BM stromal cells in various models. A large number of studies have reported that osteogenic potential of MSC is greatly enhanced by genetic engineering using osteoinductive growth factors such as BMP2 (*Lieberman* et al. 1999), BMP4 (*Gysin* et al. 2002), BMP9 (*Dumont* et al. 2002) and combination of BMP4 and VEGF (*Peng* et al. 2002).

Adipose-derived stem/stromal cells

Compared with BMD-MSC, adipose-derived stem cells (ADSC) or AD stromal cells are easier to isolate, have a relatively lower risk of donor site morbidity, and are available in large populations (*Li* et al. 2007). These fat tissue-derived cells can exhibit stable growth and sufficient cell proliferation and have been successfully differentiated in vitro toward osteogenic, adipogenic, myogenic, and chondrogenic pathways using established factors (*Rodriguez* et al. 2005). Several recent studies have applied ADSC or AD stromal cells in rodent models to induce ectopic bone formation and bone repair in animal models (*Li* et al. 2007) by adenoviral gene transfers of BMP2 (*Kang* et al. 2007), BMP7 (*Yang* et al. 2005), and Runt-related transcription factor 2 (Runx2) (*Zhang* et al. 2006). The relative osteoinductive ability between the stem cells sourced from fat and bone marrow tissues has not been comprehensively elucidated.

Muscle-derived stem cells

Skeletal muscle represents another abundant and easily accessible tissue as a source of carrier cells for ex vivo gene therapy. Several groups have identified a population of muscle-derived stem cells (MDSC) in skeletal muscles which shown to undergo multilineage differentiation into bone, and cartilage, neuron, endothelial, and hematopoietic tissues (*Qu-Petersen* et al. 2002). Unlike BMD-MSC, muscle-derived inducible osteoprogenitors do not express osteogenic markers until exposed to BMP2 (*Bosch* et al. 2000) therefore, the MDSC should be used in combination with osteoinductive factors to achieve full osteogenic potential, such as BMP2 or BMP4 (*Bosch* et al. 2000, *Peng* et al. 2004).

Dermal fibroblasts

Skin is one of the most readily available and easily accessible tissues in the body. Dermal fibroblasts (DFb) isolated from

dermis tissue are attractive carrier cells because they can be harvested by minimally invasive and less painful procedures, require less fastidious culture technique, and have rapid cell expansion. Several studies have demonstrated the osteogenic differentiation of DFb by converting DFb into bone-forming cells with the transductions of various osteogenic genes such as BMP2 (*Hirata* et al. 2003, *Hirata* et al. 2007), BMP7 (*Rutherford* et al. 2002), Runx2 (*Hirata* et al. 2007) and LMP3 (Lattanzi et al. 2008). These genetically modified DFb can accelerate bone regeneration in animals (*Hirata* et al. 2003, *Rutherford* et al. 2002).

Blood cells

The use of buffy coat cells from venous blood as a gene carrier of LMP1 gene to secrete BMP2, BMP6, and TGF-β1 proteins can successfully induce ectopic bone formation and spine fusion in rabbits (*Viggesvarapu* et al. 2001). Specifically, umbilical cord blood is known to contain multipotent cells and can serve as an alternative source of MSC. The umbilical cord blood-derived MSC has an osteogenic potential (*Rogers* et al. 2004), and can accelerate bone repair in animals (*Jang* et al. 2008). In addition, umbilical cord blood-derived MSC may be capable to evade the host immune rejection, as they have been considered as immune-privileged cells due to their surface characteristics (*Chen* et al. 2008). Therefore, an implantation of umbilical cord blood-derived MSC could have a great potential for allogenic cell-mediated gene therapy for bone regeneration.

Gene Therapy in Horses for Bone Regeneration

Cell-mediated and direct gene therapies have provided numerous promising results in recent years and may become a practical alternative solution for a potential treatment of equine bone repair and regeneration. Sufficient gene transfer can be achieved by using an adenoviral (Ad) vector in equine cells, including bone marrow-derived stem cells, synovial cells and chondrocytes (*Ishihara* et al. 2006). High vector dosages can be used in equine cells because of relative resistance to cytotoxicity in these cells compared to a human cell lines. Bone regeneration within equine metatarsal osteotomies and ostectomies in response to delayed percutaneous injection of adenoviral bone morphogenetic protein-2 (Ad-BMP2), Ad-BMP6, or beta-galactosidase protein vector control (Ad-LacZ) administered 14 days after surgery confirmed greater and earlier mineralized callus in the bone defects injected with Ad-BMP2 or Ad-BMP6. Peak torque to failure and torsional stiffness were greater in osteotomies treated with Ad-BMP2 than Ad-BMP6, and both Ad-BMP-2 and Ad-BMP6 treated osteotomies were greater than Ad-LacZ or untreated osteotomies. Gene expression of ostectomy mineralized callus 8 weeks after surgery indicated upregulation of genes related to osteogenesis compared to intact metatarsal bone. These results demonstrated a greater relative potency of Ad-BMP2 over Ad-BMP6 in accelerating osteotomy healing (*Ishihara* et al. 2008). Accelerated bone regeneration has also been proven in response to cell delivered BMP2 and BMP6 (*Ishihara* et al. 2010a). Equine metacarpal/metatarsal osteotomies responded to percutaneous injection of autologous dermal fibroblasts (DFb) genetically engineered to secrete BMP2 by greater and earlier healing of bone defects treated with DFb with BMP2 gene augumentation. The qCT and biomechanical testing revealed greater mineralized callus and torsional strength of DFb-BMP2 treated bone defects. On the histologic evaluation, the bone defects with DFb-BMP2 implantation had greater formation of mature cartilage and bone nodules within the osteotomy gap and greater mineralization activity on osteotomy edges. In subsequent studies in equine rib drill defects, both the DFb-mediated and direct adenoviral vector delivery of BMP2 had equivalent relative efficacy in bone regeneration. Equine rib drill defects were treated by percutaneous injection of either DFb-BMP2 or Ad-BMP2 vector (*Ishihara* et al. 2010b). At week 6, both the DFb-BMP2- and Ad-BMP2-treated rib defects had greater bone filling volume and mineral density, with DFb-BMP2 inducing greater bone volume and maturity in cortical bone aspect of the defect than Ad-BMP2. The transplantation of DFb alone induced modest bone formation. Increased mineral density and bone turnover were evident in the cortical and cancellous bone directly adjacent to the healing drill defects treated with either DFb-BMP2 or Ad-BMP2.

Conclusion

These results demonstrated an efficacy and feasibility of DFb-mediated BMP2 therapy to accelerate the equine bone healing. Additionally, BMP2 gene therapy appeared safe for articular fracture, because the direct intra-articular administrations of Ad-BMP2 did not cause of mineralization or ossification of articular cartilage and synovium tissues. In concert, both cell-mediated and direct BMP2 gene therapy may be considered as a potential treatment for various types of fractures and bone defects in horses.

Clinical Relevance

Clinical application of these gene delivery methods of BMP2 in horses is supported by these efficacy and safety data.

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