Myostatin Inhibitor Enhancement of Musculoskeletal Repair

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Abstract
The methods and compositions of this invention provide a means of regenerating injured musculoskeletal tissue by inhibition of myostatin function. The invention provides methods of treating a nonunion fracture in an individual comprising delivering to the fracture via a delivery system comprising biodegradable hydrogel, a pharmacological amount of myostatin propeptide effective to inhibit myostatin function. The invention also teaches compositions useful for the treatment of non-union fracture in an individual, said compositions comprising a pharmacological amount of myostatin propeptide effective to inhibit myostatin function; and a delivery system for delivering said myostatin propeptide to said fracture, wherein the delivery system comprises a biodegradable hydrogel or biodegradable nanobeads.
This nonprovisional application claims benefit of priority under 35 U.S.C. §119(e) of provisional U.S. Ser. No. 61/269,421, filed Jun. 25, 2009, now abandoned, the entirety of which is hereby incorporated by reference.

FEDERAL FUNDING LEGEND

This invention was created in part using funds from the federal government under grant AR49717 from the National Institutes of Health and Dept of Defense (Office of Naval Research) under grant N000140810197. Consequently, the federal government has certain rights in this invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention
The present invention relates to the fields of muscle physiology and therapeutics. Specifically, the present invention relates to myostatin inhibitor enhancement of musculoskeletal repair.

2. Description of the Related Art
Approximately 2 million cases of delayed and non-union fractures occur annually in the United States, and the treatment and care of these patients requires considerable time and cost. Bony nonunions are common in cases where fractures are associated with extensive muscle damage or poor muscle coverage, revealing that muscle is a primary factor driving the rate of bone healing with traumatic musculoskeletal injury (1). For example, size of the fracture callus is increased in regions alongside muscle (2) and open fractures in sites lacking muscle coverage, such as the tibia, heal much more slowly than fractures where muscle coverage is available (3-4). Healing of open bone defects is accelerated when a muscle flap is used to cover the wound, and intact muscle is more effective at promoting bone repair than injured muscle (5-6).

It has also been observed that new bone growth can be observed when minced muscle tissue is implanted alongside bone, but minced liver tissue does not have the same osteogenic effect (7). As noted by Stein et al. (2) “muscle is perhaps the most crucial factor in the physiological process of fracture healing”. It is therefore clear that improving muscle regeneration and muscle coverage in cases of orthopedic trauma has significant potential to accelerate bone repair.

Myostatin (GDF-8), a member of the TGF-beta superfamily of growth and differentiation factors, is most well-known as a potent suppressor of muscle growth, development, and regeneration. Mice lacking myostatin show a significant increase in muscle mass (8), and congenital absence of myostatin is associated with increased muscle mass in both humans and dogs (9-11). It has also been shown that factors which inhibit myostatin, such as follistatin, can improve muscle regeneration and decrease fibrosis in injured muscle (12-13).

It has been demonstrated that the receptor for myostatin, ActRIIB, is expressed in bone marrow derived stem cells (14) and mice lacking myostatin show increased bone density and strength (15-16). These data are further supported by genetic studies showing that myostatin gene polymorphisms are associated with variation in peak bone mineral density (17) and that inhibition of normal myostatin signaling by transgenic overexpression of myostatin propeptide increases bone mineral density in mice (18). The mechanism(s) by which myostatin regulates bone formation and bone density is not yet well understood.

Myostatin is normally bound to a propeptide from which it must be cleaved to form an active ligand (22-23). A recombinant myostatin propeptide effectively inhibits active myostatin in vitro and in vivo, and overexpression of the propeptide increases muscle mass (24-25). The potential of a myostatin inhibitor to improve muscle and bone repair in a model of deep penetrating injury remains unknown.

There is still, therefore, a recognized need in the art for improved therapeutics to improve muscle and bone repair. Specifically, the prior art is deficient in novel strategies to utilize myostatin inhibitor enhancement of musculoskeletal repair. The present invention fulfills this long-standing need in the art.

SUMMARY OF THE INVENTION

Myostatin (GDF-8) is known as a potent inhibitor of muscle growth and development, and myostatin is also expressed early in the fracture healing process. The invention demonstrated that a myostatin inhibitor, a recombinant myostatin propeptide, enhanced the repair and regeneration of both muscle and bone in cases of deep penetrating injury. Blocking myostatin signaling in the injured limb improves fracture healing and enhances muscle regeneration. These data suggest that myostatin inhibitors are effective for improving wound repair in cases of orthopedic trauma and extremity injury.

The present invention is directed to a method of regenerating injured musculoskeletal tissue comprising contacting the injured tissue with a myostatin inhibitor, wherein said contact results in musculoskeletal tissue regeneration by inhibition of myostatin function.

In certain embodiments of the above, the myostatin inhibitor is myostatin propeptide. In certain other embodiments, the myostatin inhibitor is a soluble myostatin receptor. In certain embodiments, the myostatin inhibitor is delivered to the injured tissue by means of a biodegradable delivery system.

The present invention is also directed to methods of treating a nonunion fracture in an individual. One such method comprises delivering to the fracture via a delivery system comprising biodegradable hydrogel, a pharmacological amount of myostatin propeptide effective to inhibit myostatin function, thereby treating the fracture. In addition, the present invention also provides a method which comprises delivering to the fracture via a delivery system comprising biodegradable nanobeads, a pharmacological amount of myostatin propeptide effective to inhibit myostatin function, thereby treating the fracture.

The present invention is also directed to a composition useful for the treatment of non-union fracture in an individual. The composition comprises a pharmacological amount of myostatin propeptide effective to inhibit myostatin function; and a delivery system comprising a biodegradable hydrogel for delivering the myostatin propeptide to the fracture.

Other and further aspects, features and advantages of the present invention will be apparent from the following
description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions and certain embodiments of the invention briefly summarized above are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

[0019] FIGS. 1A-1E show the design and results of an experiment using myostatin propeptide. FIG. 1A: Mice received treatments on the day of surgery, 5 days post-op, 10 days post-op, and were euthanized 15 days following the initial treatment. FIG. 1B: A fibril osteotomy procedure was used (arrow) and the lateral compartment muscles were cut. Fibula, Tib-fibia, TA-tibialis anterior. FIG. 1C: Histological sections at the osteotomy site were stained using Masson trichrome and 0.80 mm2 region of interest lateral to the fracture callus examined for fraction of fibrotic tissue (blue). FIG. 1D: Body weight and (FIG. 1E) muscle mass (b. triceps brachii+quadriceps femoris) in saline (VEH) and propeptide (PROP; 20 mg/kg) treated mice. Error bars represent S.D.

[0020] FIGS. 2A-2C show MicroCT reconstructions of the osteotomy site in control and propeptide-treated animals. (FIG. 2A) MicroCT images of the fibula osteotomy site in saline (VEH) and propeptide (PROP; 20 mg/kg) treated mice. Note extensive bridging across the osteotomy gap in the propeptide-treated animals. (FIG. 2B) Bone bridging across the osteotomy gap is increased significantly in the propeptide (PROP) treated mice, and (FIG. 2C) bone volume of the fracture callus is increased significantly in the propeptide (PROP) treated mice.

[0021] FIGS. 3A-3B show histological preparations of cal- lus bone after propeptide treatment. (FIG. 3A) Cartilage area, as indicated by safranin-O staining, is increased in fracture callus of propeptide (PROP)-treated mice (FIG. 3B).

[0022] FIGS. 4A-4B show histological sections in control (vehicle) treated mice and propeptide (PROP) treated animals. (FIG. 4A) Masson trichrome staining of the soft-tissue injury site lateral to the fracture callus showing greater fibrotic tissue staining (blue, left panel) in the vehicle (VEH) treated animal compared to greater muscle staining (red, right panel) in the propeptide (PROP) treated animal. (FIG. 4B) Quantification of red and blue pixel fractions, where a value of 250 is either pure red or pure blue, indicates a significant increase in the fraction of red (muscle) pixels and decrease in blue (fibrous tissue) pixels with propeptide (PROP) treatment.

[0023] FIGS. 5A-5D describe the nanobeads of the present invention, (FIG. 5A) DMOG-loaded nanobeads with (FIG. 5B) 45 nm average particle size. (FIG. 5C) Collagen IV (green color) synthesis was inhibited by DMOG-loaded nanobeads (FIG. 5D) when compared with blank nanoparticles encapsulated in hollow fiber device (FIG. 5C). Scale bar=75 um and implantation time was 4 weeks. Det lines indicate the borders of hollow fiber.

DETAILED DESCRIPTION OF THE INVENTION

[0024] As used herein, the term “a” or “an”, when used in conjunction with the term “comprising”, in the claims and/or
delivery system comprising biodegradable nanobeads, a pharmacological amount of myostatin propeptide effective to inhibit myostatin function, thereby treating the fracture.

[0032] In certain embodiments of the instant invention, are provided compositions useful for the treatment of non-union fracture in an individual. One embodiment recites a composition comprising a pharmacological amount of myostatin propeptide effective to inhibit myostatin function; and a delivery system comprising a biodegradable hydrogel for delivering the myostatin propeptide to the fracture.

[0033] Other and further aspects, features and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure. The following example(s) are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

Example 1

Animals, Treatments, & Surgical Procedures

[0034] An initial dose-response study was performed to evaluate efficacy of the myostatin inhibitor in male CD-1 mice four months of age. Mice were treated with the propeptide at 0 mg/kg, 10 mg/kg, 20 mg/kg, or 50 mg/kg at day 0, day 5, and 10 and then sacrificed one week after the last treatment (FIG. 1A).

[0035] Results showed that three injections of the propeptide over a 15 day treatment period increased fore- and hind-limb muscle mass by 10% at the 10 mg/kg dose and increase muscle mass by more than 15% at the 20 mg/kg dose. The 50 mg/kg dose did not increase muscle mass beyond the increase observed in the 20 mg/kg group and so the 20 mg/kg dose was used. Adult CD-1 mice were separated into two groups: those receiving the propeptide (PRO) or saline (VEH). Each treatment group included 10-12 male and 10-12 female mice, for a total of 20-24 mice per treatment group. Fibula osteotomy was performed on the left leg under isoflurane anesthesia as described (21), and the lateral compartment muscles fibularis longus and brevis severed in the region overlaying the osteotomy site (FIG. 1B). The skin incision was closed using VetBond™ skin glue. Treatments were administered immediately following osteotomy, 5 days following surgery, and 10 days following surgery. Animals were weighed at each of these timepoints and euthanized according to IACUC-approved procedures 5 days after the last treatment (15 days after surgery; FIG. 1). Mice were weighed and the left quadriceps femoris and triceps brachii muscles weighed. The left leg was removed and fixed in 10% buffered formalin for 24-48 hrs, washed, and then stored in 70% ETOH (FIG. 1C).

Example 2

MicroCT, Histology, & Histomorphometry

[0036] Intact legs with surrounding muscle were first imaged using a FAXITRON small-animal x-ray cabinet at 35 kVP, 2.5 mA for 45 seconds to verify that the fibula osteotomy was successful and that the tibia was not damaged. Specimens were sent to the Savannah River Site National Laboratory (Aiken, S.C.) for micro-computed tomography using a 160 kV micro-focus X-ray machine (Kevex Inc., Model 16010), a four-axis positioning system (New England Affiliated Technologies series 300), and an amorphous silicon imager (Varian Inc, Paxscan 4030) at 12 micron resolution. Measurements of total callus volume and callus bone mineral density were calculated 0.5 mm either side of the callus center. MicroCT images were then scored by a technician blind to the treatments as either having bone crossing the osteotomy site (‘bridged’) or showing no bone crossing the fracture gap (‘unbridged’). Specimens were then decalcified using EDTA, embedded in paraffin, and sectioned at 6-8 µm. Paraffin sections were stained with safranin-O and fast green for measurement of cartilage area (Cg.Ar) in the callus. Histomorphometric nomenclature follows recommended standards (26). Alternate sections were stained using Masson trichrome, which stains fibrous collagen-rich tissue blue and skeletal muscle red. A 0.80 mm² region of interest was examined lateral to the fibula fracture callus, 90° from an axis running through the center of the tibia and fibula (FIG. 1). The image was captured using a QImaging digital camera at 100x, and the relative fraction of red and blue pixels in each image quantified using SigmaScan software.

Example 3

Statistical Analysis

[0037] Experiments were performed in two blocks, with osteotomy performed in half the mice (n=20-24) for Block 1 and then a second group of 20-24 mice included for Block 2 approximately six weeks later. Single-factor ANOVA was used to detect significant effects of treatment and block on the outcome measures described above. Chi-square test was used to test for differences between treatment groups in the frequency of bridged or unbridged osteotomy sites.

Example 4

Mice Assigned to Vehicle or Propeptide Treatment did not Differ (<5%) in Body Weight at the Time of Surgery

[0038] Mice treated with the propeptide were slightly (~7%) but not significantly (p=0.12) larger than saline-treated mice at the end of the treatment period (FIG. 1D), and the propeptide significantly (p<0.001) increased muscle mass in the mice by almost 20% (FIG. 1E).

[0039] This increase is significant not only in absolute terms but also when the data are normalized by body mass (p<0.001). MicroCT reconstructions of the osteotomy site show that bone is observed to bridge the osteotomy gap in approximately 40% of cases among the control mice whereas bridging is observed in 80% of the mice treated with the propeptide (p<0.01, FIGS. 2A-2B). MicroCT quantification of bone volume 0.05 mm either side of the osteotomy center shows that propeptide treatment significantly increases the volume of bone in the fibula fracture callus (FIG. 2C). Histological preparations reveal that the increase in callus bone volume with propeptide treatment is accompanied by an increase in callus cartilage volume as well (FIGS. 3A-3B).

[0040] Examination of histological sections stained with Masson trichrome indicates that collagen-rich fibrous tissue is abundant in the area of injured muscle lateral to the osteotomy site in control (vehicle) treated mice, whereas fibrous tissue is less prolific and regenerative muscle fibers more common in the area of injury among propeptide treated mice (FIG. 4A). Quantification of blue (fibrous) versus red (muscle) staining using image analysis shows that propeptide treatment significantly increases the fraction of muscle staining and decreases the fraction of fibrous tissue in the injury
site (FIG. 4B). In vehicle-treated mice the fraction of red-staining tissue is approximately 15% greater than the region staining positive for fibrous (blue) tissue, whereas in proteptide treated mice the fraction of red tissue is 35% greater than the blue-positive area (FIG. 4B).

Discussion

[0041] The effects of myostatin inhibitors on muscle mass are now relatively well known, but the effects of these molecules on bone formation and regeneration have only recently been investigated. Myostatin deficiency directly increases the osteogenic potential of bone-marrow derived stromal cells (7) and mice lacking myostatin show increased bone density in the limb and spine (8-9). Absence of myostatin increases size and bone volume in the fracture callus (14) and another group has demonstrated that a soluble decoy myostatin receptor increases bone formation and trabecular bone volume (27).

[0042] Previous work has indicated that myostatin is highly expressed in the earliest stages of fracture healing (12), suggesting that this factor may play a key role in the recruitment and proliferation of progenitor cells in the fracture callus. This hypothesis is supported by the data presented here, showing that cartilage area and bone volume in the fibula fracture callus are both increased with proteptide treatment. These findings point to a role for myostatin in regulating the early sequence of events in endochondral ossification, such that inhibition of myostatin increases the number and/or proliferative capacity of these cells. This increase in the progenitor cell population appears to then have subsequent downstream effects, such that during the chondrogenic and osteogenic phases of fracture healing the soft- and hard-callus remains relatively large. It is also possible that this increase in the progenitor cell population with inhibition of myostatin function enhances the rate of endochondral ossification, so that by two weeks post-fracture the formation of new bone across the fracture gap is accelerated (FIG. 2A).

[0043] To date, the therapeutic potential of myostatin inhibitors has received greatest attention in the area of muscular dystrophy treatment (28-29). Inhibitors such as a myostatin antibody (MYO-029), decoy soluble myostatin receptor (ActRIIB-Fc), and myostatin propeptide can enhance muscle regeneration, increase myofiber hypertrophy, and decreases fibrosis in healing muscle (30). Other factors that can inhibit myostatin, such as follistatin and decorin, may also have potential for treating muscle injury and congenital muscular disorders (6,31). The realization that myostatin inhibitors may also enhance bone healing, together with the substantial evidence for a role of these inhibitors in improving muscle regeneration, suggest that myostatin inhibitors represent novel therapeutic molecules in the treatment of musculoskeletal injuries in which both muscle and bone are damaged.

[0044] Perhaps the most immediate application of these inhibitors would be in treating the penetrating extremity injuries frequently encountered in the battlefield setting. Musculoskeletal injuries are the most common wounds encountered in modern warfare, representing 60-70% of all combat-related injuries (32). The Joint Theater Trauma Registry indicates that out of 3,575 extremity wounds in Iraq, 53% were penetrating soft-tissue wounds and 26% were bone fractures (33). Most of these injuries are caused by fragments from detonating explosives, which produce extensive soft-tissue damage, bone fractures, and frequently lead to secondary infections (34). As noted, impaired muscle healing and damage to skeletal muscle has a direct effect on bone healing, as muscle serves as a local source of stem cells, growth factors, and vascular supply for bone (13,35). Myostatin inhibitors may therefore represent novel therapeutic agents for enhancing the repair of battlefield injuries, but also for improving bone and muscle healing in cases of orthopedic trauma resulting from non-combat injuries such as motor vehicle accidents.

[0045] First, the surgical model used, while involving muscle damage, does not attempt to simulate the introduction of foreign particles and debris that often occurs with blast trauma. The extensive wound debridement and irrigation that must be performed when treating such wounds is likely to affect the rate of soft tissue healing and regeneration, as is the potential application of antibiotics either locally or systemically. Second, traumatic musculoskeletal injuries frequently occur alongside injuries to other organ systems such as the liver, lungs, or brain (36) and the damage to multiple organ systems as well as the extensive blood loss that can occur complicates bone healing possibly having both negative and even positive effects (37). Finally, mice are relatively small mammals and they have they ability to regenerate bone rapidly. We have observed that by two weeks following osteotomy the callus is already more than 50% bone (21). Thus, the present invention shows that blocking myostatin function with various recombinant peptides is likely to have significant potential in the area of musculoskeletal tissue regeneration and repair.

Efficacy and Safety of a Soluble Myostatin Receptor Using Systemic Treatments

[0046] Adult mice 4-6 months of age are included. 12 males and 12 females per treatment group (soluble receptor or vehicle), for a total of 48 mice. Bone and muscle injury is induced by cutting the mouse fibula and surrounding musculature. There are several well-established protocols for inducing bone fractures in rodents, including cutting the tibia at the midshaft, which requires inserting a pin internally to stabilize the fracture, or fracturing the femur by applying blunt force to the thigh, which also requires an internal fixator pin. The fibula protocol from Midura et al. (38) is used because 1) the fibula is a small bone that bears little weight during locomotion, and so the animals can walk relatively normally post-surgery to access food and water, 2) the fibula has more muscle coverage than the tibia, and this protocol involves cutting muscle around the fracture to simulate a deep penetrating injury, and 3) no internal fixator pin is required because the fibula is small and plays a minor role in weight-bearing.

[0047] Mice are anesthetized using isoflurane, and analgesia (ketorolac, 15 mg/kg) is delivered by IP injection at the time of surgery and 24 hours later. For fibular osteotomy, a 5 mm incision is made in the skin and in the biceps femoris, exposing the fibula and peroneus longus muscle, and then cutting the peroneus longus muscle and fibula with microtodontomy scissors. The incision is closed using skin glue. Mice are then treated systemically with either the soluble myostatin receptor (ActRIIB; 10 mg/kg body weight, or approximately 0.3-0.4 mg per injection) or saline at 2, 7, 12, 17, and 22 days post-surgery. This dose is selected because 10 mg/kg of soluble receptor significantly increases muscle mass in mice. Mice are euthanized by CO2 overdose & thoracotomy four weeks after surgery when tissue is collected and the fracture site and surrounding muscle analyzed radiographically and histologically.
Prior to being sacrificed, recovery of musculoskeletal function is assessed in each animal at 1, 2, 3, and 4 weeks post-surgery using rotarod testing, measurement of hindlimb grip strength, and quantification of open field locomotor activity. After mice are killed, the leg is disarticulated at the knee and ankle, and the leg skinned and fixed in 4% ice cold paraformaldehyde for 24 hours. Specimens are then transferred to 70% ETOH, and radiographed using a FAXITRON x-ray cabinet at 35 kV and 2.5 Ma for 15 seconds. Specimens are sent for microCT imaging (12 µm resolution) of the fracture callus. Fracture callus dimensions are quantified from FAXITRON and microCT (.CT) images.

In adult male normal and myostatin-deficient mice (5 per genotype), loss of myostatin function improves fracture healing by increasing size and bone volume of the fracture callus. These data further suggest that myostatin inhibitors accelerate bone and tissue repair following musculoskeletal injury. Following fcatron and microCT imaging, specimens are decalcified in EDTA, embedded in paraffin, and sectioned transversely to yield intact cross-sections of the calf musculature and fracture callus. Alternate sections are stained with safranin-O and fast green to detect bone and cartilage in the callus (39), hematoxylin and eosin to label muscle and nuclei, and with an antibody (Santa Cruz SC-7557) to vimentin to label fibrotic areas in muscle (40).

Quantitative parameters measured from histological sections include: cartilage area relative to total area, bone area relative to total area, number of regenerating myofibers (identified by centrally placed nuclei), diameter of regenerating myofibers, and fibrotic surface area. Statistical analyses of behavioral and microstructural variables include two-factor ANOVA with treatment (soluble receptor or vehicle) and sex (male vs. female) as the factors. Mice treated with the soluble receptor show improved musculoskeletal function (e.g., grip strength), a larger fracture callus, increased bone area and volume, increased size and number of regenerating muscle fibers, and decreased fibrotic surface area in muscle compared to normal mice.

Efficacy and Safety of a Myostatin Propeptide Using Systemic Treatments

Surgery and sample sizes are the same as described above. Mice are treated systemically with either the myostatin propeptide (10 mg/kg body weight, or approximately 0.3-0.4 mg per injection) or saline at 2, 7, 12, 17, and 22 days post-surgery. Motor function and strength testing, microradiographs and high-resolution microCT images of fracture sites, histomorphometric analysis of muscle and bone tissue, serum markers, and toxicity analyses are as described above for Task 1. Two-factor ANOVA with sex and treatment as the factors is performed.

Example 5

Efficacy of Local Delivery of a Myostatin Inhibitor Using a Degradeable Hydrogel

Extracellular matrix (ECM)-derived polymeric hydrogels with a wide array of physiological functions represent ideal substrates for protein and peptide delivery and tissue regeneration. In addition, heparin in the ECM protects growth factors, peptides, or proteins from enzymatic degradation and thermal denaturation, and extend their releasing durations in vivo while retaining their bioactivity (41). An injectable, in situ-crosslinkable ECM-based hydrogel by crosslinking thiol-modified hyaluronan (HA) and gelatin (Gtn) using polyethylene glycol (PEG) diacrylate (PEG-DA) was used. This hydrogel facilitates the long-term release of growth factors and peptides, promoting continuous and sustained tissue regeneration. For example, the in vitro release of hepatocyte growth factor (HGF) was used as a model system to demonstrate the feasibility of this approach. Hepatocyte growth factor was allowed to release from the hydrogels at 37° C. into a PBS buffer solution. Over 26 days in vitro, the hyaluronan-gelatin hydrogels without heparin released a total of 35% of the loaded growth factor, as compared to 18% by the hyaluronan-gelatin with heparin hydrogels. Addition of heparin to the hydrogels significantly prolonged the releasing duration of the loaded hepatocyte growth factor.

The myostatin inhibitor identified is loaded into a degradable hydrogel. Thiol-modified hyaluronan and gelatin are mixed in a 1:2 ratio, and 5% heparin is added to the gel. The preliminary data was obtained at the hyaluronan to gelatin ratio of 1:1. Heparin was added to 0.3% final concentration of HA-gelatin (1:1) mixture for HA-Gtn-HF-DTPH curve (red color). The same ratios are used herein. The inhibitor is added into the mixture at a concentration of 0.9-1.8 mg/ml, which equals a constant release rate of 1-2 ng per day to local tissue when 0.1 ml myostatin inhibitor-loaded hydrogels are implanted in vivo. The 1 ml of hydrogel can be used for 10-20 mice, and a sample of 24 experimental mice (12 male, 12 female) treated with inhibitor-loaded hydrogel and 24 control mice (12 male, 12 female) with hydrogel carrying no peptide is used. Surgery (fibular osteotomy) is performed and approximately 60 microliters of hydrogel carrying either saline (control) or myostatin inhibitor delivered locally into the site of injury. A dose of 1-2 ng/ml was chosen for local delivery based on data from systemic administration, the weight of the mice, and the size of the bone defect.

The release profile of inhibitor from 1 ml hydrogel is expected to attain 10-20 ng release per day in 1 ml 0.1M phosphate-buffered saline (PBS). The release profile is examined using a mass spectrometer (MCN Core Genomics facility), as ELISA assays for the inhibitor are not available. PBS conditioned by the inhibitor-loaded hydrogel is collected after 10 days. The bioactivity of the inhibitor is validated by treating C2C12 myotubes with PBS conditioned by the inhibitor-loaded hydrogel. C2C12 cells are obtained from the American Type Culture Collection (ATCC Number CR1-1772), and are cultured in differentiating medium (DMEM+10% horse serum) for 7 days in the presence of either hydrogel-conditioned PBS or PBS alone. C2C12 cells treated with inhibitor-conditioned PBS shows increased differentiation and proliferation, measured as increased cell number, after 7 days compared to cells treated with PBS alone.

Efficacy of Local Delivery of a Myostatin Inhibitor Using Degradeable Nanobeads

Fabricated scaffolds, such as hollow fiber membranes, loaded with therapeutic agents using a wet-phase inversion technique are used. Biodegradable nanobeads loaded with therapeutic molecules have also been fabricated using a water-in-oil method combining vigorous sonication and low temperature slow emulsion. Using these two methods, many therapeutic agents have been loaded into the scaffolds, microbeads, or nanobeads. 4-nitrophenyl-β-D-xylopyranoside (PNPX), cyclic adenosine monophosphate (cAMP), prolyl hydroxylase inhibitors (PHIs), and brain-derived neurotrophic factor (BDNF) have been loaded into either
nanobeads, microbeads, liposome, or porous scaffolds. The release profiles of these loaded molecules were quantitatively characterized in saline at 37°C using either high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), and other assays such as mass spectrometry. Four weeks of steady release can be achieved with degradable scaffolds, microbeads and nanobeads. Released molecules maintain their bioactivities. As shown in FIGS. 5A-5D, PN-5X loaded hollow fiber scaffold can suppress giant scar formation, and PEG (such as Dimethyloctadecylglycine (DMOG) or ethyl-3,4-dihydroxybenzoate (EDH(B)) loaded nanoparticle can suppress fibrous scar formation.

[0056] Inhibitor-loaded PLGA nanobeads are prepared by a water-in-oil method combining vigorous sonication and low-temperature slow emulsion, following the techniques utilized previously. The nanoparticles are examined using a transmission electron microscopy (TEM; JEOL Inc., Japan). The particle size distribution is determined by measuring the size of each nanobead in TEM images using ImagePro. A local constant release of 1-2 ng per day is used, which will require loading 10-20 mg of myostatin into 100 mg nanobeads. Surgery, sample size, and tissue collection is described above. Approximately 1 mg of nanoparticles in 0.1 ml saline is delivered to the injury site, and mice are sacrificed for tissue collection 4 weeks later.

[0057] The release profile is examined using mass spectrometry. The release profile of inhibitor from 1 mg nanoparticles is expected to attain 1-2 ng release per day in 1 ml. 0.1M PBS. Biocytivation of the inhibitor released from nanoparticles into saline after a period of 10 days is validated by treating C2212 myotubes with inhibitor-conditioned PBS.

[0058] Hyaluronan (HA) lacks immunogenicity but since hyaluronic acid alone is unable to give hydrogels, chemical reactions must be carried out in order to obtain chemically cross-linked networks. Using an established modification method, dithiobis(propanoic dithiodyrazide) (DTP) and dithio
tetraacetic acid (DTB) are coupled to the carboxylic acid moieties of hyaluronic acid with carbodiimide chemistry, with disulfide bonds of the initially formed gel being reduced with diithothreitol to give the corresponding thiol-modified macromolecular derivatives. The modification is very mild and biocompatible and does not cause changes in polysaccharide structure. Using a similar approach, recombinant human gelatin can be thiol-modified. The risk of eliciting an immune response is eliminated with human recombinant gelatin molecules that are based on human sequences of collagen Type I alpha chains. If human recombinant gelatin is a concern, one can use Arg-Gly-Asp (RGD) adhesion peptides to replace gelatin using the same chemistry. It is well known that RGD is not immunogenic. Poly(ethylene glycol) (PEG) serves as a crosslinker to crosslink the thiol groups in modified HA and gelatin and promotes the hydrogel formation. Both thiol-modified hyaluronan and recombinant human gelatin are not immunogenic.

[0059] Diffusion of the peptide outside the local region of interest has been a problem with similar products used in bone repair, such as InFuse (rhBMP2); however, the molecular weight of InFuse is around 30 kDa (dimer and glycosylated), whereas the inhibitors being used herein are much larger (propeptide fusion 120 kDa, dimer and glycosylated, and soluble receptor fusion 110 kDa, dimer and glycosylated) and therefore have less potential for local migration. The other concern regarding InFuse is its actions at unwanted sites. InFuse promotes target cell differentiation into osteoblast cells which can be a problem in areas other than the target site. Inhibiting myostatin will promote muscle growth and is tissue specific. It might actually be advantageous if the inhibitor increases muscle development outside of the intended area, since this may aid in the recovery process by compensating for the loss of muscle function due to injury.

[0060] The following references are cited herein.

What is claimed is:

1. A method of regenerating injured musculoskeletal tissue, comprising:
   contacting the injured tissue with a myostatin inhibitor,
   wherein said contact results in musculoskeletal tissue regeneration by inhibition of myostatin function.
2. The method of claim 1, wherein the myostatin inhibitor is myostatin propeptide.
3. The method of claim 1, wherein the myostatin inhibitor is a soluble myostatin receptor.
4. The method of claim 1, wherein said myostatin inhibitor is delivered to said injured musculoskeletal tissue via a biodegradable delivery system.
5. The method of claim 4, wherein said delivery system is a biodegradable hydrogel.
6. The method of claim 5, wherein said biodegradable hydrogel comprises hyaluronan-gelatin and heparin.
7. The method of claim 4, wherein said delivery system is biodegradable nanobeads.
8. The method of claim 1, wherein said injured musculoskeletal tissue is a fractured bone.
9. A method of treating a fracture in an individual comprising:
   administering a pharmacological amount of a myostatin inhibitor effective to inhibit myostatin function, thereby treating the fracture.
10. The method of claim 9, wherein the myostatin inhibitor is myostatin propeptide.
11. The method of claim 9, wherein the myostatin inhibitor is myostatin receptor.
12. The method of claim 9, wherein said myostatin inhibitor is delivered to the fractured bone via a biodegradable delivery system.
13. The method of claim 12, wherein said delivery system is a biodegradable hydrogel comprising hyaluronan-gelatin and heparin.
14. The method of claim 12, wherein said delivery system is biodegradable nanobeads.
15. The method of claim 9, wherein said fracture is a nonunion fracture.
16. A method of treating a fracture in an individual comprising:
   contacting said fracture with a biodegradable delivery vehicle containing a pharmacological amount of a myostatin inhibitor effective to inhibit myostatin function, thereby treating the fracture.
17. The method of claim 16, wherein the myostatin inhibitor is myostatin propeptide.
18. The method of claim 16, wherein said delivery system is a biodegradable hydrogel comprising hyaluronan-gelatin and heparin.
19. The method of claim 16, wherein said delivery system is a biodegradable nanobead.
20. The method of claim 16, wherein said fracture is a nonunion fracture.
21. A method of treating a nonunion fracture in an individual comprising:
   contacting said fracture with a biodegradable delivery vehicle containing a pharmacological amount of a myostatin propeptide effective to inhibit myostatin function, thereby treating the fracture.
22. The method of claim 21, wherein the biodegradable delivery vehicle comprises biodegradable nanobeads.
23. A biodegradable composition containing a pharmacological amount of myostatin propeptide.
24. The composition of claim 23, wherein said composition is a hydrogel.
25. The composition of claim 24, wherein said hydrogel comprises hyaluronan-gelatin and heparin.
26. The composition of claim 23, wherein said composition is a nanobead.

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