(54) USE OF FIBROBLAST GROWTH FACTORS TO STIMULATE BONE GROWTH

VERWENDUNG VON FIBROBLASTWACHSTUMSFAKTOREN ZUR STIMULIERUNG DES KNOCHENWACHSTUMS

UTILISATION DES FACTEURS DE CROISSANCE DE FIBROBLASTE POUR STIMULER LA CROISSANCE OSSEUSE

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(56) References cited:

- MAYAHARA ET AL: "In vivo Stimulation of Endosteal Bone Formation by Basic Fibroblast Growth Factors in Rats" GROWTH FACTORS, vol. 9, 1993, pages 73-80, XP000917937

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Description

Background of the Invention

[0001] The present invention relates to the use of fibroblast growth factors as therapeutic agents for enhancing periosteal bone formation.

Field of the Invention

[0002] Living bone tissue is continuously being replenished by the processes of resorption and deposition of bone matrix and minerals. This temporally and spatially coupled process, termed bone remodeling, is accomplished largely by two cell populations, the osteoclasts and osteoblasts. The remodeling process is initiated when osteoclasts are recruited from the bone marrow or the circulation to the bone surface and remove a disk-shaped packet of bone. The bone matrix and mineral is subsequently replaced by a team of osteoblasts recruited to the resorbed bone surface from the bone marrow. Among the pathological conditions associated with abnormal bone cell function is osteoporosis, a disease characterized by reduced amounts of bone (osteopenia) and increased bone fragility. These changes can be the result of increased recruitment and activity of osteoclasts, often in combination with reduced recruitment or activity of osteoblasts. It is believed that the development of excess or deficient populations of osteoclasts or osteoblasts may result from a corresponding lack or excess of specific protein factors called cytokines.

[0003] Cytokines have been identified by their biological characteristics and their unique amino acid sequences. Each cytokine presents a unique spectrum of characteristics that distinguish it from other cytokines. In general, the cytokines stimulate the growth and/or differentiation of specific types of cells. Some cytokines can also target cancerous cells for destruction. Examples of cytokines include granulocyte-colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), macrophage CSF (M-CSF), interleukin-1 beta, interleukin-3, interleukin-6, interferon-gamma, tumor necrosis factor, lymphotoxin, leukemia inhibitory factor, fibroblast growth factors, transforming growth factor-alpha and transforming growth factor-beta.

[0004] Many of the known cytokines stimulate or inhibit blood cells. Several growth regulatory cytokines, such as M-CSF, transforming growth factor alpha, interleukin-1 and tumor necrosis factor, have been shown to stimulate marrow mononuclear cell proliferation. Although cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and interleukin-6 (IL-6) may influence osteoclast formation and differentiation (Mundy (1990) Trends Endo. Metab. 1: 307-311), these factors are not specific osteoclast growth regulatory factors.

[0005] Although there is much information available on the factors that influence the breakdown and resorption of bone, information is more limited on factors that can actually stimulate the formation of new bone. Bone itself contains factors that have the capacity for stimulating the growth and/or differentiation of bone cells. Thus, extracts of bone tissue contain not only structural proteins that are responsible for maintaining the structural integrity of bone, but also biologically active bone growth factors that stimulate bone cells to proliferate. Among the growth factors found in bone that are known to stimulate proliferation of bone cells are transforming growth factor β, the insulin-like growth factors (insulin-like growth factor I and insulin-like growth factor II), basic fibroblast growth factor (bFGF) and bone morphogenetic proteins (BMPs). These factors also cause proliferation of non-bone cell types.

[0006] The fibroblast growth factor (FGF) family is comprised of at least 9 structurally related proteins (FGFs 1-9), whose best known members are acidic FGF (aFGF; FGF-1) and basic FGF (bFGF; FGF-2). Members of this family stimulate mitogenesis in most cells derived from the mesoderm and neuroectoderm and influence other biological processes, including angiogenesis, neurite extension, neuronal survival, and myoblast differentiation. In general, FGFs have a high affinity for heparin (prior to resolution of their nomenclature, some FGFs were referred to as heparin-binding growth factors -1, -2, etc.), and many, but not all, are mitogens for fibroblasts. The members of the FGF family possess roughly 25-55% amino acid sequence identity within a core sequence and some FGFs possess significant extensions, either C-terminal, N-terminal, or both, outside of this core sequence. This structural homology suggests that the 9 different genes encoding known FGFs may be derived from a common, ancestral gene.

[0007] In addition to the 9 known members of the FGF family, additional complexity results from the generation of several molecular forms of FGF from a single gene. For example, the primary translation product of aFGF (FGF-1) consists of 155 residues. However, the longest form of FGF-1 found in a natural source (e.g., bovine brain) consists of 154 residues. This 154 residue form of FGF-1 lacks the NH2-terminal methionine of the 155 residue form and has an acetylated amino terminus. Proteolytic processing in vivo or during purification generates smaller active forms of FGF-1 in which either the amino-terminal 15 (des 1-15) or 21 (des 1-21) amino acids are deleted. As defined herein, FGF-1 refers to the 154 residue form of FGF-1 and shorter, biologically active forms thereof, such as the above described forms deleted of the amino-terminal 15 (des 1-15) or 21 (des 1-21) amino acids. Historically, the 154 residue
form of FGF-1 was termed β-endothelial cell growth factor (β-ECGF), the des 1-15 form was termed α-ECGF. Prior to standardization of the terminology for this group of growth factors, several additional terms were also applied to the same protein, including eye derived growth factor and heparin binding growth factor 1. Similar forms of bFGF (FGF-2) have also been described. In addition to cleaved forms, extended forms of bFGF have also been described, resulting from initiation of translation at several different GTG codons located upstream of the ATG translation initiation codon which generates the 155 residue form of bFGF. All of these alternative forms of the FGFs contain the core region of structural homology which defines the FGF family.

Reported Developments

[0008] An osteogenic role for bFGF was suggested based on in vitro studies (Hauschka et al., J. Biol. Chem 261: 12665-12674, 1986; Globus et al., Endocrinology 123:98-105, 1988; Canalis et al. J. Clin. Invest. 81:1572-1577, 1988; McCarthy et al., Endocrinology 125:2118-2126, 1989; Noff et al., FEBS Lett, 250:619-621 Y 1989). However, there has been only one report of in vivo administration of bFGF (Mayahara et al., Growth Factors 9:73-80, 1993). Intravenous administration of human bFGF to rats demonstrated only endosteal new bone formation. No increase in periosteal bone formation was evidenced. Similar systemic osteogenic potential was seen after intravenous administration of human aFGF.

Summary of the Invention

[0009] The present invention refers to the use of FGF to manufacture a medicament for treating patients suffering from pathological conditions in which bone mass is inadequate or in repairing defects in bone or dental tissue. The pharmaceutical compositions comprise certain fibroblast growth factors in amounts which stimulates the proliferation and/or differentiation of osteoblasts and/or precursors to promote bone anabolism. Preferably the FGFs are to be applied locally to stimulate periosteal bone formation. The fibroblast growth factors preferred in the practice of the present invention are FGF-1 and FGF-2, and, most preferably, full-length FGF-1 (ECGF β) and FGF des 1-21 (ECGF α).

[0010] Other and further objects, features and advantages will be apparent from the following description of the preferred embodiments of the invention given for the purpose of disclosure when taken in conjunction with the following drawings.

Brief Description of the Figures

[0011] Figure 1 is a photomicrograph demonstrating new bone growth in the calvaria of the nude mouse adjacent to WISH tumor cells.

Figure 2 is a photomicrograph demonstrating new periosteal bone growth in the mouse calvaria after administration of acidic FGF (panel A) and basic FGF (panel B).

Figure 3 demonstrates the effects of acidic and basic FGF on calvaria bone growth.

Figure 4 demonstrates the time response of bone growth to aFGF.

Figure 5 demonstrates the time response of aFGF on periosteum proliferation and osteoblast differentiation to aFGF.

Figure 6 demonstrates the dose response of the effects of aFGF and bFGF on bone formation of the calvaria.

Figure 7 demonstrates the effects of daily administration of aFGF on tibial bone loss in ovariectomized rats.

Figure 8 demonstrates the effects of daily administration of aFGF on vertebral bone density in ovariectomized rats.

Figure 9 demonstrates the levels of serum fluoride following methoxyflurane administration.

Figure 10 demonstrates the effects of cyclic administration of aFGF on bone density in rats.

Figure 11 demonstrates the effects of aFGF on bone mass in ovariectomized rats.

Description of the Preferred Embodiments

[0012] A preferred aspect of the present invention provides a use of FGF for stimulating periosteal bone formation, useful in treatment of pathological conditions in which bone mass is inadequate or in repairing defects in bone and dental tissue. The present invention is useful in enhancing periosteal bone formation following local application to promote a net increase in cortical bone.

The invention encompasses the use of naturally occurring fibroblast growth factors in partially purified, as well as substantially homogeneous, form, as well as synthetically or recombinantly produced fibroblast growth factors, biolog-
Definitions:

[0013] "Substantially purified" is used herein as "substantially homogeneous" which is defined as a proteinaceous material which is substantially free of compounds normally associated with it in its natural state (e.g., other proteins or peptides, carbohydrates, lipids). Most preferably, it means a polypeptide, which may be glycosylated or non-glycosylated, which is characterized by a single molecular weight and/or multiple set of molecular weights, chromatographic response and elution profiles, amino acid composition and sequence and biological activity. "Substantially purified" is not meant to exclude artificial or synthetic mixtures with other compounds. The term is also not meant to exclude the presence of impurities which do not interfere with biological activity, and which may be present, for example, due to incomplete purification or compounding with a pharmaceutically acceptable preparation.

[0014] The term "animal" includes, mammals, such as dogs, cats, horses, cows, pigs, rates, mice, simians, and humans.

[0015] The term "biologically active polypeptide" means naturally occurring polypeptide per se, as well as biologically active analogues thereof, including synthetically produced polypeptides and analogues thereof, as well as natural and pharmaceutically acceptable salts and pharmaceutically acceptable derivatives thereof.

[0016] The term biologically active polypeptide also encompasses biologically active fragments thereof, as well as biologically active sequence analogues thereof. Different forms may exist in nature. These variations may be characterized by differences in the nucleotide sequence of the structural gene coding for proteins of identical biological function.

[0017] The term "biologically active sequence analogue" includes naturally and non-naturally occurring analogues having single or multiple amino acid substitutions, deletions, additions, or replacements. All such allelic variations, modifications, and analogues resulting in derivatives which retain one or more of the native biologically active properties are included within the scope of this invention.

[0018] The term "FGF-1" encompasses the 154 residue form of FGF-1 acidic growth factors (also known as ECGF \( \beta \)), the des 1-15 form and the des 1-21 form (also known as ECGF \( \alpha \)). FGF-1 may be prepared by methods known in the art. Preferably, the FGF-1 used in the present invention is prepared by recombinant technology, such as disclosed in U.S. Patent No. 4,868,113.

[0019] To purify the polypeptides useful in practicing the present invention, either from natural sources or after recombinant production, chromatographic procedures may be carried out, for example in a narrow bore column containing a fine particle resin under increased pressure to enhance the effectiveness of separation, i.e., by high pressure liquid chromatography.

[0020] Concentration and salt removal are commonly used steps in certain chromatographic or separation techniques employed in the invention.

[0021] Salt removal is generally necessary if ion exchange or other techniques which depend on ionic strength are employed. Salt removal may be performed by, for example, dialysis or gel filtration or by control pore glass (CPG) chromatography.

[0022] A number of gel filtration and concentration techniques are also used. Certain commercially available materials are especially useful. Sephacryl, Sephadex, and Bio-Gel are examples of gel filtration media commonly used to isolate and purify proteins and characterize their physical properties.

[0023] The invention encompasses using fibroblast growth factors, preferably FGF-1, bFGF and new forms of basic FGF isolated from WISH cells, in treatment of bone diseases associated with reduced skeletal mass or defective or deficient bone formation. Acidic FGF and basic FGF stimulate the growth of human cells with the osteoblast phenotype and their precursors. In one embodiment, the present invention provides a use for the treatment of human and animal disorders characterized by an abnormally reduced level of osteoblastic cell proliferation.

[0024] Administration of the fibroblast growth factors to effect the therapeutic objectives of the present invention may be by local, parenteral, intravenous, intramuscular, subcutaneous, rectal or any other suitable means. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the bone pathology being treated.

[0025] The compositions of the present invention may be employed by administering fibroblast growth factors in such forms as liquid solutions, suspensions, elixirs, or sterile liquid forms. Suitable carriers include diluents or fillers, sterile aqueous media and various non-toxic organic solvents. The compositions may be formulated in the form of powders, aqueous suspensions, or solutions, injectable solutions, elixirs, syrups and the like and may contain one or more stabilizing agents such as human serum albumin, sugar (including, but not limited to, glycosaminoglycans such as heparin or heparin fragments) or amino acid, antibacterial, and preserving agents in order to provide a pharmaceutically acceptable preparation. Any inert carrier is preferably used, such as saline, or phosphate-buffered saline, or any such carrier in which the factors used in the method of the present invention have suitable solubility properties. Carriers...
such as devitalized bone powder, hydroxyapatite or fibrin sealant may also be used to practice the present invention.

The particular carrier and the ratio of active compound to carrier are determined by the solubility and chemical properties of the proteinaceous factors, the particular mode of administration and standard pharmaceutical practice. For parenteral administration, solutions or suspensions of these factors in aqueous alcoholic media or in sesame or peanut oil or aqueous solutions of the soluble pharmaceutically acceptable salts can be employed.

The dosage regimen in carrying out the invention is that which insures maximum therapeutic response until improvement is obtained and thereafter the minimum effective level which gives relief. Doses may vary, depending on the age, severity, body weight and other conditions of the patients but are ordinarily in the area of 0.1 mg/kg to 500 mg/kg, preferably 1 mg/kg to 250 mg/kg, and most preferably 5 mg/kg to 100 mg/kg of body weight in injectable form; such may, of course, be given in divided doses. With other forms of administration equivalent or adjusted doses will be administered depending on the route of administration.

The following examples describe the isolation, purification and measurement of biological activity of the fibroblast growth factors and their use as therapeutic agents for the prevention and treatment of pathological conditions of bone defects and injuries.

Example 1

Assay of Proliferative Effects of Fibroblast Growth Factors on Osteosarcoma Cells and Osteoblast Bone Cells

The proliferative effects of fibroblast growth factors on MG-63 human osteosarcoma cells and MC3T3 bone cells is assessed by measuring the incorporation of tritiated thymidine into cellular DNA.

MG-63 human osteosarcoma cells, obtained from the American Type Culture Collection (Rockville, MD), are cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). MC-3T3EI mouse osteoblasts, provided by Dr. T. Suda (Showa University, Tokyo), are cultured in alpha modification of Eagle's medium (αMEM) supplemented with 10% FBS. All cultures are maintained at 37°C, in a humidified atmosphere of 5% CO2 in air.

MG-63 and MC3T3 cells are seeded at 5×10³ cells/100 µl/well in 96 well plates in EMEM or αMEM, respectively, supplemented with 10% FBS. After incubation for approximately 18-24 hours, the cells are washed with 200 µl/well of phosphate buffered saline (PBS), re-fed with 50 µl/well of serum free 0.1% BSA in EMEM or αMEM and 50 µl/well of a sample containing growth factor activity diluted in 0.1% BSA in DMEM/F12 is added. Baseline controls (50 µl/well of 0.1% BSA in DMEM/F12 50/50) as well as positive controls (50 µl/well of 20% FBS in DMEM/F12 50/50) are run in each plate in parallel with the test sample. About 44 hours after the addition of the sample and controls, the cells are pulsed with 1 µCi/well of [methyl ³H] thymidine for 4 hours. After 4 hours, the cells are collected and rinsed on filter paper discs using a PHD cell harvester (Cambridge Technology, Watertown, MA). The radioactivity retained by the filters is measured as cpm using a liquid scintillation counter (Beckman, Fullerton, CA).

The proliferative activity of each sample (run in duplicate) is expressed as percentage of the stimulation of incorporation of ³H thymidine caused by the positive control, by using the formula:

\[
\text{Percentage of stimulation} = \left( \frac{\text{cpm sample} - \text{cpm baseline control}}{\text{cpm positive control} - \text{cpm baseline control}} \right) \times 100
\]

Example 2

Purification of the Biological Activity of Extended bFGF from WISH Cells

A. Growth of tumor in vivo

Human amniotic tumor cells (2×10⁷) were injected into the right hind limb area adjacent to the femoral shaft of fourteen 4 week old male nude mice. Solid tumors were isolated from the animals after 3-5 weeks. At this time, there was evidence of new bone growth as assessed radiologically and histologically. In 10 mice, tumor was closely adhered to the bone surface and in each of these there was evidence of new bone growth. New bone growth was easily recognized by its woven, rather than lamellar, structure, and was extensive on periosteal bone surfaces adjacent to the tumor mass. Solid tumor was removed from the bone and immediately frozen in liquid nitrogen for subsequent protein purification. For quantitative histomorphometry, the cells (1×10⁷ cells/0.2 ml PBS) were injected subcutaneously over the calvariae of nude mice (n=8). The mice were sacrificed after two weeks. In six mice, tumor closely adhered to the bone surface. Histological methods for preparing the bone samples were as described in Example 3 below. There was extensive new bone formation over the calvaria of these mice (Figure 1) and the cross-sectional area of the calvarial bone was increased 96 ± 37 (S.D.)%.
B. Isolation and purification of extended bFGF

[0034] Twenty-five grams of solid tumor tissue isolated from femora of tumor-bearing mice were pulverized in liquid nitrogen and extracted by stirring at 4°C for 72 hours in 125 ml of extraction buffer containing 10 mM EDTA, 50 mM Tris- HCl pH 7.0, 1.5 M NaCl, 25 mM benzamidine, 1 µg/ml each of leupeptin and aprotinin, and 1 mM PMSF (dissolved in 100% isopropanol), adjusted to a final pH of 7.0. After 72 hours, the extract was centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was removed and frozen at -70°C.

[0035] A second extraction of the insoluble precipitate from the initial extraction was performed in the same buffer by adding 250 ml of fresh extraction buffer to the pellet and stirring the mixture at 4°C for another 24 hours. After this period, the extract was centrifuged at 6000 rpm for 20 min at 4°C. Supernatants from the first and second extractions were combined and centrifuged at high speed at 15,000 g for 1 hr at 4°C. The supernatant was removed, and dialyzed against 10 mM Tris- HCl, 0.1 M NaCl, pH 7.0. Protein concentration was determined by the absorbance at 280 and 260 nm.

[0036] A 4.8 x 30 cm column was packed with heparin Sepharose CL-6B and equilibrated with a buffer containing 0.1 M NaCl, 10 mM Tris- HCl, pH 7. The final extract (approx. 200 ml) was applied to the column. The column was washed with the same buffer and eluted with a linear gradient from 0.4-3.0 M NaCl. Twelve ml fractions were collected. Aliquots of each fraction were assayed in the MG-63 and MC3T3 proliferative assays described in Example 1. Fractions containing MG-63 proliferative activity eluted at 1.5 M NaCl and were rechromatographed on heparin-Sepharose under the same conditions. Fractions containing MG-63 proliferative activity eluted at 1.8 M NaCl. These fractions (34-44) were pooled, adjusted to 0.05% CHAPS ([3-(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; a mild zwitterionic detergent) and dialyzed against 50 mM sodium phosphate pH 6.0, 0.05% CHAPS. The dialysate was chromatographed on a MonoS FPLC column in a gradient from 0 to 1.0 M NaCl in 50 mM sodium phosphate pH 6.0, 0.05% CHAPS. Fractions of 0.8 mls were collected. The MG.63 positive fractions (26-32) were pooled and injected into a C4 or C18 HPLC column and chromatographed in a gradient from 7% to 63% acetonitrile in 0.1% TFA. For biological activity measurements, 10% of the material was run in a C18 RPHPLC column and the fractions were collected in a neutralizing solution (24 mM ammonium bicarbonate pH 8, 0.1% BSA, 2 µg/ml heparin and 0.01% CHAPS). Protein eluting at 37% acetonitrile was subjected to SDS-PAGE (15% gel), and transblotted to a nitrocellulose membrane (0.45 µm, Schleicher & Schuell, Keene, NH). Amino acid sequence was obtained by automated Edman degradation using an Applied Biosystems Model 477A protein sequencer equipped with an online Model 120A PTH Analyzer.

[0037] The amino acid sequence of the polypeptide isolated and purified from the WISH cells is similar, but not identical, to the amino acid sequence of bFGF. The novel sequence appears to define a heterofore unknown extended form of basic FGF, having the additional amino acid sequence GSRPGAGT (SEQ ID NO: 1) extending the N-terminal end MAAGSIT (SEQ ID NO: 2) of the known 18 kD form of bFGF.

Example 3

In Vivo Assay of Effects of Polypeptides on Bone Growth

[0038] The effects of the polypeptide factors on bone growth were tested in male ICR Swiss white mice, aged 4-6 weeks and weighing 12-26 gms using 4-5 mice per group. The polypeptide or a control vehicle was injected into the subcutaneous tissue over the right calvarium of normal mice. Among the polypeptides tested were the crude extract from WISH tumors containing the extended form of bFGF isolated as described in Example 2, basic FGF and FGF-1 which comprises acidic FGF. These test proteins were injected either alone or together with heparin.

[0039] Unless otherwise specified, the control vehicle was PBS supplemented with 1% BSA. Heparin was administered at a dose of 50 units/ml. The animals were sacrificed on day 14 and bone growth measured by histomorphometry.

[0040] Bone samples for quantitation were cleaned from adjacent tissues and fixed in 10% buffered formalin for 24-48 hours, decalcified in 14% EDTA for 1-3 weeks, processed through graded alcohols and embedded in paraffin wax. Three µm sections of the calvaria and femurs were prepared. Representative sections were selected for histomorphometric assessment of the effects of the tumors, tumor extracts or fibroblast growth factors comprising acidic or basic FGF on bone formation and bone resorption. Sections were measured by using a camera lucida attachment to directly trace the microscopic image onto a digitizing plate. Bone changes were measured on sections collected 200 µm apart, over 4 adjacent 1X1 mm fields on both the injected and noninjected sides of the calvaria. New bone was identified by its woven, rather than lamellar structure, and osteoclasts and osteoblasts were identified by their distinctive morphology. Histomorphometry software (Osteo measure, Osteometrix, Inc., Atlanta) was used to process digitizer input to determine cell counts and feature areas or perimeters.

[0041] To identify whether mineralization of newly formed bone occurred normally, calvaria bone samples were also taken after animals were administered two doses of tetracycline (25 mg/kg i.p.). Tetracycline is deposited at the sites of active bone formation and functions as a timed-tissue marker to calculate bone formation rates and assess miner-
alization. These bone samples were fixed as described above, processed through graded alcohols and embedded undecalcified in a methacrylate based plastic to preserve the tetracycline labels. Sections were cut at five µm intervals and were assessed for the degree of mineralization and for tetracycline uptake. The results of these experiments are described in Example 4.

Example 4

In Vivo Assay of Effects of Fibroblast Growth Factors on Bone Growth

[0042] The effects of the polypeptide factors on bone growth were tested in male ICR Swiss white mice, aged 4-6 weeks and weighing 13-26 gms using 4-5 mice per group. The polypeptide or a control vehicle is injected into the subcutaneous tissue over the calvarium of normal mice. Among the polypeptides tested were the extended bFGF isolated from WISH cells as described in Example 2, full length (155 residue) fibroblast growth factor-1 and basic FGF. These test proteins were injected either alone or together with heparin.

[0043] In one experiment, the control vehicle was PBS supplemented with 1% BSA. Heparin was administered at a dose of 50 units/ml. Table 1 summarizes the results of four, when included, injections per day for 3 days of either vehicle alone, vehicle supplemented with heparin, 5 µg of IGF1 or IGF2, 1 µg of aFGF together with heparin, and crude extract from the first ("WISH crude") and second ("WISH partially purified") extractions of WISH cells. The animals were sacrificed on day 14 and bone growth measured by histomorphometry.

1All experiments utilizing aFGF or FGF-1 herein utilize the 154 amino acid form of the protein, also termed ECGF-B. The terms aFGF and FGF-1 are used interchangeably throughout this application and in the Figures attached hereto.
Table 1b

<table>
<thead>
<tr>
<th></th>
<th>PBS+BSA n=5</th>
<th>PBS+BSA +Heparin n=5</th>
<th>IGF-1 5µg/inj n=5</th>
<th>IGF-2 5µg/inj n=5</th>
<th>aFGF+hep 1µg/inj n=5</th>
<th>WISH crude n=5</th>
<th>WISH pp n=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. New Bone Width %</td>
<td>12.3±1.8</td>
<td>14.4±4.1</td>
<td>19.9±2.6</td>
<td>17.4±1.4</td>
<td>35.1±9.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.4±1.5</td>
<td>13.7±1.6</td>
</tr>
<tr>
<td>New Bone (% total bone)</td>
<td>1.8±0.5</td>
<td>2.6±0.8</td>
<td>3.2±0.6</td>
<td>3.3±0.8</td>
<td>20.3±3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±0.6</td>
<td>2.9±0.7</td>
</tr>
<tr>
<td>Osteoblasts (% periosteal surface)</td>
<td>20.7±7.0</td>
<td>25.3±4.5</td>
<td>20.4±2.1</td>
<td>30.2±1.5</td>
<td>93.0±3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.2±6.9</td>
<td>34.3±6.9</td>
</tr>
<tr>
<td>Osteoclasts/mm&lt;sup&gt;2&lt;/sup&gt; Bone Area</td>
<td>7.9±1.8</td>
<td>6.4±2.2</td>
<td>9.7±0.9</td>
<td>8.8±1.0</td>
<td>10.5±2.6</td>
<td>8.1±2.8</td>
<td>8.5±2.0</td>
</tr>
<tr>
<td>Marrow Area (% total)</td>
<td>6.8±2.2</td>
<td>4.5±1.7</td>
<td>6.0±1.3</td>
<td>6.6±1.6</td>
<td>4.9±1.2</td>
<td>6.4±1.3</td>
<td>7.2±0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Statistically significant as compared to PBS+BSA+heparin, p<0.001 (Bonferroni t-test).

<sup>b</sup> Data expressed as mean ± S.E.
As demonstrated on Table 1, FGF-1, injected with heparin over the calvaria of mice, was able to induce new periosteal bone formation. In this experiment no other treatments produced significant new bone formation relative to the control. The proliferative activity of injected WISH extracts, administered in the absence of heparin, was probably only equivalent to 10-100 ng of FGF. There was no evidence of increased bone resorption in any of the treated groups.

In order to further examine the effect of FGF's on bone growth and the effect of heparin on the stimulation of bone growth, 1 µg of FGF-1 or bFGF in the presence of absence of heparin, in a volume of 10 µl, were administered to Swiss ICR white male mice aged 4-6 weeks (five for each treatment group) by injection over the calvaria 4 times per day for 3 days. The control vehicle was 0.1% BSA in PBS. Heparin was administered at a dose of 50 units/ml. Demeclocycline (25mg/Kg) was given IP on days 4, 8 and 12 to calculate bone formation rates and assess mineralization.

The mice were sacrificed on day 14 and the posterior half of the calvaria was decalcified and paraffin embedded. The anterior half of the calvaria, the tibial and femoral metaphysis and lumbar vertebra were embedded undecalcified in plastic. Mice receiving aFGF and bFGF showed extensive new bone formation over the upper surface of the calvaria (Figure 2). No effects were detected in the femoral metaphysis or the vertebra. The new bone formed in the more recently formed superficial layers. It was well mineralized and showed diffuse uptake of the demeclocycline labels, typical of woven bone.

Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Total Bone Area (mm²)</th>
<th>Max. New Bone Width (% orig.)</th>
<th>% New Periosteal Bone</th>
<th>% New Endosteal Bone</th>
<th>Osteoblasts (% periosteal surface)</th>
<th>Osteoclasts/mm² Bone Area</th>
<th>% Marrow Space in Original Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.76±.06</td>
<td>26±3</td>
<td>3.6±0.5</td>
<td>0</td>
<td>33±5</td>
<td>20.3±3.3</td>
<td>7.1±2.1</td>
</tr>
<tr>
<td>PBS+ heparin</td>
<td>0.79±.02</td>
<td>26±2</td>
<td>4.0±0.8</td>
<td>0</td>
<td>35±10</td>
<td>14.8±6.2</td>
<td>6.6±1.6</td>
</tr>
<tr>
<td>aFGF</td>
<td>0.98±.05</td>
<td>62±10*</td>
<td>20.4±6.1*</td>
<td>3.9±1.4</td>
<td>71.2±10.3</td>
<td>10.7±1.6</td>
<td>4.25±1.0</td>
</tr>
<tr>
<td>aFGF+ heparin</td>
<td>1.04±.02</td>
<td>74±5*</td>
<td>29.1±4.2*</td>
<td>1.3±0.9</td>
<td>76.8±9.7*</td>
<td>18.7±3.6</td>
<td>8.27±2.0</td>
</tr>
<tr>
<td>bFGF</td>
<td>1.26±.06</td>
<td>122±20*</td>
<td>59.6±9.3*</td>
<td>24.2±6.3*</td>
<td>81.8±10.3*</td>
<td>19.4±2.2</td>
<td>2.97±1.0</td>
</tr>
<tr>
<td>bFGF+ heparin</td>
<td>1.12±.06</td>
<td>118±20*</td>
<td>46.9±2.7*</td>
<td>1.1±1.1</td>
<td>88.7±4.3*</td>
<td>33.0±4.4</td>
<td>5.62±2.2</td>
</tr>
</tbody>
</table>

**Mean ± S.E.  
*statistically significant difference as compared to control p< 0.01

Table 2 and Figure 3 demonstrate that both aFGF and bFGF stimulate the formation of new bone, increase osteoblast surface area, periosteal bone area and total bone area, both in the presence and absence of heparin. However, the increase in new bone under the calvaria (% new endosteal bone) effected by bFGF is inhibited by heparin (Figure 3).

The time course of the bone changes following stimulation by 1 ug/injection of acidic FGF given with heparin was examined. The experimental design was as described above, except eight animals were injected for each time point. Four were injected with vehicle alone and four with FGF-1 and heparin. The mice were sacrificed on days 3, 7, 14, 21 and 36 and bone changes measured at two levels at least 200 um apart.

Table 3

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>Total Bone Area (mm²)</th>
<th>New Bone Area % Original</th>
<th>Osteoblasts (% periosteal surface)</th>
<th>Prolif. Cell Width (µm)</th>
<th>Osteoclasts/mm² Bone Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>aFGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.85±0.04</td>
<td>2.0±0.5</td>
<td>45±10</td>
<td>23.7±2.8*a</td>
<td>18±3</td>
</tr>
<tr>
<td>7</td>
<td>1.06±0.06</td>
<td>13.2±2.9*a</td>
<td>78±10</td>
<td>21.7±3.1*a</td>
<td>12±3</td>
</tr>
</tbody>
</table>

*aStatistically different from control, p < 0.01
As can be seen on Table 3, FGF-1 produces a sustained stimulation of bone formation lasting 21 days after a three day pulse of treatment (Figure 4). The initial response is one of cell proliferation, with differentiation of osteoblasts and matrix formation occurring only after the 3 days of treatment (Figure 5). There is no evidence of enhanced osteoclast formation at any of the time periods examined.

The dose response of bone changes following four times daily stimulation by 0.5, 5.0, 50 and 500 ng/injection of FGF-1 or basic FGF given with and without heparin (50 U/ml) was also determined. Each treatment group comprised 4 Swiss ICR white male mice aged 4-6 weeks. Fourteen mice served as controls, 7 receiving vehicle alone (PBS + 0.1% BSA) and 7 receiving vehicle and heparin.

The mice were sacrificed on day 14, and the calvaria prepared as described above. Figure 6 demonstrates that both acidic FGF and basic FGF were active at the nanogram range in vivo. While bone responses were unchanged by heparin when administered alone, heparin enhanced the effects of both aFGF and bFGF particularly on the non-injected side of the calvaria.

Example 5

In vivo Effects of Systemic Administration of FGF-1 in the Ovariectomized Rat

The ovariectomized rat is accepted as an animal model of human post-menopausal osteoporosis. To assess the effects of systemic administration of aFGF on skeletal tissues in an animal model of acute bone loss related to estrogen deficiency similar to that seen in post-menopausal women, female Sprague-Dawley rats (250gms) were either sham-operated or surgically ovariectomized. Commencing seven days after surgery, rats were administered either vehicle (PBS), aFGF (0.2mg/kg i.v. via tail vein) or estrogen (160ug/kg s.c.) for 28 days. All injections were performed under methoxyflurane anesthesia. Prior to termination, all rats were administered single doses of tetracycline and demeclocyline to assess bone formation and mineralization. The tibias and lumbar vertebrae were removed, fixed, and processed, both decalcified and undecalcified, for histomorphometric evaluation.

Cancellous bone area (expressed as a % of tissue area) was quantitated on decalcified sections of tibial metaphysis and lumbar vertebrae. When compared with vehicle-treated ovariectomized rats, bone area in the region

Table 3 (continued)

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>Total Bone Area (mm²)</th>
<th>New Bone Area % Original</th>
<th>Osteoblasts (% periosteal surface)</th>
<th>Prolif. Cell Width (µm)</th>
<th>Osteoclasts/mm² Bone Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>aFGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.16±0.03ᵃ</td>
<td>32.1±3.0ᵃ</td>
<td>92±4ᵃ</td>
<td>15.5±1.8ᵃ</td>
<td>25±6</td>
</tr>
<tr>
<td>21</td>
<td>1.40±0.07ᵃ</td>
<td>52.4±7.9ᵃ</td>
<td>90±4ᵃ</td>
<td>11.8±2.1</td>
<td>22±3</td>
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<tr>
<td>36</td>
<td>1.50±0.12ᵃ</td>
<td>64.1±8.5ᵃ</td>
<td>46±16</td>
<td>10.9±1.9</td>
<td>10±5</td>
</tr>
<tr>
<td>control</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.83±0.06</td>
<td>0.1±0.1.</td>
<td>36±11</td>
<td>5.7±1.5</td>
<td>20±7</td>
</tr>
<tr>
<td>7</td>
<td>0.88±0.03</td>
<td>1.8±0.4</td>
<td>35±6</td>
<td>11±2.1</td>
<td>18±6</td>
</tr>
<tr>
<td>14</td>
<td>0.89±0.06</td>
<td>3.6±1.2</td>
<td>34±13</td>
<td>4.0±0.9</td>
<td>17±5</td>
</tr>
<tr>
<td>21</td>
<td>0.95±0.04</td>
<td>5.0±1.9</td>
<td>29±6</td>
<td>4.6±0.6</td>
<td>7±2</td>
</tr>
<tr>
<td>36</td>
<td>1.09±0.06</td>
<td>10.5±2.3</td>
<td>28±7</td>
<td>2.8±0.5</td>
<td>15±5</td>
</tr>
</tbody>
</table>

ᵃStatistically different from control, p < 0.01
of the secondary spongiosa of the tibia was significantly increased in both sham-operated rats administered vehicle or aFGF and in ovariectomized rats administered FGF-1 or estrogen (Figure 7). Similar but non-significant changes in cancellous bone area were observed in the lumbar vertebrae (Figure 8). These data indicate that FGF-1 blocked the cancellous bone loss in the secondary spongiosa associated with estrogen deficiency. Additionally, periosteal bone formation was increased in FGF-1 treated sham-operated rats.

Qualitative assessment of sections of the tibia and vertebrae revealed that daily injection of aFGF induced new woven bone formation in the vertebrae, tibial diaphysis, and tibial epiphysis. Evaluation of undecalcified sections revealed that aFGF produced new bone which was poorly mineralized. This mineralization defect was likely a result of fluoride accumulation from the daily exposure to fluoride-containing gas anesthetic. This is supported by the increase in serum fluoride observed in these rats (Figure 9).

To determine the response of the skeleton to cyclic administration of systemic FGF-1, 250 gm Sprague-Dawley rats were either sham-operated or surgically ovariectomized and were administered either vehicle or FGF-1 commencing 2 months post-surgery. Treatment was initiated 2 months following surgery because approximately 50% of the cancellous bone in the tibial metaphysis is lost in response to estrogen deficiency by this time. Rats were administered daily injections of either vehicle or FGF-1 (0.5mg/kg/day i.v.) for three days followed by 6 days without treatment. This regimen was repeated 3 times. Rats were terminated on day 28. Prior to termination, rats were administered single doses of tetracycline and demeclocycline. Tibias, femurs, and lumbar vertebrae were removed for histomorphometric and dual energy X-ray absorptiometric analyses.

Bone mineral density (BMD), an assessment of bone mass, was determined with a Lunar DEXA. BMD was increased in the tibial metaphysis, lumbar vertebrae, and femur of the aFGF-treated rats compared with the vehicle treated group (Figure 10). These data were corroborated by increases in cancellous bone area as determined by histomorphometric analysis in the tibial and femoral metaphyses in the FGF-1-treated rats (Figure 11). Qualitative assessment revealed that cyclic systemic administration of FGF-1 induced some woven bone formation in the long bone shafts but not in the vertebrae or tibial epiphysis. Evaluation of the tetracycline labelling revealed that the new bone formed in response to aFGF was mineralized normally. Serum fluoride was not elevated in these rats. These data indicate that aFGF can maintain and increase bone mass in a model with established and progressive osteopenia.

Example 6

Established post-menopausal osteoporosis is a disease characterized by reduced cancellous and cortical bone mass, low bone turnover, and reduced connectivity of vertebral cancellous bone. To assess the potential effects of FGF-1 on these alterations, FGF-1 is administered systemically for 28 days to aged ovariectomized rats, an animal model of established cortical and cancellous osteopenia, low bone turnover, and reduced connectivity of vertebral cancellous bone. Eight 10-month old retired breeder Sprague-Dawley rats were either sham-operated or surgically ovariectomized. Six months following surgery, either vehicle or FGF-1 (0.2mg/kg/day i.v.) was administered to ovariectomized rats. Sham-operated rats received vehicle alone. All injections were performed under isoflurane anesthesia. Half of the rats in each treatment group were terminated after 28 days of treatment. The remaining half is maintained an additional 28 days without treatment to determine the long-term consequences of FGF-1 treatment. Prior to termination, all rats received 2 injections of calcine green as a timed-tissue marker in bone to calculate bone formation rates. Femurs, tibias, and lumbar vertebrae were removed for DEXA and histomorphometric analysis as described above in Example 5. To perform biomechanical analyses, isolated lumbar vertebrae are compressed in a material testing machine at a constant compression rate to failure. Failure is designated as the point when the load deformation curve decreases after achieving maximal compressive strength.

DEGA analysis revealed that FGF-1 treatment caused a small but not significant increase in bone mineral density in femur, tibia and vertebrae at day 28. In the long bones, this increase is evident in the diaphysis and metaphysis, indicating an increase in both cortical and cancellous bone. Vertebral cancellous bone connectivity is likely to be increased in the FGF-1-treated rats at day 28 and 56. These changes will translate into an increase in vertebral compressive strength in the vertebrae from the FGF-1-treated rats. These data indicate that systemic administration of FGF-1 increased both cortical and cancellous bone mass and increases vertebral cancellous bone connectivity and strength in and animal model of established post-menopausal osteoporosis.

One skilled in the art will readily appreciate the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The peptides, antibodies, methods, procedures and techniques described herein are presented as representative of the preferred embodiments.
SEQUENCE LISTING

[0061]

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(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Macintosh
(C) OPERATING SYSTEM: System 7.1
(D) SOFTWARE: Word 5.0 (PatentIn)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/207,985
(B) FILING DATE: 08-MAR-1994

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Goodman, Rosanne
(B) REGISTRATION NUMBER: 32,534
(C) REFERENCE/DOCKET NUMBER: A1325-WO

(ix) TELECOMMUNICATION INFORMATION:

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(B) TELEFAX: (610) 454-3808

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
Claims

1. Use of a human fibroblast growth factor to manufacture a medicament for enhancing periosteal bone formation.

2. The use according to claim 1, wherein the human fibroblast growth factor is FGF-1.

3. The use according to claim 2, wherein said FGF-1 is selected from ECGFα, aFGF, and ECGFβ.

4. The use according to claim 3, wherein said FGF-1 is ECGFα.

5. The use according to claim 3, wherein FGF-1 is the full length FGF-1.

6. The use according to any one of claims 1-6, wherein the medicament comprises heparin or heparin fragments.

Patentansprüche

1. Die Verwendung eines humanen Fibroblasten-Wachstumsfaktors zur Herstellung eines Medikamentes, dass zur Förderung einer periostalen Knochenbildung eingesetzt wird.

2. Die Verwendung nach Anspruch 1, wobei der humane Fibroblasten-Wachstumsfaktor FGF-1 ist.

3. Die Verwendung nach Anspruch 2, worin gesagt ist, dass FGF-1 von ECGFα, aFGF und ECGFβ selektiert wurde.

4. Die Verwendung nach Anspruch 3, worin gesagt ist, dass FGF-1 ECGFα ist.

5. Die Verwendung nach Anspruch 3, worin FGF-1 die vollständige FGF-1 Sequenz ist.

6. Die Verwendung nach sämtlichen Ansprüchen 1-6, worin das Medikament Heparin oder Heparin-Fragmente ent-
Recommandations

1. Utilisation d'un facteur humain de croissance de fibroblaste pour la préparation de médicament destiné à stimuler la croissance osseuse périostal.

2. Utilisation selon la recommandation 1, caractérisé en ce que le facteur humain de croissance de fibroblaste est FGF-1.

3. L'utilisation selon la recommandation 2, caractérisé en ce que le FGF-1 est sélectionné parmi ECGFα, aFGF, et ECGFβ.

4. L'utilisation selon la recommandation 3, caractérisé en que le FGF-1 est le ECGFα.

5. L'utilisation selon la recommandation 3, caractérisé en ce que le FGF-1 est la séquence complète de FGF-1.

6. L'utilisation selon l'une quelconque des recommandations 1 à 6, caractérisé en ce que le médicament comprend de l'héparine ou de fragments d'héparine.
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6

- Basic FGF
- Acidic FGF

NEW BONE WIDTH (μm)

<table>
<thead>
<tr>
<th>FGF DOSE GIVEN 4/DAY FOR 3 DAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>No heparin</td>
</tr>
</tbody>
</table>
FIGURE 7

* Different to OVX–PBS p<0.01, # different to SHAM–PBS P<0.01
FIGURE 9
BONE MINERAL DENSITY BY DEXA OF RATS TREATED WITH 3x3 DAY TREATMENT CYCLES WITH αFGF OVER 28 DAYS. (GROUP n=4)

FIGURE 10
\* Different to untreated control p<0.05

**FIGURE 11**