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(54) Title: IGF/IGFBP COMPLEX FOR PROMOTING BONE FORMATION AND FOR REGULATING BONE REMODELING

(57) Abstract

IGF and IGFBP are administered to stimulate new bone formation in subjects with bone loss due to bone marrow disorders, connective tissue disorders, drugs, pregnancy, lactation, chronic hypophosphatemia, hyperphosphatasia, insulin-dependent diabetes mellitus, anorexia nervosa, cadmium poisoning, juvenile osteoporosis, Paget's disease of bone, osteoarthritis and periodontal disease. IGF-I and IGFBP-3 are optionally combined with agents that inhibit bone resorption.

-1-

5   IGF/IGFBP COMPLEX FOR PROMOTING BONE FORMATION
                 AND FOR REGULATING BONE REMODELING

     Technical Field
           The instant invention relates generally to
10   polypeptide factors and their use in bone growth and
     maturation. Specifically, the invention relates to the
     use of a complex of insulin-like growth factor (IGF) and
     insulin-like growth factor binding protein (IGFBP) with
     or without an inhibitor of bone resorption to stimulate
15   bone formation.

     Background Art
           Growth factors are polypeptides which stimulate
     a wide variety of biological responses (e.g., DNA
20   synthesis, cell division, cell differentiation,
     expression of specific genes, etc.) in a defined
     population of target cells. A variety of growth factors
     have been identified including transforming growth
     factor-β1 (TGF-β1), TGF-β2, TGF-β3, epidermal growth
25   factor (EGF), platelet-derived growth factor (PDGF),
     fibroblast growth factor (FGF), insulin-like growth
     factor-I (IGF-I), and IGF-II.
           IGF-I and IGF-II are related in amino acid
     sequence and structure, with each polypeptide having a
30   molecular weight of approximately 7500 daltons. IGF-I
     mediates the major effects of growth hormone and thus is
     the primary mediator of skeletal growth after birth.
     IGF-I has also been implicated in the actions of various
     other growth factors, since treatment of cells with such
35   growth factors leads to increased production of IGF-I.

-2-

Both IGF-I and IGF-II have insulin-like activity (hence
the name) and are mitogenic (stimulating cell division)
for various types of cells involved in the growth and
differentiation of skeletal tissues such as muscle and
5   bone, as well as non-skeletal tissues.

IGF can be measured in blood serum to diagnose
abnormal growth-related conditions, e.g., gigantism,
acromegaly, dwarfism, various growth hormone
deficiencies, etc. Although IGF is produced in many
10   tissues, most circulating IGF is believed to be
synthesized in the liver.

Unlike most growth factors, the IGFs are
present in substantial quantity in the circulation, but
only a very small fraction of this IGF is free in the
15   circulation or in other body fluids. Most IGF is
complexed with IGF-binding proteins. IGF in the blood is
mainly complexed with IGFBP-3, the major circulating IGF-
binding protein.   Almost all IGF circulates in a non-
covalently associated ternary complex composed of IGF-I
20   or -II, an IGF specific binding protein termed IGFBP-3,
and a larger protein termed the Acid Labile Subunit
(ALS). This ternary complex is composed of equimolar
amounts of each of the three components. The ALS has no
direct IGF binding activity and appears to bind only a
25   pre-formed IGF/IGFBP-3 complex. The ternary complex of
IGF + IGFBP-3 + ALS has a molecular weight of
approximately 150,000 daltons. This ternary complex
likely functions in the circulation "as a reservoir and a
buffer for IGF-I and IGF-II preventing rapid changes of
30   free IGF." Blum, W.F., et al., "Plasma IGFBP-3 Levels as
Clinical Indicators", In Modern Concepts in Insulin-Like
Growth Factors, E. M. Spencer, ed., Elsevier, New York,
pages 381-393, 1991.

Having most circulating IGF in complexes is
35   beneficial. Excess free IGF can cause serious

-3-

hypoglycemia because IGF has insulin-like effects on
circulating glucose levels. In contrast to the low
levels of free IGFs and IGFBP-3, there is a substantial
pool of free ALS in plasma which assures that IGF/IGFBP-3
5   complex entering the circulation immediately forms a
ternary complex.

IGF Binding Proteins

        IGFBP-3 is the most abundant IGF binding
10   protein in the circulation. Recently, Wood et al. (Mol.
Endocrin. (1988), 2:1176-85) and Spratt et al. (Growth
Factors (1990), 3:63-72) described the cloning and
expression of human IGFBP-3, whose structure is
incorporated herein by reference. The gene for IGFBP-3
15   codes for 291 amino acids, the first 27 of which
represent a characteristic signal sequence. Thus, the
mature protein comprises 264 amino acids and has a
predicted molecular weight of 28,749 (without
glycosylation or other post-translational changes). When
20   the human IGFBP-3 gene was expressed in Chinese hamster
ovary ("CHO") cells and the conditioned culture medium
was subjected to SDS electrophoresis and transferred to
nitrocellulose membrane for ligand binding analysis,
Spratt et al. reported "the presence of a 43-45 kd
25   doublet, a 28 kd band and a minor 31 kd band" protein
bands (p. 69), indicating there were post-translational
changes. A side-by-side comparison revealed that the 43-
45 kd doublet was also present in human serum.
        Figures 12-15 show coding sequences and deduced
30   amino acid sequences of IGFBP-3 suitable for various
forms of use in the present invention.
        It is unclear which tissue is the primary
source of circulating IGFBP-3, although synthesis has
been demonstrated in numerous cell types, including human
35   fibroblasts, liver cells (most likely Kupfer cells) and

-4-

osteoblasts. cDNA libraries that include the IGFBP-3
cDNA have been obtained from liver and other tissues.
Vascular endothelial cells produce IGFBP-3 and may be the
major source for systemic IGFBP-3.

5   IGFBP-3 has been purified from natural sources
and produced by recombinant means. For instance, IGFBP-3
can be purified from natural sources using a process such
as that shown in Martin and Baxter (J. Biol. Chem. (1986)
261:8754-60) IGFBP-3 also can be synthesized by
10   recombinant organisms as discussed in Sommer, A. et al.,
In Modern Concepts of Insulin-Like Growth Factors, E. M.
Spencer, ed., Elsevier, New York, pp. 715-728, 1991.
This recombinant IGFBP-3 binds IGF-I with a 1:1 molar
stoichiometry.

15   At least five other distinct IGF binding
proteins have been identified in various tissues and
body fluids. Although all these proteins bind IGFs, they
each originate from separate genes and they have distinct
amino acid sequences. Thus, the binding proteins are not
20   merely analogs of a common precursor. For example,
Spratt et al. compared the amino acid sequences of IGFBP-
1, -2 and -3. Of the total 264 amino acids in the mature
protein, only 28% of the amino acids are identical
between IGFBP-3 and IGFBP-1, and 33% are identical
25   between IGFBP-3 and IGFBP-2. Spratt et al. suggested
that the similar portions of the binding proteins are the
region(s) that bind IGF. Unlike IGFBP-3, the other
IGFBPs in the circulation are not saturated with IGFs.
All six known IGFBPs are reviewed and compared by
30   Shimasaki and Ling, Prog. Growth Factor Res. (1991)
3:243-66.

Spencer et al. (1991) Bone 12:21-26; and Tobias
et al. (1992) Endocrinology 131:2387-2392 report the
stimulation of bone formation by IGF-I.

35

-5-

The trabecular bone of rats, like that of humans, shows a coupling of bone formation to bone resorption, such that the increased resorption that occurs with estrogen deficiency entrains increased bone
5   formation, which can be suppressed by inhibition of bone resorption. It has been established that in adult humans this coupling of formation and resorption involves a site specific sequence of events, in which bone resorption is normally followed, at the same site, by bone formation.
10 Frost (1985) <u>Clin. Orthop. Rel. Res.</u> <u>200</u>:198-225.

There is also some evidence that bone formation can occur without previous bone resorption, primarily in those situations where demands for mechanical support of the skeleton are increased (modeling). Parfitt, A.M., et
15 al. (1984) <u>Calcif. Tissue Int.</u> <u>36</u>:5123-5128.

The present invention offers <u>in vivo</u> single or combination therapy for stimulating new bone formation through the administration of the IGF/IGFBP complex or through the administration of the IGF/IGFBP complex and
20 an agent which inhibits bone resorption. These combinations provide more effective therapy for prevention of bone loss and replacement of bone.

<u>Disclosure of the Invention</u>
25   The present invention discloses the use of IGF and its binding protein for the initiation and promotion of bone formation and the regulation of bone remodeling. The IGF/IGFBP-3 complex can be used alone or in conjunction with an inhibitor of bone resorption.
30   The present invention discloses a method for stimulating bone formation in a subject who has a bone marrow disorder causing bone loss. The method comprises administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3, with or without an inhibitor
35 of bone resorption.

-6-

In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has a connective tissue disorder causing bone loss. The method comprises administering to the subject

5   pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

In another embodiment, the present invention discloses a method for treating a subject who has drug-related osteoporosis. The method comprises administering

10   to the subject pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

In another embodiment, the present invention discloses a method of stimulating bone formation in a subject who has bone loss due to pregnancy, lactation,

15   chronic hypophosphatemia, hyperphosphatasia, insulin-dependent diabetes mellitus, anorexia nervosa, cadmium poisoning, juvenile osteoporosis. The method comprises administering to the subject pharmaceutically effective doses of the IGF-I/IGFBP-3 complex.

20   In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has periodontal bone loss. The method comprises administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

25   In another embodiment, the present invention discloses a method for stimulating bone formation in a subject who has bone loss associated with osteoarthritis, disuse, or prolonged exposure to reduced gravitational field. The method comprises administering to the subject

30   pharmaceutically effective doses of IGF-I and IGFBP-3.

In another embodiment, the present invention discloses a composition for inducing bone formation in a subject. The composition comprises IGF-I, IGFBP-3 and an inhibitor of bone resorption in a pharmaceutically

35   acceptable excipient.

-7-

Brief Description of the Figures

Figure 1 shows a schematic of the experimental design used in experiments carried out in support of the invention (Example 2);

Figures 2A and 2B show bar graphs of mean body weights (2A) and lean body mass (2B) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF1/ IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line);

Figure 3 shows a bar graph of endochondral bone growth) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF-I/IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line);

Figures 4A and 4B show bar graphs of tibial periosteal bone formation rate (4A) and tibial endocortical resorption (4B) of ovariectomized (OVX) animals treated for 8 weeks with rhIGF-I or rhIGF-I/IGFBP-3 complex at the doses of IGF-I indicated, compared to no treatment (solid bars) and sham operated controls (solid line);

Figures 5A-5CC show matching UV micrographs (5A-5C, 3X magnification; 5CC, 12X magnification of periosteal envelope region shown in 5C) of ground 40 μm thick tibial cross-sections from sham (5A), OVX (5B), and OVX rats treated with 7.5 mg/kg rhIGF-I complexed with rhIGFBP-3 (5C, 5CC) where new bone formation on the periosteal envelope is indicated by the arrows;

Figures 6A-6D show polerized light micrographs (12.5 X magnification) of unstained, 40 μm thick cross sections from Sham operated animals (6A), OVX animals (6B), and from animals treated with 7.5 mg/kg rhIGF-I complexed with rhIGFBP-3 (6C, 6D), where outer lamellar

-8-

layers are indicated by arrowheads and inner lamellar
layers are indicated by arrows;

Figures 7A-7D show bar graphs of bone formation
rates of cancellous bone in the metaphyses (7A),
5   epiphyses (7B), lumbar vertebral bodies (7C) and femoral
neck region (7D), of ovariectomized (OVX) animals treated
for 8 weeks with rhIGF-I or rhIGF-I/IGFBP-3 complex with
the doses of IGF-I indicated, compared to no treatment
(solid bars) and sham operated controls (solid line),
10 where an asterisk indicates $p< 0.05$ compared to OVX
group;

Figures 8A-8C show UV micrographs of cancellous
trabeculae in distal femoral epiphysis from sham-operated
control (8A), OVX (8B) and 7.5 ml/kg rhIGF-I/IGFBP-3
15 complex (8C) treated rats, where arrows indicate
resorption pits and arrowheads indicate newly formed
bone;

Figures 9A-9C show matching micrographs (3X
magnification) of 4 μm section taken from distal femurs
20 stained by the mineral-staining method of von Kossa from
sham-operated controls (9A) OVX (9B) and 7.5 mg/kg
rhIGF-I/IGFBP-3 complex treated rats (9C);

Figures 10A-10C show cross-sectional images
(2.5X magnification) of sections taken from femoral mid-
25 neck region of sham-operated control (10A), OVX rats
(10B) and from rats treated with 7.5 mg/kg rhIGF-I
complexed with IGFBP-3 (10C);

Figure 11 shows the amino acid sequences of
human IGF-I used in the present invention (SEQ ID NO:1);
30 Figure 12 shows the amino acid sequence of
human IGFBP-3 (SEQ ID NO:2), where an asterisk at
position 5 indicates substitution of glycine for alanine
according to naturally occurring heterogeneity in the
sequence);
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-9-

Figure 13 shows a nucleotide sequence of human
IGFBP-3 (SEQ ID NO:3 and SEQ ID NO:4) used to produce
rhIBFBP-3 for use in accordance with the invention;

Figure 14 shows an alternate nucleotide
5   sequence (SEQ ID NO:5) for production of rhIBFBP-3 for
use in accordance with the invention; and

Figure 15 shows a second alternate nucleotide
sequence (SEQ ID NO:6 and SEQ ID NO:7) for production of
rhIGFBP-3 for use in accordance with the invention.
10

Modes for Carrying Out the Invention
A.   Definitions

It must be noted that as used in this
specification and the appended claims, the singular forms
15   "a", "an" and "the" include plural referents unless the
context clearly dictates otherwise.

The term "connective tissue disorder" includes
conditions such as osteogenesis imperfecta, Ehlers-Danlos
syndrome, Marfans syndrome, cutis laxa, homocystinuria,
20   Mankes's syndrome and scurvy. These have been reported
to be associated with and cause bone loss.

"Drug-related osteoporosis" means osteoporosis
whose only identified cause is a drug. The definition
includes all known drugs which cause osteoporosis, as
25   well as those drugs later discovered to cause
osteoporosis. Examples of drugs known to cause
osteoporosis include corticosteroids, heparin, oral
anticoagulants, anticonvulsants, methotrexate, thyroid
hormone, lithium and gonadotrophin-releasing analogs.
30   "Insulin-like growth factor (IGF)" comprises a
family of factors, including but not limited to IGF-I and
IGF-II. IGF is a polypeptide having a molecular weight
of about 7500 daltons. IGF can be obtained from natural
sources or prepared by recombinant means. Preferably IGF
35   is IGF-I from human sources. Most preferably, IGF is

-10-

is IGF-I from human sources. Most preferably, IGF is
human IGF-I made by recombinant means, such as by the
methods detailed in Example 3 herein, and designated
rhIGF-I.

5   "Insulin-like growth factor binding protein
(IGFBP)" comprises a family of binding proteins,
including but not limited to IGFBP-1, IGFBP-2, IGFBP-3,
IGFBP-4, IGFBP-5 and IGFBP-6. Each IGFBP can be obtained
from natural sources or prepared by recombinant means,
10   such as detailed in Example 4 herein. At least one form
of IGFBP (for example, IGFBP-3) complexes with IGF and
with a third molecule known as ALS. IGFBP also can be a
mixture of any combination of the six IGFBP's. Such a
mixture would take advantage of the different binding
15   affinities for IGF-I and/or IGF-II, the ability of some
IGFBP's to bind to cell surfaces, and the different half-
lives.

          A "therapeutic composition" as used herein is
defined as comprising the IGF binding protein (IGFBP)
20   with IGF and a bone resorption inhibitor. The
therapeutic composition can also contain excipients such
as water, minerals and carriers such as protein.

          The method of the present invention
contemplates treating a subject in need of local bone
25   repair or replacement with IGFBP in combination with IGF
with or without a bone resorption inhibitor. The IGF may
be any of the IGF family, including but not limited to,
IGF-I and IGF-II, or a combination thereof. When IGF-I
and IGF-II are combined, the ratio of IGF-I to IGF-II
30   ranges from 0.01:1 to 99:1.

          "IGFBP-1" is an IGF-binding protein whose
molecular structure was disclosed by Brewer et al.,
Biochem. Biophys. Res. Comm. (1988) 152(3):1289-1297, and
by Drop et al. in PCT Publication No. WO 89/98667,
35   published on September 21, 1989, and is incorporated

-11-

herein by reference. Human IGFBP-1 has 234 amino acids
and a molecular weight of about 28 kd.

   "IGFBP-2" comprises 289 amino acids (human) and
has a molecular weight of 36 kd under nonreducing
conditions. The amino acid sequence of human IGFBP-2 was
determined from cDNA clones isolated from a human fetal
liver library by Binkert et al. EMBO J. (1989) 8:2493-
2502, and is incorporated herein by reference. IGFBP-2
also may bind to cell surfaces. IGFBP-2 has a preference
for IGF-II, and thus is preferred in formulations
comprising IGF-II.

   "IGFBP-3" is the preferred IGFBP in the
IGF/IGFBP complex. Native and recombinant IGFBP-3, as
well as some N-terminal and C-terminal fragments, bind
IGF-I and IGF-II. Human IGFBP-3 comprises 264 amino
acids and has three potential N-linked glycosylation
sites. IGFBP-3 is the major IGFBP in blood.

   Nearly all IGF-I or IGF-II in blood is bound to
IGFBP-3. The IGF/IGFBP-3 complex normally circulates in
the form of a complex in humans and other mammals and
avians. This complex associates with a third protein
(ALS), which is present in excess over the concentration
of IGF and IGFBP-3. Therefore, in the circulation, ALS
is found both associated with the IGF/IGFBP-3 complex and
in free form. The resultant ternary complex has a size
of about 150 kD. Administration of a pre-formed complex
of IGF and IGFBP-3, either from natural or recombinant
sources, results in the formation of the ternary complex
with the normally excess ALS. This type of treatment
appears to produce a long term increase in the level of
circulating IGF, which is gradually released from the
ternary complex. This mode of administration avoids the
detrimental side effects associated with administration
of free IGF-I, e.g., hypoglycemia, suppression of growth
hormone and ALS production, and release of endogenous

-12-

IGF-II since administered exogenous free IGF-I replaces endogenous IGF-II in normally circulating IGF-II/IGFBP-3 complexes.

"IGFBP-4" and "IGFBP-6" are glycosylated proteins which are widely distributed in the body. The primary structure of IGFBP-4 was reported by Shimasaki et al. Mol. Endocrinol. (1990) 4:1451-1458, and is incorporated herein by reference. IGFBP-6, whose cDNA has been isolated by Shimasaki et al. (Mol. Endocrinol. (1991) 5:938-48), has a much greater affinity for IGF-II than for IGF-I and may be preferred with formulations containing IGF-II. This reference is incorporated herein by reference.

"IGFBP-5" is a 252-amino acid protein which is not glycosylated. Shimasaki et al. (J. Biol. Chem. (1991) 266:10646-53) cloned human IGFBP-5 cDNA from a human placenta library, and is incorporated herein by reference.

Depending on the binding, metabolic and pharmacokinetic characteristics required in the IGF/IGFBP complex formulation, these binding proteins can be added to the complex formulation in various proportions. These IGFBP's can be combined in a wide variety of ratios with IGF-I and/or IGF-II.

The term "inhibition of bone resorption" refers to prevention of bone loss, especially the inhibition of removal of existing bone either from the mineral phase and/or the organic matrix phase, through direct or indirect alteration of osteoclast formation or metabolism. Thus, the term "inhibitor of bone resorption" as used herein refers to agents that prevent bone loss by the direct or indirect alteration of osteoclast formation or metabolism.

The term "bone remodeling" refers to that process which renews the skeleton and repairs microdamage

-13-

before it accumulates to the point of loss of skeletal
integrity.   Most adult human metabolic bone disease
results from derangement of remodeling processes.   The
individual remodeling packets are known as "basic
5   multicellular units" (BMU).   Remodeling which occurs in
BMUs follows a preprogrammed sequence: activation →
resorption →   formation (ARF).   (Recker, R.R., "Bone
Histomorphometry: Techniques and Interpretation."   Boca
Raton, FL, 1983, pp. 37-57.)
10   "Bone formation" includes increases in the
number and/or activity of BMU (Basic Multicellular Units)
producing new bone.   This process can take place anywhere
in the skeleton and affect cancellous and/or cortical
bone with no regard to functional differences and
15   turn-over rates which particular bone sites might have in
the skeleton.   (Frost (1986) Intermediary Organization of
the Skeleton, Vols. I and II. CRC Press, Boca Raton, FL.)
           The term "osteogenically effective" means that
amount which affects the formation and differentiation of
20   mature bone or early bone progenitor cells.   As used
herein, an osteogenically effective dose is also
"pharmaceutically effective."
           The term "subject" as used herein refers to a
living vertebrate animal such as a mammal or bird in need
25   of treatment, i.e., in need of bone repair or
replacement.   Such need arises in connective tissue
disorders such as osteogenesis imperfecta, Ehlers-Danlos
syndrome, Marfans syndrome, cutis laxa, homocystinuria,
Menkes's syndrome and scurvy; osteoporosis related to
30   such drugs as corticosteroids, heparin, oral
anticoagulants, anticonvulsants, methotrexate, thyroid
hormone, lithium and gonadotrophin-releasing analogs;
pregnancy; lactation; chronic hypophosphatemia;
hyperphosphatasia; insulin-dependent diabetes mellitus;
35   anorexia nervosa; Paget's disease of bone; cadmium

-14-

poisoning; periodontal disease; osteoarthritis, disuse of
the musculoskeletal system such as occurs during
prolonged bed rest, paralysis or semi-paralysis; and
prolonged exposure to reduced gravitational field.

5   The term "treatment" as used herein shall mean
(1) providing a subject with an amount of a substance
sufficient to act prophylactically to prevent the
development of bone loss; or (2) providing a subject with
a sufficient amount of a substance so as to alleviate or
10   eliminate the problems associated with the bone loss.

B.   General methods
          Drugs which prevent bone loss and/or add back
lost bone have been evaluated in the ovariectomized rat.
15   This animal model is well established in the art (see,
for example, Wronski et al. (1985) Calcif. Tissue Int.
37:324-328; Kimmel et al. (1990) Calcif. Tissue Int.
46:101-110; Durbridge et al. (1990) Calcif. Tissue Int.
47:383-387; and Miller et al. (1991) Bone 12:439-446;
20   these references are hereby incorporated in their
entirety). Wronski et al. ((1985) Calcif. Tissue Int.
43:179-183)) describe the association of bone loss and
bone remodeling in the ovariectomized rat.
          Examples of inhibitors of bone resorption
25   include estrogen, such as conjugated estrogen, tamoxifen,
bisphosphonates, calcitonins, or other small peptides or
molecules that may inhibit bone resorption. (Turner et
al. (1987) J. Bone Mineral Res. 2:115-122; Wronski et al.
(1988) Endocrinology 128:681-686; and Wronski et al.
30   (1989) Endocrinology 125:810-816; Pfeilshifter et al.
(1987) Proc. Natl. Acad. Sci. U.S.A. 84:2024-2028; Turner
et al. (1988) Endocrinology 122:1146-1150). An example
of a small peptide is echistatin, which includes the
arginine-glycine-aspartate (RGD) sequence which is
35   recognized by some cell surface adhesion receptors and

-15-

apparently disrupts osteoclast interaction (Fisher et al. (1993) _Endocrinology_ 132: 1411-13). Another example of a bone resorption factor is OPF or osteoclastpoietic factor (PCT Publication WO 93/01827 published 4 February 1993).

5   Other agents that are under investigation or have been proposed to inhibit bone resorption include mithramycin, gallium nitrate, glucocorticoids, transforming growth factor-β (TGF-β), interferon-γ and amylin. (Zaidi et al. (October 1992) _Curr. Opin. Therapeutic Patents_, pp. 1517-

10   38).

The entire molecule of a particular inhibitor may be used, or alternatively, only a functional part of the inhibitor molecule may be used. Bisphosphonates include, but are not limited to, pamidronate acid,

15   alendronate, tiludronate, risedronate and other experimental compounds.

Certain growth factors can inhibit bone resorption, or are anti-resorptive. Examples of such growth factors include but are not limited to the

20   transforming growth factors-β.

In accordance with the method of the present invention, the formulation comprises a complex of IGF and IGFBP administered with or without a bone resorption inhibitor.

25   Preferably, the IGF is IGF-I, although IGF-II can be useful. Preferably, the IGFBP is IGFBP-3. Because IGF and IGFBP-3 naturally bind in a 1:1 molar ratio, a composition of equimolar amounts of IGF and IGFBP-3 is preferred. Nevertheless, the composition can

30   be formulated with IGF:IGFBP-3 molar ratios ranging from 0.5:1 to 1.5:1. More preferably, the molar ratio is 0.9:1 to 1.3:1; and most preferably, the composition is formulated with approximately a 1:1 molar ratio.

In accordance with the method of the present

35   invention, IGF and IGFBP-3 are human proteins obtained

-16-

from natural or recombinant sources. Most preferably,
IGF and IGFBP-3 are human IGF-I and IGFBP-3 made by
recombinant means and designated rhIGF-I and rhIGFBP-3,
respectively. rhIGFBP-3 can be in glycosylated or non-

5 glycosylated form. *E. coli* is a source of the non-
glycosylated IGFBP-3. Glycosylated IGFBP-3 can be
obtained from CHO-cells.

The method of the present invention provides
for formulating the complex in modes which are readily

10 apparent to those skilled in the art. Preferably, the
IGF and IGFBP-3 are complexed prior to administration to
the treated individual. Preferably, the complex is
formed by mixing approximately equimolar amounts of IGF-I
and IGFBP-3 dissolved in physiologically compatible

15 carriers such as normal saline solution or phosphate
buffered saline solution. Most preferably, a
concentrated solution of rhIGF-I and a concentrated
solution of IGFBP-3 are mixed together for a sufficient
time to form an equimolar complex.

20   Pharmaceutical compositions of the invention
which include IGF, IGFBP and an inhibitor of bone
resorption for administration include osteogenically
effective amounts of IGF and IGFBP to promote bone
formation and an inhibitory amount of a bone resorption

25 inhibitor, in addition to a pharmaceutically acceptable
excipient. Suitable excipients include most carriers
approved for parenteral administration, including water,
saline, Ringer's solution, Hank's solution, and solutions
of glucose, lactose, dextrose, ethanol, glycerol,

30 albumin, plasma, other protein-containing solutions and
the like. These compositions may optionally include
stabilizers, antioxidants, antimicrobials, preservatives,
buffering agents, surfactants, and other accessory
additives. The IGF/IGFBP complex and inhibitor of bone

35

-17-

resorption may also be delivered in a slow release form
from a suitable carrier.

        Various vehicles may be used with the present
invention. A thorough discussion of suitable vehicles
5   for parenteral administration may be found in E.W.
Martin, "Remington's Pharmaceutical Sciences" (Mack Pub.
Co., current edition). Sections relating to the
excipient vehicles and formulating are incorporated
herein by reference. Such formulations are generally
10 known to those skilled in the art and are administered
systemically to provide systemic treatment.

        The IGF/IGFBP complex and an agent which
inhibits bone resorption may be administered
simultaneously, as a single composition to the subject,
15 or sequentially. If administered sequentially, the
period between the administration of the IGF/IGFBP
complex and the inhibitor of bone resorption is typically
one day to one year, and preferably, one week to six
months. If the IGF/IGFBP complex and the agent which
20 inhibits bone resorption are administered as a single
composition, the molar ratio of IGF/IGFBP complex to
inhibitor of bone resorption varies considerably
depending on the type of bone resorption inhibitor and
the formulation of the IGF/IGFBP complex. The ratio for
25 most compounds is between about 100:1 to 1:100.
Furthermore, if administered as a single composition, the
IGF/IGFBP complex and the inhibitor of bone resorption
may be administered as separate molecules in the
composition, or the respective molecules may be
30 conjugated or fused according to techniques well known in
the art.

        The precise dosage necessarily varies with the
age, size, sex and condition of the subject, the nature
and severity of the disorder to be treated, and the like.
35 Thus, a precise effective amount cannot be specified in

-18-

advance and must be determined by the care giver.
However, appropriate amounts have been and can be
determined by routine experimentation with animal models,
as described below.

5   In general terms, an effective dose of
IGF/IGFBP complex for systemic treatment will range from
about 1 μg to about 10 mg IGF/kg of body weight. An
effective dose for an inhibitor of bone resorption
depends upon the particular inhibitor selected for

10   administration. For example, an effective dose of
estrogen is about 0.25 to 1.5 mg/day. An effective dose
for bisphosphonates varies but is generally between about
0.05 μg/kg to about 15 mg/kg of body weight. An
effective dose for calcitonin is about 0.05 IU

15   (International Units or Medical Research Council
Units)/kg to about 2.5 IU/kg of body weight.
         Effective doses for local administration range
from about 0.01 μg to 1 mg of IGF/IGFBP complex.
         The methods and compositions of the invention

20   are useful for treating bone fractures, defects, and
disorders which result in weakened bones. Among these
disorders are bone marrow dyscrasias such as plasma cell
dyscrasias, leukemia, lymphomas, systemic mastocytosis,
anemias, lipidoses and mucopolysaccharidoses; connective

25   tissue disorders such as osteogenesis imperfecta, Ehlers-
Danlos syndrome, Marfans syndrome, cutis laxa,
homocystinuria, Menkes's syndrome and scurvy;
osteoporosis related to such drugs as corticosteroids,
heparin, oral anticoagulants, anticonvulsants,

30   methotrexate, thyroid hormone, lithium and gonadotrophin-
releasing analogs; pregnancy; lactation; chronic
hypophosphatemia; hyperphosphatasia; insulin-dependent
diabetes mellitus; anorexia nervosa; Paget's disease of
bone; juvenile osteoporosis; cadmium poisoning;

35   periodontal disease; and osteoarthritis. In addition,

-19-

methods and compositions of the present invention are
useful in treating weakened bones resulting from disuse
of the musculoskeletal system, such as occurs as a
consequence of prolonged bed rest or exposure to
5   decreased gravitational fields.

          In accordance with one method of use, the
IGF/IGFBP complex may be administered locally to a
specific area in need of bone growth or repair, with
either the concomitant administration of the inhibitor of
10   bone resorption at the site, or the administration of the
inhibitor of bone resorption in a separate vehicle.
Thus, the IGF/IGFBP complex and/or inhibitor of bone
resorption may be implanted directly at the site to be
treated, for example, by injection or surgical
15   implantation in a sustained-release carrier. Suitable
carriers include hydrogels, controlled- or sustained-
release devices (e.g., an Alzet® minipump), polylactic
acid, and collagen matrices. Presently preferred
carriers are formulations of atelopeptide collagen
20   containing particulate calcium phosphate mineral
components, such as combinations of homologous or
xenographic fibrillar atelopeptide collagen (for example
Zyderm® Collagen Implant, available from Collagen
Corporation, Palo Alto, California) with hydroxyapatite-
25   tricalcium phosphate (HA-TCP, available from Zimmer,
Inc., Warsaw, IN). It is presently preferred to
administer implant compositions containing an IGF/IGFBP
complex and/or an inhibitor of bone resorption in a
collagen/mineral mixture implant.
30   IGF/IGFBP complex and/or an inhibitor of bone
resorption, delivered in sustained-release vehicles is
also particularly useful for improving implant fixation,
for example, for improving ingrowth of new bone into a
metal prosthesis in joint reconstruction and dental or
35   orthopedic implants. Alternatively, the IGF/IGFBP

-20-

complex may be delivered in the implant, with the
inhibitor delivered in a separate vehicle, and vice
versa.

Dental and orthopedic implants can be coated

5   with the IGF/IGFBP complex in combination with an
inhibitor of bone resorption to enhance attachment of the
implant device to the bone. Alternatively, the IGF/IGFBP
complex can be used to coat the implant, and the
inhibitor of bone resorption can be administered

10 concomitantly or sequentially in a separate vehicle, and
vice versa.

In general, implant devices may be coated with
the IGF/IGFBP complex and/or an inhibitor of bone
resorption as follows. The IGF/IGFBP complex (and the

15 inhibitor of bone resorption, if desired) is dissolved at
a concentration in the range of 0.01 mg/ml to 200 mg/ml
in phosphate-buffered saline (PBS) containing 2 mg/ml
serum albumin. The porous end of an implant is dipped in
the solution and is air dried (or lyophilized) or

20 implanted immediately into the bony site. The viscosity
of the coating solution is increased, if desired, by
adding hyaluronate at a final concentration of 0.1 mg/ml
to 100 mg/ml or by adding other pharmaceutically
acceptable excipients. Alternatively, the solution

25 containing the IGF/IGFBP complex (and the inhibitor of
bone resorption, if desired) is mixed with collagen gel
or human collagen (e.g. Zyderm® Collagen Implant,
Collagen Corp., Palo Alto, CA) to a final collagen
concentration of 2 mg/ml to 100 mg/ml to form a paste or

30 gel, which is then used to coat the porous end of the
implant device. The coated implant device is placed into
the bony site immediately or is air dried and rehydrated
with PBS prior to implanting, with the objective of
maximizing new bone formation into the implant while

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-21-

minimizing the ingrowth of soft tissue into the implant
site.

C.   Examples

    The following examples are put forth so as to
provide those of ordinary skill in the art with a
complete disclosure and description of how to extract,
isolate, formulate and use the compositions and methods
of the invention and are not intended to limit the scope
of what the inventors regard as their invention. Efforts
have been made to insure accuracy with respect to numbers
used (e.g., amounts, times, temperature, etc.), but some
experimental error and deviations should be accounted
for. Unless indicated otherwise, parts are parts by
weight, temperature is in degrees centigrade, pressure is
at or near atmospheric, and other parameters are
conventional and in accordance with those normally
accepted by those skilled in the art.

                      Example 1
    This example shows the use of free IGF-I and
the IGF-I/IGFBP-3 complex in female rats with
ovariectomy-induced osteoporosis. In this experiment,
rats were treated with human recombinant IGF-I and
IGFBP-3. The rhIGF-I (Ciba-Geigy) was synthesized in
yeast and provided in sterile water and stored at -70°C.
The rhIGFBP-3 was made according to the procedure
described in Example 4 (Celtrix Pharmaceuticals, Inc.,
Santa Clara, CA) and was dissolved in phosphate-buffered
saline and stored at -70°C until use. Prior to
administration, these solutions were thawed, and
sufficient amounts of IGF-I and IGFBP-3 were mixed to
provide equimolar amounts of the two proteins. This
experiment demonstrates the ability of the IGF-I/IGFBP-3

-22-

complex to increase trabecular bone mass and other parameters in ovariectomized rats.

In this example, young female rats of 90-100 g body weight were ovariectomized by the dorsal route and
5   were divided into six groups of eight rats each. An additional group consisted of eight intact, age-matched sham operated control rats. Six weeks after ovariectomy, rats were treated with vehicle or with one of the following combinations of IGF-I and IGFBP-3 or IGF alone,
10   as indicated. In these experiments, the amounts of IGF and IGFBP used were calculated to provide a 1:1 molar ratio of IGF:IGFBP:

	Group 1:	Sham Operated Controls; Vehicle
	Group 2:	Ovariectomized Controls; Vehicle
15	Group 3:	Ovariectomized; 2.5 mg/kg IGF-I
		complexed to IGFBP-3 (9.5 mg/kg)
	Group 4:	Ovariectomized; 0.25 mg/kg IGF-I
		complexed to IGFBP-3 (0.95 mg/kg)
	Group 5:	Ovariectomized; 0.025 mg/kg IGF-I
20		complexed to 0.095 mg/kg IGFBP-3
	Group 6:	Ovariectomized; 2.5 mg/kg IGF-I
	Group 7:	Ovariectomized; 0.25 mg/kg IGF-I

The complex was formed by mixing equimolar
25   amounts of IGFBP-3 (dissolved in phosphate buffered · saline (PBS), pH 6.0) and IGF-I (dissolved in 10 mm sodium acetate, pH 5.5) in the minimum volume feasible, and incubating the mixture overnight at 4°C. The complex was then diluted with PBS, pH 6.0, containing 0.1% rat
30   serum albumin. The solutions were divided into aliquots containing the amount of material needed for one day, and stored at -70°C until needed. The controls received the dilution buffer.

The rats were treated for 22 days. The test
35   substances were administered six times per week by one

-23-

daily subcutaneous injection. One day before treatment
was started and on the 17th day of treatment, 20 mg/kg of
calcein was given by intraperitoneal injection. Calcein
and tetracycline are markers for bone mineralization and
5   are used to estimate the amount of bone formation between
administrations. Similarly, on the tenth day 20 mg/kg of
demeclocycline was administered. On day 23, 24 hours
after the last injection, the rats were killed by
anesthesia with carbon dioxide.
10   Body weights were recorded throughout the
experiment. At autopsy, 0.1 ml of blood was taken for
the determination of blood glucose. Serum was prepared
from the rest of the blood, and total serum IGF-I levels
were determined by radioimmunoassay (RIA). Gastrocnemius
15   muscle, periuterine fat and uterus were removed,
dissected free of connective tissue, and weighed.
            The amount of trabecular and cortical bone was
determined according to Gunness-Hey ((1984) _Metab. Bone
Dis. & Rel. Res._ 5:177-81), incorporated herein by
20   reference. Briefly, the femurs were cut in half at the
mid-diaphysis using a dental saw. The proximal halves
were discarded. The epiphysis of the distal half was cut
off using a scalpel, and the bone was split into sagittal
halves. The marrow was flushed out with water. With a
25   dental curette, the metaphyseal trabecular bone was
scraped out of both cortical shells, combined and put
into 5% trichloroacetic acid (TCA). The two pieces of
the remaining cortical bone were also combined and put
into a separate tube with 5% TCA. After this preparation
30   stood 16 hours at room temperature, the TCA extract was
used for the determination of calcium by atomic
absorption spectroscopy. The remaining demineralized
matrix was washed successively with ethanol and methylene
chloride, and dried under vacuum. After determination of
35   the dry weight, the matrix was hydrolyzed with 6 M HCl at

-24-

120°C for 5 hours. In the hydrolysate hydroxyproline was
determined by a standard colorimetric assay (Jamall et
al. (1981) Analyt. Biochem. 112:70-75).

5   The results of this experiment are detailed in
Tables 1 and 2 and summarized below.

When ovariectomized ("Ovx" in the tables)
control rats were compared to sham operated control
animals, no significant differences were observed in
cortical bone weight or hydroxyproline (Table 2).

10   However, cortical bone calcium was significantly
increased in ovariectomized rats (7%). This increase may
be due to increased longitudinal bone growth and increase
in organ size. In the ovariectomized rats, trabecular
bone weight, calcium and hydroxyproline were reduced by

15   65% (Table 1).

In ovariectomized rats treated with IGF-I
alone, both doses (2.5 mg/kg and 0.25 mg/kg) increased
trabecular bone mass; however, the lower dose was more
effective than the higher dose. The lower dose increased

20   various parameters 79%-118%; whereas, the higher dose
increased various parameters by 57%-67%. There were
marginal changes in other parameters.

Ovariectomized rats treated with IGF-I/IGFBP-3
complex showed an increase in various trabecular bone

25   parameters (Table 1). Significant increases in
trabecular calcium, dry weight, and hydroxyproline were
observed at an IGF-I/IGFBP-3 dose of 12 mg/kg (2.5 mg/kg
IGF-I and 9.5 mg/kg IGFBP-3). Cortical bone matrix dry
weight was also increased significantly by treatment with

30   12 mg/kg IGF-I/IGFBP-3 complex (Table 2).

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Table 1

Treatment Groups	Group 1 (Sham Operated)	Group 2 (Ovx Control)	Group 3	Group 4	Group 5	Group 6	Group 7
IGF-I (mg/kg)	-	-	2.5	0.25	0.025	2.5	0.25
IGFBP-3 (mg/kg)	-	-	9.5	0.95	0.095	-	-
Trabecular Bone[1]							
Calcium (mg) (%)	4.0±0.4 (+186)*	1.4±0.1 -	2.7±0.2 (+93)	1.8±0.2 (+29)	1.6±0.1 (+14)	2.2±0.2 (+57)*	2.5±0.2 (+79)*
Dry Weight (mg) (%)	2.9±0.4 (+164)*	1.1±0.1 -	2.1±0.2 (+91)	1.7±0.2 (+55)	1.6±0.3 (+45)	1.8±0.1 (+64)*	2.4±0.2 (+118)*
Hydroxyproline (mg) (%)	269±35 (+186)*	94±11 -	221±20 (+135)*	122±20 (+30)	157±30 (+67)	157±30 (+67)	169±16 (+80)*

1 % are compared to ovariectomized, vehicle treated control values (Group 2)

* $p < 0.05$ compared to Ovx control

Table 2

Treatment Groups	Group 1 (Sham Operated)	Group 2 (Ovx Control)	Group 3	Group 4	Group 5	Group 6	Group 7
IGF-I (mg/kg)	-	-	2.5	0.25	0.025	2.5	0.25
IGFBP-3 (mg/kg)	-	-	9.5	0.95	0.095	-	-
Cortical Bone[1]							
Calcium (mg) (%)	24.6±0.5 (-7)*	26.5±0.7 -	26.1±0.9 (-2)	26.7±0.7 (+1)	23.2±0.5 (-12)*	24.0±0.6 (-9)*	23.5±0.6 (-11)*
Dry Weight (mg) (%)	52.8±3.4 (-7)	56.8±2.7 -	64.7±2.7 (+14)*	56.8±3.2 (±0)	59.1±2.0 (+4)	6.81±3.6 (+20)*	55.1±0.2 (-3)
Hydroxyproline (mg) (%)	2679±177 (-8)	2925±143 -	3144±146 (+7)	2978±157 (+2)	3050±107 (+4)	3331±102 (+14)*	3141±194 (+7)

1 % are compared to ovariectomized, vehicle treated control values (Group 2)
* p < 0.05 compared to Ovx control

-27-

Example 2

Thirteen week old female Sprague Dawley rats
(Charles River Associates) were housed for approximately
three weeks before undergoing either ovariectomy (OVX) or
5   sham operation (Sham). All OVXs were successful as
determined by uterine weights measured at sacrifice.
Eight weeks after surgery (designated day 0) the rats
underwent a Dual Energy X-ray Absorptometry (DEXA) spine
scan for baseline measurements of bone mineral density of
10   vertebrae L3 through L6 (BMD) and were assigned into
groups (see below). Attempts were made to exclude the
"outliers" and have similar mean spine BMD values and
body weights among the various groups.

The rhIGF-I/IGFBP-3 was prepared by mixing
15   equimolar quantities of rhIGF-I and rhIGFBP-3. The
vehicle employed for both rhIGF-I and rhIGF-I/IGFBP-3 was
phosphate buffered saline (PBS), pH. 6.0.

Treatments (by daily subcutaneous injection)
began the day after the baseline DEXA spine measurements
20   (day 1) and continued for 56 days. Two additional
"pretreatment" groups of rats (OVX and Sham) were
sacrificed on day 0 to provide baseline data.

The rats were divided into seven groups
according to the experimental paradigm illustrated in
25   Fig. 1:

(1)   Sham rats treated with saline ("Sham
control"; n=9).

(2)   OVX rats treated with saline ("OVX
control"; n=11).

30   (3)   OVX rats treated with 0.9 mg/kg/day of
rhIGF-I (n=7).

(4)   OVX rats treated with 2.6 mg/kg/day of
rhIGF-I (n=7).

(5)   OVX rats treated with 0.9 mg/kg/day of
35   rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

-28-

(6)   OVX rats treated with 2.6 mg/kg/day of
rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

(7)   OVX rats treated with 7.5 mg/kg/day of
rhIGF-I in equimolar ratio with IGFBP-3 (n=8).

5   a.   Serum Measurements

On day 22 food was withdrawn from the rats at
about 7 a.m. and 150 μl of blood was obtained from the
tail of each rat under isoflurane anesthesia between
10 a.m. and noon.   Immediately after the blood was
10   collected, each rat received its normal daily treatment
injection, and a second blood sample was obtained exactly
two hours after the injection.   Food was returned to the
rats only after this second bleeding.   Plasma glucose
levels were measured by a standard colorimetric assay
15   involving the oxidation of O-dianisidine by the peroxide
produced from glucose as a result of treatment with
glucose oxidase according to standard procedures well
known in the art.   (Table 3)

During the seventh week of the study, blood was
20   again obtained from the tail during the normal DEXA scan.
These samples were analyzed for glycosylated hemoglobin
levels using an affinity chromatographic procedure,
specifically the "GLYCO-TEK" assay kit (Helena
Laboratories, Beaumont, TX) with the eluted hemoglobins
25   detected by their absorbances at 405 nm.

Serum obtained at sacrifice was analyzed for
levels of total IGF-I, rhIGF-I and rhIGFBP-3.   Serum
IGF-I levels were determined by two separate assays.
Each assay was performed once on the entire set of
30   samples.

First, a Nichols Institute RIA procedure was
employed to quantitate the combined concentrations of
endogenous rat IGF-I and the injected rhIGF-I.   Samples
of 80 μl were extracted with 900 μl of 87.5% 2N HCl/12.5%
35   ethanol, centrifuged, and then 200 μl of the supernatant

-29-

was neutralized with 100 μl of 855 mM Tris buffer pH 11.0. This neutralized extract was kept at -20°C for one hour, centrifuged, and then diluted 31-fold with phosphate buffered saline (PBS) prior to analyzing 50 μl

5   by RIA. A preliminary assay indicated that serum obtained from hypophysectomized rats (n=6) gave values below the limit of detection (66 ng/ml) and the assay gave a linear response when the volume of serum was varied from 10 to 100 μl.

10   The second assay quantitated the serum levels of rhIGF-I by immunoradiometric assay (IRMA), according to protocols provided by the manufacturer, with a sample size of 10 μl. A pooled rat serum sample containing 555 ng/ml IGF-I by the Nichols Institute RIA procedure gave

15   values below the limit of detection (50 ng/ml) of this assay. In addition, serum levels of rhIGFBP-3 on 10 μl samples were determined by RIA. The assay kits and procedures for both rhIGF-I and rhIGFBP-3 were obtained from Diagnostic Systems Laboratories (Webster, TX).

20   Serum IGF-I and IGFBP-3 levels and blood glycosylated hemoglobin values are shown in Table 4.

25

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Table 3

Fasting Plasma Glucose (mg/dl)
(Day 22 of Study)

Group	Treatment	Time = 0 hours Glucose	SEM	Time = 2 hours Glucose	SEM	Student's P Value
1	Sham	191	6	162	6	0.003
2	OVX-Saline	183	8	197	4	0.120
3	0.9 mg/kg IGF-I	179	8	146	24	0.223
4	2.6 mg/kg IGF-I	163	6	73	11	0.001
5	0.9 mg/kg IGF-I + IGFBP-3	175	7	195	9	0.093
6	2.6 mg/kg IGF-I + IGFBP-3	192	6	204	10	0.349
7	7.5 mg/kg IGF-I + IGFBP-3	174	6	161	14	0.432

Table 4

Serum IGF-I and IGFBP-3 Levels and Blood Glycosylated Hemoblobin Values

Group	Treatment	Total IGF-I (ng/ml)	rhIGF-I (ng/ml)	rhIGFBP-3 (ng/ml)	Glycosylated Hemoglobin (%)
1	Sham-operated + Saline	not measured	not measured	not measured	6.7±0.3 (9)
2	OVX-Saline	487±33 (9)	<50 (8)	<250 (8)	7.2±0.2 (11)
3	0.9 mg/kg rhIGF-I	619±73 (5)	204±56 (5)	<250 (5)	7.3±0.3 (7)
4	2.6 mg/kg rhIGF-I	633±75 (7)	329±61 (7)	<250 (7)	7.3±0.3 (7)
5	0.9 mg/kg rhIGF-I/ IGFBP-3	1026±162 (6)	1141±294 (6)	2441±775 (6)	7.3±0.4 (8)
6	2.6 mg/kg rhIGF-I/ IGFBP-3	988±63 (7)	1391±10 (7)	2372±525 (7)	7.3±0.4 (8)
7	7.5 mg/kg rhIGF-I/ IGFBP-3	1783±273 (8)	2103±123 (8)	8217±2247 (8)	7.3±0.4 (8)
	ANOVA P Value (Dose)	<0.001	-	-	0.83
	ANOVA P Value (Treatment)	0.014	-	-	0.69

Data are presented as means ± SEM for the number of values indicated in parentheses. A two-factor ANOVA was performed to determine the effects of dose (rhIGF-I and rhIGF-I/IGFBP-3 groups) and treatment (rhIGF-I versus rhIGF-I/IGFBP-3 groups.)

TABLE 4A
(page 1 of 2)

Group	Treatment	Day 0 BMD	Day 0 SEM	Day 14 BMD	Day 14 SEM	Day 28 BMD	Day 28 SEM
				Spine BMD (mg/cm2)			
A	Pretreatment Sham	237	4	ND	ND	ND	ND
B	Pretreatment OVX	215	4	ND	ND	ND	ND
1	Sham - Saline	239	5	242	6	243	3
2	OVX - Saline	216	2	212	3	212	3
3	0.9 mg/kg IGF-I	211	4	204	5	201	2
4	2.6 mg/kg IGF-I	214	3	201	4	206	3
5	0.9 mg/kg IGF-I + IGFBP-3	213	4	210	5	211	4
6	2.6 mg/kg IGF-I + IGFBP-3	215	5	209	5	204	6
7	7.5 mg/kg IGF-I + IGFBP-3	219	5	215	4	214	5

TABLE 4A
(Page 2 of 2)

	Day 42		Day 56		Change Days 0-56	
	BMD	SEM	BMD	SEM	BMD	SEM
	ND	ND	ND	ND	ND	
	ND	ND	ND	ND	ND	
	243	6	241	5	3	5
	213	3	208	4	-8	3
	199	4	199	5	-12	2
	202	5	201	5	-12	7
	212	4	211	4	-2	4
	206	5	210	4	-5	3
	213	4	219	4	0	3

-34-

b.   Bone Measurements

        Bone surfaces undergoing active mineralization
were labeled in all animals included in this study with
the fluorescent bone marker declomycin and calcein by
5   injecting those markers on days 9 and 2 prior to
sacrifice, respectively.

        The rats were sacrificed by exsanguination
under ketamine/xylazine anesthesia. Numerous soft
tissues were collected for histological analyses and some
10   of these tissues were also weighed to examine organ
hypertrophy. The long bones (tibia and femur), the spine
and jaws were fixed or frozen for DEXA, mechanical and
bone histomorphometric analyses according to the
procedures detailed below.
15

        1.   DEXA Measurements.

        Spine DEXA scans were performed on days 0
(described above), 14, 28, 42, and 56 (the day before
sacrifice). DEXA whole-body scans (excluding the head
20   and tail) for measurements of body composition (lean body
mass and fat mass) were performed on days 7, 21, 35, and
49.

        The DEXA measurements were made with a Hologic
QDR-1000/W instrument according to directions provided by
25   the manufacturer. The rats were anesthetized with a
gaseous anesthetic of 2% isoflurane in oxygen during the
scans and body weights were also obtained while the rats
were anesthetized.

        Parameters measured on the tibia and femurs
30   during the DEXA analyses included length (mm), projected
area ("global area") (cm^2), global bone mineral content
(BMC) (mg), global bone marrow density (BMD) (mg/cm^2),
global bone mineral apparent density (global BMAD = BMC
divided by projected area to the 1.5 power) (mg/cm^3),
35   metaphyseal BMD (met BMD) (mg/cm^2), cortical BMD

-35-

(mg/cm^2), and epiphyseal BMD (epiph BMD) (mg/cm^2). The
results are shown in Table 5 (tibia) and Table 6 (femur).
In these tables, the numerical group designations
correspond to those shown in Fig. 1. Groups A and B were
5   taken for baseline measurements prior to treatment.

DEXA bone measurements were validated by
comparing mineral content of the tibia as measured by
both global tibia BMC values and ash weight. DEXA body
composition measurements were validated by comparing the
10   changes in body weight measured by both weighing on a
balance and DEXA determinations of total body mass.

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Table 5

DEXA Parameters on Excised Tibia

Group	Treatment	Length Mean	Length SEM	Global Area Mean	Global Area SEM	Global BMC Mean	Global BMC SEM	Global BMD Mean	Global BMD SEM	Global BMAD Mean	Global BMAD SEM	Met BMD Mean	Met BMD SEM	Cortical BMD Mean	Cortical BMD SEM	Epiph BMD Mean	Epiph BMD SEM
A	Sham - Pretreatment	39.8	0.4	1.41	0.02	323	7	229	3	193	2	241	3	216	3	287	6
B	OVX - Pretreatment	40.4	0.1	1.39	0.02	286	6	206	2	175	2	187	4	211	3	235	2
1	Sham - Saline	40.3	0.4	1.44	0.04	310	9	221	2	184	2	217	6	209	3	276	5
2	OVX - Saline	40.7	0.6	1.46	0.02	313	7	215	3	178	2	198	3	221	3	231	3
3	0.9 mg/kg IGF-I	40.4	0.4	1.39	0.04	284	12	205	4	174	2	192	5	210	6	217	6
4	2.6 mg/kg IGF-I	41.7	0.4	1.48	0.02	316	7	214	3	176	3	196	5	221	4	226	4
5	0.9 mg/kg IGF-I + IGFBP-3	41.2	0.3	1.46	0.03	306	7	210	2	174	3	197	4	215	3	223	5
6	2.6 mg/kg IGF-I + IGFBP-3	40.8	0.5	1.50	0.04	332	10	223	3	182	3	200	4	227	2	237	4
7	7.5 mg/kg IGF-I + IGFBP-3	42.0	0.2	1.59	0.02	355	4	223	3	177	3	204	3	233	3	238	4

-37-

Table 6

Group	Treatment	Length Mean	Length SEM	Global Area Mean	Global Area SEM	Global BMC Mean	Global BMC SEM	Global BMD Mean	Global BMD SEM	Global BMAD Mean	Global BMAD SEM	Met BMD Mean	Met BMD SEM
					DEXA Parameters on Excised Femurs								
A	Sham - Pretreatment	36.1	0.3	1.68	0.02	419	10	250	3	193	2	226	4
B	OVX - Pretreatment	35.9	0.3	1.60	0.02	360	7	223	3	175	3	172	5
1	Sham - Saline	36.5	0.3	1.71	0.03	429	15	250	5	191	2	212	5
2	OVX - Saline	36.9	0.3	1.75	0.03	401	10	229	2	173	1	177	3
3	0.9 mg/kg IGF-I	36.2	0.4	1.69	0.04	370	15	218	4	168	2	167	4
4	2.6 mg/kg IGF-I	37.6	0.4	1.78	0.02	411	10	231	3	173	1	173	3
5	0.9 mg/kg IGF-I + IGFBP-3	37.0	0.3	1.76	0.03	394	10	224	4	169	3	169	5
6	2.6 mg/kg IGF-I + IGFBP-3	37.4	0.3	1.80	0.04	424	13	235	4	175	2	174	4
7	7.5 mg/kg IGF-I + IGFBP-3	37.9	0.2	1.86	0.02	451	7	243	2	178	2	174	3

-38-

2. Bone Histomorphometry and Structure

Right femurs, tibias and lumbar vertebral
bodies (Lvb; L_4-L_5) were collected at necropsy, cleaned
of soft tissues, fixed in 70% ethanol for 48h, dehydrated
5   through upgraded ethanols, and embedded undecalcified in
methyl methacrylate. Longitudinal sections of distal
femoral epiphyses, metaphyses, Lvb, and cross-sections of
the mid femoral neck and the distal tibial diaphyses
proximal to the tibio-fibular junction were cut with a
10 Reichert-Jung supercut microtome (Reichert-Jung,
Heidelberg, Germany) to 15 μm in thickness. Three
consecutive sections were left unstained for dynamic
histomomorphometric analyses and the other three sections
were stained by the von Kossa method for mineral,
15 counterstained with toluidine blue, and analyzed for
static histomorphometic parameters. The 100 μm thick
cross-sections from femoral midshaft and mid-neck region
were cut with a precision low-speed bone saw (Isomet,
Buehler, Lake Bluff, IL), glued to plastic slides, ground
20 and polished to ~40 μm in thickness (Ecomet 3, Buehler,
Lake Bluff, IL) and analyzed for bone dynamic parameters.
The same set of slides were used for static bone
histomorphometry after being stained with the von Kossa
method and covered with cover slips. Bone dynamic
25 measurements were performed under UV light and 20-fold
magnification by using a semi-automated software program
called "Stereology" (KSS Computer Engineers, Magna, UT)
described earlier by Miller et al. (1989) Bone 7:283-287.
Static bone analyses were performed with an automated
30 television microscope image analysis system and analyzed
using "Image Analysis" software (KSS Computer Engineers,
Magna, UT).

For the fedoral neck only, internal structure
of trabecular bone, interconnectivity, and connectivity
35 between endocortical surface and trabecular network, the

-39-

"Nodal" and "Star volume" analyses were performed as described by Miller and Wronski (1993) <u>Anat. Record</u> <u>236</u>:433-441 and Bagi and Miller (1994) <u>Anat. Record</u> <u>239</u>:1-12. The marrow star volume is a direct measure of
5   trabecular separation, while the nodal analysis describes the number of nodes and struts, type of struts, and number of inter-trabecular connectivity. Connections between endocortical surface and trabecular network are important determinants of bone strength linking cortical
10   and cancellous bone into one anatomico-functional unit. The total number of endocortico-trabecular connections was counted and the percentages of free ending trabeculae versus those connected with other trabeculae and/or the endocortical surface were calculated. All measurements
15   and derived parameters describing bone dynamic and structure were performed as recommended by ASBMR Histomorphometric Committee (Parfitt et al. (1987) <u>J.</u> <u>Bone Min. Res.</u> <u>2</u>:595-610.

20   3.   <u>Bone Failure Strength and Internal</u>
                      <u>Cortical Bone Structure</u>
              Left femurs were cleaned of soft tissue and were used in DEXA measurements of bone mineral density (BMD) and bone mineral content (BMC). To test the
25   biomechanical properties of the cortical bone, the   · proximal and distal ends of each femur were secured in test fixtures, designed to allow freedom of motion parallel to the long axis of the bone. The fixtures were mounted in an MTS 858 Bionix System (MTS System Corp.,
30   Minneapolis, MN) and torque was measured with an externally conditioned miniature torque transducer (range 0-50 in-lbs; Model QWFK-8M/1941, Sensotec, Columbus, OH) as described earlier (van der Meulen, M.C.H., "Bone Strength in Young, Suspended Rats, Stanford University
35   Thesis, 1993). (Mechanical tests were performed at the

-40-

Department of Mechanical Engineering, Stanford
University, Stanford, CA; and Rehabilitation Research and
Development Center, Palo Alto, CA; and VA Medical Center,
Palo Alto, CA). After mechanical testing, the same
5   specimens were dehydrated in graded ethanols, embedded in
methyl methacrylate, and cut with a low speed bone saw
(Isomet, Buehler, Lake Bluff, IL) to ~100 μm in
thickness. The cross-sections (2-3) from the femoral
proximal midshafts were glued on plastic slides, ground
10   and polished to ~40 μm thickness and analyzed under
polarized light for cortical bone internal structure.
The measurements included thickness of the inner and
outer lamellar bone layer and thickness of the medially
placed woven bone. Following these measurements, samples
15   were stained by the von Kossa method, counterstained with
toluidine blue and cover-slipped. Static bone analyses
were performed including the calculation of the second
moment of inertia parameter.

20   4.   Cellular Bone Analyses
Right tibias were fixed in 10% neutral buffered
formalin for 48h, demineralized in Cal-Rite decalcifying
solution (Richland-Allan Medical, Richland, MI) for two
weeks and processed for paraffin embedding. Three μm
25   thick longitudinal sections through the proximal tibial
metaphysis were stained with hematoxylin and eosin and
Gomori's trichrome and were used for cellular bone
analyses.

30   5.   Statistical Analyses
Differences between plasma glucose values at
zero and two hours in Table 3 were analyzed by Student's
t-test. A two-factor analysis of variance was employed
in Table 3 for comparing differences between treatment
35   groups. Differences between the groups shown in Tables

-41-

7-16 or Figures 2a-b, 3, 4, 7a-d were tested for significance in a one-way analysis of variance. When the analysis of variance indicated significant differences among means, the differences were evaluated using

5   Dunnat's t-test and Fisher's Protected Least Significant Difference method for multiple comparisons (Netter et al. (1982) "Applied Statistics," Allyn and Bacon, Boston.) Statistical significance was considered at $P<0.05$, and results were expressed as the mean $\pm$ standard error (SE).

10

c.   Results

            Analysis of data revealed site-specific alterations of cortical and cancellous bone to ovariectomy and treatment with rhIGF-I and rhIGF-I/IGFBP-

15   3 complex. As shown in Table 3, the 2.5 mg/kg dose of rhIGF-I caused acute hypoglycemia which did not occur with any of the doses of rhIGF-I/IGFBP-3. The data presented in Table 4 indicate that no treatment had any influence on glycosylated hemoglobin values and that

20   increasing doses of rhIGF-I and rhIGFBP-3 led to higher serum levels of total IGF-I, rhIGF-I and rhIGFBP-3. Combining rhIGF-I with rhIGFBP-3 resulted in higher serum values of these agents than observed in rats treated with only rhIGF-I.

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            Spine BMD data throughout the study along with means of the individual changes are presented in Table 4A. As expected, ovariectomy led to a decline in spine BMD which was prevented by the 7.5 mg/kg dose of

30   rhIGF-I/IGFBP-3. Some protection was observed with the two lower doses of rhIGF-I/IGFBP-3 but not with the two doses of rhIGF-I. The DEXA data obtained on excised tibia (Table 5) and femurs (Table 6) show dose-related increases in global BMC and global BMD for both rhIGF-I

35   and rhIGF-I/IGFBP-3.

-42-

1.   Cortical Bone

Treatment with both formulations tested caused
a dose dependent increase in the body weight and in the
lean body mass of treated rats (Figs. 2A and 2B).

5   Periosteal bone formation activity was higher in all
treated groups and at all sites measured relative to Sham
or OVX controls (Tables 7, 8, 9, 11). However, the group
of rats treated with 7.5 mg/kg of rhIGF-I/IGFBP-3 complex
exhibited the highest increase in the periosteal bone

10   formation rate parameter (Figs. 4A and 5C, 5CC).

Endocortical bone resorption in treated rats,
which is a hallmark of cortical bone changes in estrogen
deficient women and rats, were not different from values
obtained for the Sham-op controls (Fig. 4B), showing

15   significantly lower values compared to OVX rats at the
femoral neck location (Table 11). Also, bone formation
at the endocortical envelope was increased at both bone
sites measured (femoral diaphyses, Table 8).

Internal structural analyses showed normal

20   "lamellar" configuration of the bone formed on both
cortical envelopes after rhIGF-I/IGFBP-3 treatment (Table
9; Fig. 6). Increased modeling-dependent bone formation
on the periosteal and endocortical bone envelopes and
moderate or decreased bone resorption on the endocortical

25   envelope resulted in thicker cortical bone in the treated
rats (Table 7, 9, 11; Figs. 5 and 6). Increased cortical
thickness and newly formed lamellar bone which is
mechanically superior to woven bone resulted in the
higher failure torque and polar moment of inertia

30   parameters in rats treated with 7.5 mg/kg of rhIGF-I/
IGFBP-3 complex (Table 10).

All treated animals exhibited similar increases
in longitudinal bone growth when compared to OVX of Sham
rats (Fig. 3). In general, treatment with rhIGF-I/

35   IGFBP-3 caused cortical bone thickening by adding

-43-

"lamellar" bone on both envelopes. The increase in lean
body component might potentiate periosteal bone formation
by increasing the muscle pull on the periosteum. Both
"lamellar" structure and increased cortical thickness
improved cortical bone strength after rhIGF-I/IGFBP-3
treatment.

2.   Cancellous Bone
        Cancellous bone dynamics and structure were
examined at four different locations in the rat skeleton.
Differences in different locations in the bone turnover
rates and functional anatomy between the designated sites
(distal femoral epiphysis, distal femoral metaphysis,
femoral mid-neck, and lumbar vertebral bodies) were
measured. Treatment with rhIGF-I/IGFBP-3 increased bone
formation parameters at all four sites (Tables 12, 13,
14, 15; Figs. 7 and 8). The increase in bone formation
rates (volume referents; Figs. 7A-7D) was the highest in
the group treated with 7.5 mg/kg of rhIGF-I/IGFBP-3
complex. Bone resorption had similar or lower values
compared to OVX rats (Tables 12-15). Such turnover
activity resulted in increased trabecular thickness and
preservation of the trabecular number in treated rats at
all sites measured, except in the femoral metaphysis.
        Primary and secondary spongiosa at the femoral
metaphyseal site was highly affected by the endochondral
bone elongation, particularly in the OVX rats and after
treatment with rhIBF or rhIBF-I/IBFBP-3 (Figs. 9A-9C).
This bone site has the highest bone turnover rate. Ovx
caused disappearance of "metabolic" trabeculae in the
central metaphyseal region with no mechanical value
(Fig. 9B) while treatment with rhIGF-I/IGFBP-3 complex
helped the restoration (Fig. 9C).
        Structural trabecular bone analyses were
performed at the femoral neck location, which is the most

-44-

relevant bone site for fractures in humans (Table 16;
Figs. 10A-10C). Besides having a very unique anatomy,
this bone site combines cortical and cancellous bone
interconnected at the endocortical surface. Cortical
5   bone thickness, cancellous bone mass, structure, and
connectivity between endocortical surface and trabecular
network are all important determinants of the bone
strength in the femoral neck region. The treatment with
the rhIGF-I/IGFBP-3 improved trabecular bone structure
10   and connectivity between the trabecular network and
endocortical surface. In general, data presented here
revealed benefits of systemically administered rhIBF-I/
IBFBP-3 on the entire musculo-skeletal system in
previously ovariectomized rats.

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Table 7: Cortical bone morphometry measured at the tibio-fibular junction in rats treated with rhIGF-I alone, or with equimolar doses of rhIGF-I/IGFBP-3 complex for eight weeks.

Parameter	Sham	Ovx	rhIGF-I(mg/kg)		rhIGF-I(mg/kg)+rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Periosteal perimeter (mm)	8.64±0.12	9.24±0.15†	9.19±0.18†	9.29±0.27†,*	9.21±0.21†	9.43±0.17†,*,*	9.55±0.17†,*
Endocortical perimeter (mm)	3.56±0.06	4.03±0.18†	3.8±0.15	4.12±0.2†	3.73±0.14	3.69±0.09b	3.71±0.11†
Organ area (mm²)	5.52±0.09	5.69±0.18†	5.59±0.27	5.83±0.24†	5.63±0.18	5.78±0.14*	6.27±0.16†,*
Marrow area (mm²)	0.95±0.02	1.08±0.05†	0.92±0.09	1.03±0.06†	0.67±0.05†,*	0.81±0.05*	0.93±0.04*
Cortical area (mm²)	4.57±0.07	4.62±0.13†	4.68±0.29*	4.8±0.17†,*	4.95±0.14†	4.97±0.13†	5.34±0.14†,*
Percent periosteal double labeled surface (%)	11.76±4.6	10.33±2.9†	43.09±8.4†,*	67.27±9.6†,*	44.12±3.8†,*	73.09±4.5†,*	90.04±2.2†,*
Periosteal new bone area (μm²)	0.004±0.002	0.002±0.001†	0.02±0.01†,*	0.03±0.01†,*	0.02±0.004†,*	0.07±0.01†,*,b	0.09±0.004†,*
Periosteal mineralized surface (%)	12.16±4.7	8.33±3.2†	43.52±8.8†,*	52.29±9.0†,*	44.68±4.1†,*	63.65±5.5†,*	84.26±2.8†,*
Periosteal mineral apposition rate (μm/d)	0.33±0.17	0.17±0.12†	0.71±0.05†,*	1.03±0.09†,*	0.62±0.04†,*	1.42±0.1†,*,b	1.43±0.02†,*
Periosteal bone formation rate (μm²/μm/d)	0.06±0.03	0.02±0.02	0.27±0.08†,*	0.4±0.11†,*	0.21±0.04†	0.77±0.11†,*,b	1.09±0.06†,*
Periosteal bone formation rate (%/y)	5.22±2.18	1.63±3.65	18.39±14.2†,*	28.8±8.78†,*	13.67±6.8†	53.7±13.8†,*,b	72.6±12.5†,*

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by Dunnett's test; * Significantly different from Ovx group, P<0.05 by Dunnett's test; b Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by Fisher's PLSD test.

Table 8: Dynamic cortical bone parameters measured at the femoral diaphyses in control rats and rats treated with 7.5mg/kg of rhIGF-I/IGFBP-3 complex for eight weeks.

Parameter	Basal Sham	Basal Ovx	Sham	Ovx	IGF-I/IGFBP-3
Periosteal mineralizing surface (%)	50.18±5.26	58.34±3.15	41.38±3.66[b]	32.87±4.05[a,b]	86.45±4.72[a,b,c,d]
Periosteal mineral appositional rate ($\mu m/d$)	0.61±0.02	1.03±0.04[a]	0.76±0.05[a,b]	0.83±0.04[a,b]	1.29±0.07[a,b,c,d]
Periosteal bone formation rate ($\mu m^2/\mu m/d$)	0.23±0.03	0.49±0.02[a]	0.26±0.03[b]	0.2±0.04[b]	1.07±0.13[a,b,d]
Periosteal bone formation rate (%/y)	21.27±2.5	45.85±0.9[a]	24.89±1.48[b]	21.4±3.02[b]	88.81±10.3[a,b,c,d]
Endocortical mineralizing surface (%)	1.02±0.43	4.03±0.69	1.69±0.27	2.14±0.4[c]	17.79±4.22[a,b,c,d]
Endocortical mineral appositional rate ($\mu m/d$)	0.009±0.01	0.425±0.19[a]	0.068±0.06[b]	0.052±0.05[b]	0.807±0.13[a,b,c,d]
Endocortical bone formation rate ($\mu m^2/\mu m/d$)	0.008±0.05	0.023±0.01	0.002±0.001	0.001±0.001	0.173±0.046[a,b,c,d]
Endocortical bone formation rate (%/y)	0.04±0.02	2.16±0.86	0.42±0.42	0.32±0.32	7.79±2.35[a,b,c,d]
Endocortical resorption surface (%)	1.01±0.25	4.14±0.8[a]	1.79±0.23[b]	6.33±0.79[a,b,c]	2.71±0.33[a,d]

Data are expressed as the mean ± SE; Significant diference $P<0.05$ by Fisher's PLSD test.
[a] Significantly different from Basal Sham group; [b] Significantly different from Basal Ovx group;
[c] Significantly different from Sham group; [d] Significantly different from Ovx group.

Table 9: Static cortical parameters measured at the femoral midshafts, and structural parameters measured at the postero-lateral 1/4 of the cross-sectional areas in control rats and rats treated with 7.5mg/kg of rhIGF-I/IGFBP-3 complex for eight weeks.

Parameter	Basal Sham	Basal Ovx	Sham	Ovx	IGF-I/IGFBP-3
Periosteal perimeter (mm)	9.54±0.09	9.65±0.23	9.76±0.11	10.51±0.19[a,b,c]	11.61±0.41[a,b,c,d]
Endocortical perimeter (mm)	5.4±0.13	5.79±0.18	5.38±0.12	5.98±0.18[a,c]	5.69±0.15
Organ area (mm²)	6.02±0.13	5.89±0.22	6.05±0.16	6.65±0.19[a,b,c]	7.03±0.15[a,b,c]
Marrow area (mm²)	1.76±0.05	2.0±0.13	1.82±0.09	2.18±0.13[a,c]	1.86±0.09[d]
Cortical area (mm²)	4.26±0.09	3.89±0.1	4.23±0.12	4.46±0.09[b]	5.17±0.09[a,b,c,d]
Average cortical width (µm)	601.4±7.9	568.6±1.5[a]	597.5±6.1[b]	574.8±3.8[a,c]	614.8±5.6[b,c,d]
Percent cortical area (%)	70.7±0.5	69.16±0.77	69.9±1.1	67.3±1.2[a]	73.6±0.9[a,b,c,d]
Structural parameters:					
Outer lamellar layer (µm)	277.1±8.9	273.5±8.4	274.5±11.3	316.8±14.6[a,b,c]	280.3±10.1[d]
Central woven layer (µm)	119.8±5.1	114.6±6.0	120.5±6.4	103.7±14.5	114.2±6.2
Inner lamellar layer (µm)	213.3±8.1	145.4±11.2[a]	202.6±9.2[b]	70.4±8.8[a,b,c]	242.6±13.7[a,b,c,d]

Data are expressed as the mean ± SE; Significant diference $P<0.05$ by Fisher's PLSD test.
a Significantly different from Basal Sham group; b Significantly different from Basal Ovx group;
c Significantly different from Sham group; d Significantly different from Ovx group.

Table 10: Femoral failure strength and general parameters in control rats and rats treated with 7.5mg/kg of rhIGF-I/IGFBP-3 complex for eight weeks.

Parameter	Basal Sham	Basal Ovx	Sham	Ovx	IGF-I/IGFBP-3
Failure torque (N/mm)	461.9±526.8	387.0±41.1[a]	390.6±31.2	443.6±21.2[b,c]	471.5±15.3[b,c,d]
Torsional rigidity (Ncm²/rad)	454±58	439±66	468±98	479±93	468±39
Linear stiffness (N·m/degrees)	43.4±2.6	40.7±2.5	42.6±2.5	43.1±3.9	44.9±2.8
Polar moment inertia (mm⁴)	6.6±0.7	6.3±0.9	6.5±0.6	6.8±1.3[b]	9.3±1.8[a,b,d]
Femoral length (mm)	36.0±0.3	35.8±0.3	36.6±0.4	36.8±0.4[b]	37.9±0.2[a,b,c,d]
Femoral area (cm²)	1.67±0.03	1.66±0.04	1.73±0.03	1.74±0.04	1.86±0.02[a,b,c,d]
Femoral BMC (g)	0.41±0.01	0.37±0.01[a]	0.44±0.02[b]	0.34±0.01[c]	0.45±0.01[a,b,d]
Femoral BMD (g/cm²)	0.25±0.003	0.23±0.004[a]	0.25±0.01[b]	0.23±0.003[a,c]	0.24±0.002[b,d]

Data are expressed as the mean ± SE; Significant diference $P<0.05$ by Fisher's PLSD test.
a Significantly different from Basal Sham group; b Significantly different from Basal Ovx group;
c Significantly different from Sham group; d Significantly different from Ovx group.

Table 11: Cortical bone morphometry measured at __femoral neck__ cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Entire organ							
Cortical bone area (%)	83.3±1.4	78.9±1.7*	84.3±1.0@	84.9±1.3@	84.1±1.5@	84.7±2.2@	87.3±1.4@
Bone marrow area (%)	13.9+0.1	13.1±0.1*	8.4±0.1*,@	6.4±0.1*,@	7.8±0.1*,@	7.9±0.1*,@	3.3±0.02@,c
Average cortical with (μm)	472±9	447±8	465±11	471±12	453±12	466±14	497±8*,@,c
Periosteal envelope							
Mineralized surface (%)	4.2±0.4	7.1±1.3*	7.9±1.4*	11.8±1.0*,@	8.0±0.9*	9.7±1.7*	15.4±1.3*,@,c
Mineral appositional rate (μm/d)	0.24+0.01	0.24±0.01	0.38±0.03*,@	0.48±0.02*,@	0.36±0.01*,@	0.35±0.01*,@	0.48±0.02*,@,b
Bone formation rate (μm²/μm/d)	0.01±0.001	0.01±0.003	0.02±0.01*	0.04±0.01*,@	0.02±0.003*	0.02±0.01*,b	0.05±0.01*,@,c
Bone formation rate (%/y)	7.3±0.8	9.6±1.3	34.7±5.2*,a	55.4±4.5*,@	37.47±6.1*,@	37.0±5.8*,@,b	59.0±6.2†,@,c

Table 11 (cont'd): Cortical bone morphometry measured at femoral neck cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Endocortical envelope							
Mineralized surface (%)	6.5+0.6	8.8±1.3	12.2±1.5*	11.7±1.6*	19.7±0.9*,@,a	17.9±2.3*,@,b	27.6±2.4*,@,c
Mineral appositional rate (μm/d)	0.23±0.01	0.24+0.01	0.49±0.02*,@	0.49±0.03*,@	0.6±0.01*,@,a	0.53±0.03*,@	0.57±0.02*,@,c
Bone formation rate (μm2/μm/d)	0.01±0.001	0.01±0.002	0.05±0.02*,@	0.04±0.01*,@	0.07±0.01*,@	0.08±0.01*,@,b	0.1±0.01*,@,c
Bone formation rate (%/y)	3.2±0.3	5.8±1.1	27.8±4.7*	27.4±5.4*,@	46.7±6.9*,@,a	60.6±11.1*,@,b	79.9±8.5*,@,c
Resorption perimeter (%)	4.8±0.2	7.5±0.6*	5.2±0.6@	3.9±0.4*,@	4.8±0.4	3.9±0.5@,b	3.2±0.3*,@,c

Data are expressed as the mean ± SE; *P<0.05 Significantly different from Sham group; @P<0.05 Significantly different from Ovx group; aP<0.05 Significantly different from rhIGF-I (0.9 mg/kg) group; bP<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group; cP<0.05 Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.

Table 12: Cancellous bone morphometry measured at distal femoral metaphysis after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Percent double labeled perimeter (%)	6.05±0.64	17.41±1.56†	14.21±0.77†	17.32±1.33†	18.02±0.84†	16.23±1.11†	26.61±2.26†.*
Percent resorption perimeter (%)	5.89±0.3	11.01±1.46†	9.61±0.67†	14.01±2.07†	9.08±1.01†	10.16±0.56†.b	14.45±0.97†.*
Corrected mineral appositional rate (μm/d)	0.17±0.03	0.47±0.06†	0.38±0.03†	0.41±0.03†	0.44±0.02†	0.44±0.03†	0.48±0.04†
Mineralized surface (%)	2.52±0.36	8.54±1.5†	5.35±1.79†.*	6.35±2.44†	10.48±3.24†.*	13.56±3.53†	19.89±4.09†.
Bone formation rate (μm²/μm/d)	0.001±0.001	0.021±0.006†	0.014±0.001†	0.012±0.002†	0.015±0.002†	0.014±0.002†	0.036±0.002†.*
Endochondral growth (μm/d)	3.77±0.21	3.81±0.29	5.57±0.33†.*	6.44±0.4†.*	5.89±1.33†.*	5.89±0.26†.*	6.15±0.28†.
Percent trabecular area (%)	25.02±1.58	14.95±0.92†	19.48±0.78†.*	14.73±1.33†	16.15±0.99†.a	17.86±1.41†	11.64±0.9†.*
Trabecular thickness (μm)	52.35±2.44	58.8±1.29†	66.24±0.58†	56.37±1.88	55.11±1.59	68.2±1.2†.*.b	50.0±2.12†.*
Trabecular number (No/mm)	4.78±0.21	2.53±0.12†	2.97±0.1†.*	2.54±0.17†	2.85±0.13†	2.74+0.13†	2.3±0.15†
Trabecular separation (μm)	229.8±12.7	492.69±26.2†	393.9±16.9†.*	501.1±39.2†	429.8±22.4†	447.3±37.1†	571.7±43.7†
Perimeter/Area ratio (mm/mm²)	32.43±1.54	28.48±0.64†	25.38±0.37†.*	29.21±1.0	29.7±0.89a	25.9±0.8†.*.b	33.23±0.83

Data are expressed as the mean ± SE; † Significantly different from Sham group, $P<0.05$ by analysis of variance followed by Dunnett's test; * Significantly different from Ovx group, $P<0.05$ by analysis of variance followed by Dunnett's test; [a] Significantly different from rhIGF-I (0.9 mg/kg) group, $P<0.05$ by analysis of variance followed by Fisher's PLSD method; [b] Significantly different from rhIGF-I (2.6 mg/kg) group, $P<0.05$ by analysis of variance followed by Fisher's PLSD method.

Table 13: Cancellous bone morphometry measured at distal femoral epiphysis after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Percent double labeled perimeter (%)	6.07±0.49	18.72±2.02†	9.35±0.62†,*	13.43±1.45†*	22.89±1.32†,a	24.12±2.06†,b	28.24±1.36†,*
Percent resorption perimeter (%)	5.93±0.48	7.18±0.57	5.11±0.39*	5.26±0.54*	4.73±0.49*	5.19±0.33*	7.13±0.52
Corrected mineral appositional rate (μm/d)	0.17±0.05	0.51±0.04†	0.57±0.01†	0.39±0.04†,*	0.55±0.02†	0.58±0.07†,b	0.7±0.02†,*
Mineralized surface (%)	1.56±0.09	9.65±2.58†	6.01±2.12†,*	9.28±3.47†	12.31±3.2†,*,a	17.71±4.05†,b	21.39±3.87†,*
Bone formation rate (μm²/μm/d)	0.001±0.003	0.016±0.004†	0.008±0.001†	0.008±0.002†	0.016±0.003†	0.023±0.005†,b	0.035±0.003†,*
Percent trabecular area (%)	37.19±1.44	31.01±0.74†	41.28±2.06*	37.8±1.67	36.3±1.54*,a	34.11±0.69	40.11±1.73*
Trabecular thickness (μm)	83.9±2.6	82.68±3.0	92.43±4.13	96.5±2.87†,*	92.04±2.33†,*	92.01±0.98†,*	98.79±4.13†,*
Trabecular number (No/mm)	4.33±0.16	3.79±0.16†	4.45±0.14*	3.71±0.13†	4.42±0.2*	3.89+0.1	4.07±0.17
Trabecular separation (μm)	206.4±11.8	265.5±11.6†	191.9±11.9*	241.1±10.9†	207.1±11.9	240.0±5.7†,*	210.9±10.9*
Perimeter/Area ratio (mm/mm²)	20.03±0.61	20.43±0.89	18.2±0.76	16.52±0.97†,*	19.17±1.15	18.45±0.41	17.06±0.84†,*

Data are expressed as the mean ± SE; † Significantly different from Ovx group, $P < 0.05$ by analysis of variance followed by Dunnett's test;
* Significantly different from Sham group, $P < 0.05$ by analysis of variance followed by Dunnett's test;
a Significantly different from rhIGF-I (0.9 mg/kg) group, $P < 0.05$ by analysis of variance followed by Fisher's PLSD method;
b Significantly different from rhIGF-I (2.6 mg/kg) group, $P < 0.05$ by analysis of variance followed by Fisher's PLSD method.

Table 14: Cancellous bone morphometry measured at lumbar vertebral bodies (L₄-L₅) after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	0.9	2.6	7.5
Percent double labeled perimeter (%)	8.17±0.99	14.2±1.16†	11.23±0.68	14.31±1.47†	12.0±0.79†	14.12±1.3†	16.06±1.21†		
Percent resorption perimeter (%)	6.19±0.48	8.88±0.97†	5.37±0.59*	10.1±1.26†	6.46±0.57	11.34±0.99†	8.57±0.71†		
Corrected mineral appositional rate (μm/d)	0.13±0.04	0.33±0.04†	0.4±0.01†	0.32±0.03†	0.29±0.03†,a	0.35±0.02†	0.35±0.04†		
Mineralized surface (%)	2.17±0.84	7.38±2.46†	6.28±1.25†	7.86±1.68†	7.21±2.15†	8.56±2.03†	11.97±4.37†		
Bone formation rate (μm²/μm/d)	0.001±0.001	0.008±0.002†	0.009±0.001†	0.007±0.002†	0.011±0.001†	0.007±0.002†	0.013±0.003†		
Percent trabecular area (%)	36.53±1.89	28.46±1.32†	30.06±1.32†	35.04±0.97*	32.95±1.46*	34.11±2.99*	37.65±1.57*		
Trabecular thickness (μm)	57.73±2.56	57.26±1.58	58.62±0.99	65.87±3.01†,*	63.66±3.34†,*	64.44±3.5†,*	66.42±2.51†,*		
Trabecular number (No/mm)	6.32±0.09	4.96±0.17†	5.12±0.18†	5.37±0.13*	5.2±0.15	5.25+0.24	5.67±0.09*		
Trabecular separation (μm)	145.1±6.05	210.2±11.1†	198.8±10.4†,*	174.8±3.15*	186.2±6.7*	185.0±15.0*	158.7±16.0*		
Perimeter/Area ratio (mm/mm²)	29.51±0.36	29.32±0.84	28.51±0.48	25.69±1.16†,*	26.74±1.46	26.46±1.52	25.39±1.05†,*		

Data are expressed as the mean ± SE; † Significantly different from Sham group, P<0.05 by analysis of variance followed by Dunnett's test;
* Significantly different from Ovx group, P<0.05 by analysis of variance followed by Dunnett's test;
a Significantly different from rhIGF-I (0.9 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method;
b Significantly different from rhIGF-I (2.6 mg/kg) group, P<0.05 by analysis of variance followed by Fisher's PLSD method.

Table 15: Cancellous bone morphometry measured at femoral neck cross-sectional areas after eight weeks of treatment with rhIGF-I alone or rhIGF-I/IGFBP-3 complex.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Percent resorption perimeter (%)	4.5±0.2	10.1±0.8*	6.1±0.5@	5.2±0.4@	6.1±0.6@	6.9±0.7*,@	6.6±0.7*,@
Corrected mineral appositional rate (μm/d)	0.23±0.01	0.32±0.01*	0.52±0.04*,@	0.54±0.01*,@	0.5±0.04*,@,a	0.52±0.02*,@,b	0.64±0.04*,@,c
Mineralized surface (%)	5.8±0.7	11.9±1.3*	12.3±0.9*	13.8±0.9*	14.9±1.4*	15.9±2.6*	19.1±2.2*
Bone formation rate (μm²/μm/d)	0.001±0.001	0.006±0.002*	0.012±0.002*	0.014±0.003*	0.014±0.003*	0.014±0.002*	0.015±0.003*
Percent trabecular area (%)	12.8±1.9	8.1±0.6*	7.4±1.1*	8.6±1.3*	8.1±1.3*	7.4±0.7*	9.4±0.8*
Trabecular thickness (μm)	83.5±6.2	69.5±5.4	79.9±2.6	75.1±3.8	69.3±8.6	78.3±2.2	88.2±6.1*,@,c
Trabecular number (No/mm)	10.8±1.4	5.6±0.5*	6.9±0.9*	8.1±1.4	7.8±0.6*	5.3+0.6*	8.5±0.2@,c
Trabecular separation (μm)	119.4+12.6	263.3±17.1*	206.8±15.2*,@	188.3±24.6*,@	183.1±20.3*,@	186.1±10.3*,@	158.4±7.9@
Perimeter/Area ratio (mm/mm²)	15.7±1.6	23.9±2.5*	19.6±0.8	20.7±1.0	20.0±2.2	18.3±1.3	21.9±2.6

Data are expressed as the mean ± SE; *$P<0.05$ Significantly different from Sham group; @$P<0.05$ Significantly different from Ovx group; a$P<0.05$ Significantly different from rhIGF-I (0.9 mg/kg) group; b$P<0.05$ Significantly different from rhIGF-I (2.6 mg/kg) group; c$P<0.05$ Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.

Table 16: Structural analyses of cancellous bone performed on von Kossa stained cross-sections of the femoral neck from rats treated for 8 weeks with rhIGF-I alone or the rhIGF-I/IGFBP-3 complex. A nodal analysis was employed for trabecular and endocortico-trabecular connectivities and star volume analysis for trabecular separation.

Parameter	Sham	Ovx	rhIGF-I (mg/kg)		rhIGF-I (mg/kg)+equimolar rhIGFBP-3		
			0.9	2.6	0.9	2.6	7.5
Nodal analysis							
Number of nodes	12.9±1.7	6.1±0.5*	5.5±0.3*	5.9±0.3*	7.0±0.7*	7.6±0.5*	6.5±0.3*
Number of struts	16.0±1.3	9.9±0.4*	7.3±0.4*,@	8.5±0.6*	8.3±0.7*	8.6±0.4*	9.3±0.6*
Type of strut (% of total)							
Free-free	5.8±1.2	20.6±1.8*	5.3±2.6@	8.7±2.9@	6.8±1.9@	7.1±2.8@	8.4±2.5@
Node-free	18.9±1.7	39.6±2.2*	29.8±3.6*	31.3±2.5*	21.2±5.4@,a	22.6±1.8@,b	24.3±1.2@
Node-node	75.4±2.0	39.7±3.1*	64.9±4.8*,@	61.7±3.1*,@	68.6±6.2@	70.3±3.5@	66.4±2.4@
Endocortico-trabecular connectivity							
Total number of endocortico-trabecular connections	11.6±0.9	6.8±0.5*	6.0±0.5*	6.4±0.5*	7.8±0.7*	6.9±0.6*	7.6±0.9*
Endocortico-endocortical (%)	95.1±1.2	42.3±1.4	50.2±2.4*	53.2±2.3*,@	54.3±1.7*,@	54.8±1.9*,@	55.1±2.3*,@
Endocortico-free ending (%)	4.9±0.3	57.7±1.4	49.8±2.1*	46.8±2.0*,@	45.7±1.3*,@	45.2±1.2*,@	44.9±2.1*,@
Star volume analysis							
Marrow volume (mm³)	0.01±0.001*	0.03±0.004*	0.01±0.002@	0.013±0.005@	0.01±0.002@	0.01±0.002@	0.006±0.001@

Data are expressed as the mean ± SE; *P<0.05 Significantly different from Sham group; @P<0.05 Significantly different from Ovx group; @P<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group;
aP<0.05 Significantly different from rhIGF-I (0.9 mg/kg) group; bP<0.05 Significantly different from rhIGF-I (2.6 mg/kg) group;
cP<0.05 Significantly different from rhIGF-I/IGFBP-3 (0.9 mg/kg and/or 2.6 mg/kg) group.

-56-

Example 3
Recombinant synthesis of IGF-I

Recombinant rhIGF-I was produced using a gene insert coding for the sequence shown in Figure 11 (SEQ ID
5   NO:1), as detailed below.

Materials

The bacterial strain used is a derivative of Escherichia coli K-12 strain W3110 which has been
10   lysogenized with DE3. (Studier, F. and Moffat, B. [1986] J. Mol. Biol. 189: 113-130). This lysogen carries the gene for T7 RNA polymerase under the control of the lacUV5 promoter.

This host strain was transformed with plasmids
15   pER10088 by selection for tetracycline-resistance.

Description of Plasmids

The three expression vectors used in this work are similar to pJU1002 and pJU1003 (Squires, C.H., et al.
20   [1988] J. Biol. Chem. 263: 16297-16302) except that the genes inserted downstream of the translational coupler are ubiquitin-IGF (pER10088). In addition, pER 10088 differs from pJU1003 in that it does not contain the synthetic 16 bp adaptor sequence at the 5' end of the tet
25   gene in that plasmid; however, it does contain DNA insertions at the unique PvuII site in the pBR322-derived backbone: pER10088 contains a linker 5'...CCTCGAGG...3' at that location.

30   ## Description of Gene Inserts

As produced for studies carried out in support of the present invention, the vector pER10088 contained an open reading frame (ORF) comprising (in order 5' to 3') an ATG triplet (initiation), the 76 codons of yeast
35   ubiquitin, 70 synthetic codons of mature human insulin

-57-

growth factor I (Figure 11), and a termination codon. In
this case, the ORF is positioned relative to the
translational coupler exactly as described by Squires et
al. (above) for fibroblast growth factor.

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IGF purification

            E. coli cells producing IGF-I were broken open
using a Gaulin mill or Microfluidizer in 5 volumes/wt
(e.g.,1 1/200 g cell paste) of 50 mM sodium acetate and 1
10   mM EDTA, pH 5.5 while its temperature was maintained at
not higher then 10°C. The lysate was checked for the
presence of whole E. coli cells using a light microscope.
To the lysate was added one-hundredth volume of a 10%
(w/v) solution of polyethyleneimine and then centrifuged
15   at 10,000 for 20 minutes to separate supernatant from
pellet. The pellet was re-extracted twice more in 2.5
volumes of 20 mM potassium phosphate, 20 mM DTT and 2 M
urea pH 5.8 and centrifuged as above. The pellet was used
for further purification steps.
20   IGF was extracted from the pellet with 5
volumes/wt of 20 mM Tris pH 8.0 containing 6 M urea, 40
mM DTT and 1 mM EDTA and filtered through a Sartorius
0.8 μ filter. The filtrate was diluted with the same
buffer to a final protein concentration of 3 mg/ml, then
25   diluted with 2 volumes of 20 mM Tris and 1 mM EDTA pH
8.0. This mixture was subjected to a DNA removal step
using protamine sulfate and Q-Sepharose, according to
standard methods. Ubiquitin protein peptidase was added
to the mixture to release IGF-I from the ubiquitin IGF-I
30   fusion protein, and IGF-I was refolded overnight at
ambient temperature. A typical refolding reaction was
carried out at about 1 mg/ml protein concentration, 1.5
to 2 M urea and pH range 8-9 in presence of Tris buffer
at 20 to 50 mM and a ratio of DTT/Cystamine of 1.
35   Refolding performed as above yields about 40% of the

-58-

correctly refolded IGF-I compared to the initial amount
of IGF-I.

The refolded IGF-I solution was clarified,
buffer was exchanged using ultrafiltration system and
5   loaded onto a cation exchange column equilibrated in 50
mM sodium acetate buffer, pH 5.5. Following the loading
of the IGF-I solution onto the column, the column was
washed with the equilibration buffer and purified IGF-I
was then eluted from the column by application of 20
10   column volumes of gradient starting with equilibration
buffer and finishing with either 0.25 or 0.5 M sodium
chloride. The appropriate fractions of pure IGF-I were
pooled.

Pooled fractions from the cation exchange
15   column were acidified to pH 2.5 with 10% trifluoroacetic
acid (TFA). The solution was filtered through a 0.2 μm
filter and loaded onto a Vydac C-4 column equilibrated in
0.1% TFA in water. The column was developed with a linear
gradient of 0 to 40% of acetonitrile in 0.1% TFA. The
20   fractions containing purified IGF were assayed by
SDS-PAGE, then pooled and lyophilized.

Example 4
Recombinant Synthesis of IGFBP-3

25   Recombinant rhIGFBP-3 was produced using as a
gene insert coding for the sequence shown in Figure 12
(SEQ ID NO:2), as detailed below. Figures 13-15 show
alternative exemplary gene sequences that can be used in
the present invention according to methods standard in
30   the art or analogous to the procedures described below.
Figure 13 is a preferred coding sequence used in the
experiments herein.

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-59-

Materials

        The bacterial strain used in experiments
carried out in support of the present invention is a
derivative of Escherichia coli K-12 strain W3110 which
5   was lysogenized with DE3. (Studier, F. and Moffat, B.
[1986] J. Mol. Biol. 189: 113-130). This lysogen carries
the gene for T7 RNA polymerase under the control of the
lacUV5 promoter. This host strain was transformed with
plasmid pDJ12833 by selection for tetracycline-
10   resistance.

Description of Plasmids

        The three expression vectors used in this work
are similar to pJU1002 and pJU1003 (Squires, C.H., et al.
15   [1988] J. Biol. Chem. 263: 16297-16302, incorporated
herein by reference) except that the gene was inserted
downstream of the translational coupler IGFBP-3
(pDJ12833). In addition, pDJ12833 differs from pJU1003
in that it does not contain the synthetic 16 bp adaptor
20   sequence at the 5' end of the tet gene in that plasmid;
however, it does contain DNA insertions at the unique
PvuII site in the pBR322-derived backbone: pDJ12833
contains a 385 bp fragment carrying the par locus of
pSC101 (Meacock, P.A., and Cohen, S.N. [1980] Cell 20:
25   529-542).

Description of Gene Insert

        pDJ12833 contains an ORF comprising an ATG
triplet followed by the 264 codons of mature human
30   IGFBP-3 (Figure 13). The amino terminal 95 codons were
synthetic; the remainder were derived from the natural
cDNA for this gene.
        In this case, the ORF was positioned relative
to the translational coupler exactly as described by
35   Squires et al. ([1988] J. Biol. Chem. 263:16297-16302,

-60-

incorporated herein by reference) for fibroblast growth
factor.

IGFBP-3 purification

5   The E. coli cells producing IGFBP-3 ("BP-3")
were broken open using a Gaulin mill or Microfluidizer in
6 volumes/wt (e.g., 1.2 1/200 g cell paste) of 20 mM
Tris-HCl and 5 mM EDTA, pH 8 while its temperature was
maintained at not higher then 10°C. The lysate was
10   checked for the presence of whole E. coli cells by light
microscopy and then centrifuged at 10,000 g for 20
minutes to separate supernatant from pellet. The pellet
was re-extracted twice more in 3 volumes of 20 mM Tris-
HCl pH 8 and centrifuged as above. The resulting pellet
15   was used for further purification.
          BP-3 was extracted from the pellet with 2.5
volumes/wt of 20 mM Tris pH 8.0 containing 6 M GdHCl, 25
mM DTT and 5 mM EDTA and filtered through a Sartorius
0.8 μ filter. The filtrate was diluted with the above
20   buffer to a final protein concentration of 4 mg/ml
followed with 1 volume of 20 mM Tris and 5 mM EDTA pH
8.0. This mixture was subjected to a DNA removal step
using protamine sulfate, according to standard procedures
known in the art. The protamine sulfate precipitate was
25   removed by filtration, and BP-3 was subjected to the
refolding reaction. A typical refolding reaction was
effected at about 0.5 to 1 mg/ml protein concentration,
1.0 to 1.5 M GdHCl at pH 8-9 in the presence of Tris
buffer at 20 to 50 mM, having reducing/oxidizing agents
30   molar ratio of 1. Refolding performed as above results
in greater than 60% of the initial amount of BP-3.
          The refolded BP-3 solution was clarified,
buffer was exchanged using an ultrafiltration system and
loaded onto a cation exchange column equilibrated in 20
35   mM sodium phosphate buffer, pH 7. Following loading of

-61-

the BP-3 post-refolding solution onto a cation exchange
column, the column was washed with the equilibration
buffer and purified BP-3 was then eluted from the column
by application of 20 column volumes of gradient starting
5   with equilibration buffer and finishing with 0.8 M sodium
chloride. The appropriate fractions of BP-3 were pooled.
        The salt concentration of the pooled BP-3-
containing fractions was increased to 0.6 M with ammonium
sulfate, and then loaded onto a hydrophobic interaction
10 chromatography matrix column equilibrated with salt
containing acetate/phosphate buffer having a pH between 5
to 6. BP-3 was eluted with a linear ammonium sulfate
gradient ending with 0% ammonium sulfate buffered at pH
5-6. The appropriate fractions were then pooled and
15 saved.
        The pooled fractions containing BP-3 were
brought to 0.1% trifluoroacetic acid (TFA), and the
solution was loaded onto a C4 RP-HPLC column equilibrated
with 0.1% TFA in water. The column was washed with
20 equilibration buffer and then eluted with a linear
gradient of acetonitrile containing 0.1% TFA. The
fractions containing BP-3 were pooled and lyophilized.

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-62-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: CELTRIX PHARMACEUTICALS, INC.

    (ii) TITLE OF INVENTION: IGF/IGFBP COMPLEX FOR PROMOTING BONE
        FORMATION AND FOR REGULATING BONE REMODELING

   (iii) NUMBER OF SEQUENCES: 7

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: MORRISON & FOERSTER
        (B) STREET: 755 Page Mill Road
        (C) CITY: Palo Alto
        (D) STATE: California
        (E) COUNTRY: USA
        (F) ZIP: 94304-1018

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: Patent In Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: New
        (B) FILING DATE: New
        (C) CLASSIFICATION:

  (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: PARK, FREDDIE K.
        (B) REGISTRATION NUMBER: 35,636
        (C) REFERENCE/DOCKET NUMBER: 2095-2072.40

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (415) 813-5600
        (B) TELEFAX: (415) 494-0792
        (C) TELEX: 706141

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 70 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe
1               5                   10                  15

Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
            20                  25                  30

Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
            35                  40                  45

Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Ala Pro Leu
    50                  55                  60
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-63-

```
Lys Pro Ala Lys Ser Ala
65                70
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 264 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ix) FEATURE:
        (A) NAME/KEY: Modified-site
        (B) LOCATION: 5
        (D) OTHER INFORMATION: /note= "There is natural
heterogeneity at this position; glycine can also occur at this
position."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Gly Ala Ser Ser Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro Cys
1               5               10              15

Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Pro Ala Val Cys Ala
        20              25              30

Glu Leu Val Arg Glu Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu
        35              40              45

Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly
    50              55              60

Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu
65              70              75              80

Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu
            85              90              95

Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu
            100             105             110

Ser Glu Glu Asp Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser
        115             120             125

Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser Lys
        130             135             140

Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys
145             150             155             160

Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu
            165             170             175

Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp
            180             185             190

Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val
        195             200             205

His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys
    210             215             220

Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys
225             230             235             240
```

-64-

```
          Tyr Gly Gln Pro Leu Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val
                      245                 250                 255

          His Cys Tyr Ser Met Gln Ser Lys
                  260
```

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 795 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ix) FEATURE:
            (A) NAME/KEY: CDS
            (B) LOCATION: 1..792

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG GGT GCA TCT TCT GCA GGT TTA GGT CCA GTT GTT CGT TGT GAA CCA          48
Met Gly Ala Ser Ser Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro
 1               5                  10                  15

TGT GAT GCT CGT GCT CTT GCT CAA TGT GCT CCA CCA GCT GTT TGT GCT          96
Cys Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Ala Val Cys Ala
                20                  25                  30

GAA CTT GTT CGT GAA CCG GGT TGT GGT TGT TGT CTG ACT TGC GCA CTT         144
Glu Leu Val Arg Glu Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu
            35                  40                  45

TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT TGT GGT TCT GGT         192
Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly
        50                  55                  60

CTG CGT TGT CAA CCA TCT CCA GAT GAA GCT CGT CCT CTG CAG GCT CTG         240
Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu
 65                  70                  75                  80

CTG GAC GGT CGT GGT CTG TGC GTT AAC GCT TCC GCT GTT TCC CGT CTG         288
Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu
                85                  90                  95

CGC GCC TAC CTG CTG CCA GCG CCG CCA GCT CCA GGA AAT GCT AGT GAG         336
Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu
            100                 105                 110

TCG GAG GAA GAC CGC AGC GCC GGC AGT GTG GAG AGC CCG TCC GTC TCC         384
Ser Glu Glu Asp Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser
        115                 120                 125

AGC ACG CAC CGG GTG TCT GAT CCC AAG TTC CAC CCC CTC CAT TCA AAG         432
Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser Lys
    130                 135                 140

ATA ATC ATC ATC AAG AAA GGG CAT GCT AAA GAC AGC CAG CGC TAC AAA         480
Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys
145                 150                 155                 160

GTT GAC TAC GAG TCT CAG AGC ACA GAT ACC CAG AAC TTC TCC TCC GAG         528
Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu
                165                 170                 175
```

-65-

```
TCC AAG CGG GAG ACA GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA GAC          576
Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp
            180             185                 190

ACA CTG AAT CAC CTG AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT GTA          624
Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val
            195             200                 205

CAC ATT CCC AAC TGT GAC AAG AAG GGA TTT TAT AAG AAA AAG CAG TGT          672
His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys
            210             215                 220

CGC CCT TCC AAA GGC AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT AAG          720
Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys
225             230             235                 240

TAT GGG CAG CCT CTG CCA GGC TAC ACC ACC AAG GGG AAG GAG GAC GTG          768
Tyr Gly Gln Pro Leu Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val
            245             250                 255

CAC TGC TAC AGC ATG CAG AGC AAG TAG                                      795
His Cys Tyr Ser Met Gln Ser Lys
            260
```

(2) INFORMATION FOR SEQ ID NO:4:

       (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 264 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Gly Ala Ser Ser Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro
1               5               10                  15

Cys Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Ala Val Cys Ala
            20              25                  30

Glu Leu Val Arg Glu Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu
        35              40                  45

Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly
    50              55                  60

Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu
65              70                  75                  80

Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu
            85              90                  95

Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu
            100             105                 110

Ser Glu Glu Asp Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser
        115             120                 125

Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser Lys
    130             135                 140

Ile Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys
145             150             155                 160
```

-66-

Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu
            165   170   175

Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp
            180   185   190

Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val
            195   200   205

His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys
    210   215   220

Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys
225   230   235   240

Tyr Gly Gln Pro Leu Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val
            245   250   255

His Cys Tyr Ser Met Gln Ser Lys
            260

(2) INFORMATION FOR SEQ ID NO:5:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 811 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGCTTCTT CTGCTGGTCT TGGACCAGTT GTTCGTTGTG AACCATGTGA TGCACGAGCT   60

TTAGCTCAAT GTGCTCCACC ACCAGCTGTT TGTGCTGAAT TAGTTCGAGA ACCAGGTTGT   120

GGTTGTTGTT TAACTTGTGC TTTATCTGAA GGTCAACCAT GTGGTATTTA TACTGAACGT   180

TGCGGTAGTG GTTTGCGTTG TCAACCAAGC CCAGATGAAG CTAGGCCTTT ACAAGCATTA   240

TTAGATGGTC GAGGTCTGTG TGTTAATGCG TCCGCTGTTT CTCGATTGCG CGCTTATTTA   300

TTACCTGCCC CACCGGCACC GGGTAACGCC TCCGAAAGCG AAGAGGATCG TTCTGCGGGT   360

TCCGTTGAAT CTCCAAGTGT GAGTTCTACC CATCGAGTTA GCGACCCGAA ATTTCATCCG   420

TTGCACTCTA AAATCATTAT TATTAAAAAG GGTCACGCAA AGGATTCTCA ACGTTATAAG   480

GTGGATTATG AAAGCCAATC TACCGACACT CAAAATTTTA GTAGTGAAAG TAAACGTGAA   540

ACCGAGTACG GCCCGTGTCG ACGTGAGATG GAGGATACCT TAAACCATTT AAAATTTTTG   600

AACGTTTTAT CCCCGCGTGG CGTTCATATC CCGAATTGCG ATAAAAAAGG CTTCTACAAA   660

AAGAAACAAT GCCGTCCGAG TAAGGGTCGT AAACGAGGTT TTTGTTGGTG CGTTGACAAA   720

TACGGTCAAC CGTTGCCGGG TTATACTACT AAAGGCAAAG AAGATGTTCA TTGTTATTCT   780

ATGCAATCTA AATAATGCAT CTCGAGAATT C   811

(2) INFORMATION FOR SEQ ID NO:6:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 876 base pairs

-67-

```
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ix) FEATURE:
                (A) NAME/KEY: CDS
                (B) LOCATION: 1..873

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
```

```
ATG CAG CGG GCG CGA CCC ACG CTC TGG GCC GCT GCG CTG ACT CTG CTG          48
Met Gln Arg Ala Arg Pro Thr Leu Trp Ala Ala Ala Leu Thr Leu Leu
265             270             275                 280

GTG CTG CTC CGC GGG CCG CCG GTG GCG CGG GCT GGC GCG AGC TCG GCG          96
Val Leu Leu Arg Gly Pro Pro Val Ala Arg Ala Gly Ala Ser Ser Ala
                285             290             295

GGC TTG GGT CCC GTG GTG CGC TGC GAG CCG TGC GAC GCG CGT GCA CTG         144
Gly Leu Gly Pro Val Val Arg Cys Glu Pro Cys Asp Ala Arg Ala Leu
                300             305             310

GCC CAG TGC GCG CCT CCG CCC GCC GTG TGC GCG GAG CTG GTG CGC GAG         192
Ala Gln Cys Ala Pro Pro Pro Ala Val Cys Ala Glu Leu Val Arg Glu
                315             320             325

CCG GGC TGC GGC TGC TGC CTG ACG TGC GCA CTG AGC GAG GGC CAG CCG         240
Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu Ser Glu Gly Gln Pro
                330             335             340

TGC GGC ATC TAC ACC GAG CGC TGT GGC TCC GGC CTT CGC TGC CAG CCG         288
Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly Leu Arg Cys Gln Pro
345             350             355             360

TCG CCC GAC GAG GCG CGA CCG CTG CAG GCG CTG CTG GAC GGC CGC GGG         336
Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu Leu Asp Gly Arg Gly
                365             370             375

CTC TGC GTC AAC GCT AGT GCC GTC AGC CGC CTG CGC GCC TAC CTG CTG         384
Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu Arg Ala Tyr Leu Leu
                380             385             390

CCA GCG CCG CCA GCT CCA GGA AAT GCT AGT GAG TCG GAG GAA GAC CGC         432
Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu Ser Glu Glu Asp Arg
                395             400             405

AGC GCC GGC AGT GTG GAG AGC CCG TCC GTC TCC AGC ACG CAC CGG GTG         480
Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser Ser Thr His Arg Val
                410             415             420

TCT GAT CCC AAG TTC CAC CCC CTC CAT TCA AAG ATA ATC ATC ATC AAG         528
Ser Asp Pro Lys Phe His Pro Leu His Ser Lys Ile Ile Ile Ile Lys
425             430             435             440

AAA GGG CAT GCT AAA GAC AGC CAG CGC TAC AAA GTT GAC TAC GAG TCT         576
Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys Val Asp Tyr Glu Ser
                445             450             455

CAG AGC ACA GAT ACC CAG AAC TTC TCC TCC GAG TCC AAG CGG GAG ACA         624
Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu Ser Lys Arg Glu Thr
                460             465             470

GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA GAC ACA CTG AAT CAC CTG         672
Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp Thr Leu Asn His Leu
                475             480             485
```

-68-

```
AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT GTA CAC ATT CCC AAC TGT    720
Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val His Ile Pro Asn Cys
    490                 495                 500

GAC AAG AAG GGA TTT TAT AAG AAA AAG CAG TGT CGC CCT TCC AAA GGC    768
Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys Arg Pro Ser Lys Gly
505                 510                 515                 520

AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT AAG TAT GGG CAG CCT CTC    816
Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys Tyr Gly Gln Pro Leu
                525                 530                 535

CCA GGC TAC ACC ACC AAG GGG AAG GAG GAC GTG CAC TGC TAC AGC ATG    864
Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val His Cys Tyr Ser Met
            540                 545                 550

CAG AGC AAG TAG                                                     876
Gln Ser Lys
        555
```

(2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 291 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Gln Arg Ala Arg Pro Thr Leu Trp Ala Ala Ala Leu Thr Leu Leu
1               5                   10                  15

Val Leu Leu Arg Gly Pro Pro Val Ala Arg Ala Gly Ala Ser Ser Ala
            20                  25                  30

Gly Leu Gly Pro Val Val Arg Cys Glu Pro Cys Asp Ala Arg Ala Leu
        35                  40                  45

Ala Gln Cys Ala Pro Pro Pro Ala Val Cys Ala Glu Leu Val Arg Glu
    50                  55                  60

Pro Gly Cys Gly Cys Cys Leu Thr Cys Ala Leu Ser Glu Gly Gln Pro
65                  70                  75                  80

Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser Gly Leu Arg Cys Gln Pro
                85                  90                  95

Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala Leu Leu Asp Gly Arg Gly
            100                 105                 110

Leu Cys Val Asn Ala Ser Ala Val Ser Arg Leu Arg Ala Tyr Leu Leu
        115                 120                 125

Pro Ala Pro Pro Ala Pro Gly Asn Ala Ser Glu Ser Glu Glu Asp Arg
    130                 135                 140

Ser Ala Gly Ser Val Glu Ser Pro Ser Val Ser Ser Thr His Arg Val
145                 150                 155                 160

Ser Asp Pro Lys Phe His Pro Leu His Ser Lys Ile Ile Ile Ile Lys
                165                 170                 175
```

-69-

```
Lys Gly His Ala Lys Asp Ser Gln Arg Tyr Lys Val Asp Tyr Glu Ser
            180             185             190

Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser Glu Ser Lys Arg Glu Thr
        195             200             205

Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu Asp Thr Leu Asn His Leu
    210             215             220

Lys Phe Leu Asn Val Leu Ser Pro Arg Gly Val His Ile Pro Asn Cys
225             230             235             240

Asp Lys Lys Gly Phe Tyr Lys Lys Lys Gln Cys Arg Pro Ser Lys Gly
            245             250             255

Arg Lys Arg Gly Phe Cys Trp Cys Val Asp Lys Tyr Gly Gln Pro Leu
            260             265             270

Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp Val His Cys Tyr Ser Met
        275             280             285

Gln Ser Lys
    290
```

-70-

CLAIMS

1.   A method for stimulating bone formation in
a subject who has a bone marrow disorder causing bone
5   loss, said method comprising administering to the subject
pharmaceutically effective doses of IGF-I and IGFBP-3.

2.   The method of claim 1 wherein the marrow
disorder is selected from the group consisting of plasma
10   cell dyscrasias, leukemia, lymphomas, systemic
mastocytosis, anemias, lipidoses and mucopoly-
saccharidoses.

3.   The method of claim 1 wherein a
15   pharmaceutically effective dose of an inhibitor of bone
resorption is also administered.

4.   The method of claim 3, wherein the IGF-I/
IGFBP and the inhibitor of bone resorption are
20   administered sequentially.

5.   The method of claim 3, wherein the
inhibitor of bone resorption is an estrogen, tamoxifen, a
calcitonin, a bisphosphonate or a growth factor having
25   anti-resorptive activity.

6.   The method of claim 5 wherein the growth
factor is a transforming growth factor-β.

30   7.   The method of claim 3, wherein the
treatment is prophylactic.

8.   A method for stimulating bone formation in
a subject who has a connective tissue disorder causing
35   bone loss, said method comprising administering to the

-71-

subject pharmaceutically effective doses of IGF-I and
IGFBP-3.

          9.   The method of claim 8 wherein the
connective tissue disorder is selected from the group
consisting of osteogenesis imperfecta, Ehlers-Danlos
syndrome, Marfans syndrome, cutis laxa, homocystinuria,
Mankes's syndrome and scurvy.

          10.   The method of claim 9 wherein a
pharmaceutically effective dose of an inhibitor of bone
resorption is also administered.

          11.   A method for stimulating bone formation in
a subject who has drug-related osteoporosis, said method
comprising administering to the subject pharmaceutically
effective doses of IGF-I and IGFBP-3.

          12.   The method of claim 11 wherein the drug
causing the osteoporosis is selected from the group
consisting of corticosteroids, heparin, oral
anticoagulants, anticonvulsants, methotrexate, thyroid
hormone, lithium and gonadotrophin-releasing analogs.

          13.   The method of claim 11 wherein a
pharmaceutically effective dose of an inhibitor of bone
resorption is also administered.

          14.   A method for stimulating bone formation in
a subject who has bone loss due to pregnancy, lactation,
chronic hypophosphatemia, hyperphosphatasia, insulin-
dependent diabetes mellitus, anorexia nervosa, cadmium
poisoning, juvenile osteoporosis or Paget's disease of
bone, said method comprising administering to the subject
pharmaceutically effective doses of IGF-I and IGFBP-3.

-72-

15.   The method of claim 14 wherein a pharmaceutically effective dose of an inhibitor of bone resorption is also administered.

16.   A method for stimulating bone formation in a subject who has periodontal bone loss, said method comprising administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

17.   A method for stimulating bone formation in a subject who has osteoarthritis-related bone loss, said method comprising administering to the subject pharmaceutically effective doses of IGF-I and IGFBP-3.

18.   A composition for inducing bone formation in a subject, comprising IGF-I, IGFBP and an inhibitor of bone resorption in a pharmaceutically acceptable excipient.

19.   The composition of claim 18, wherein the inhibitor of bone resorption is an estrogen, tamoxifen, a calcitonin, a bisphosphonate or a growth factor having anti-resorptive activity.

20.   The composition of claim 18, wherein the IGF-I or the IGFBP is conjugated to the inhibitor of bone resorption.

21.   The composition of claim 18, further comprising a sustained-release vehicle.

22.   The composition of claim 18, wherein the IGFBP is IGFBP-3.

1/21
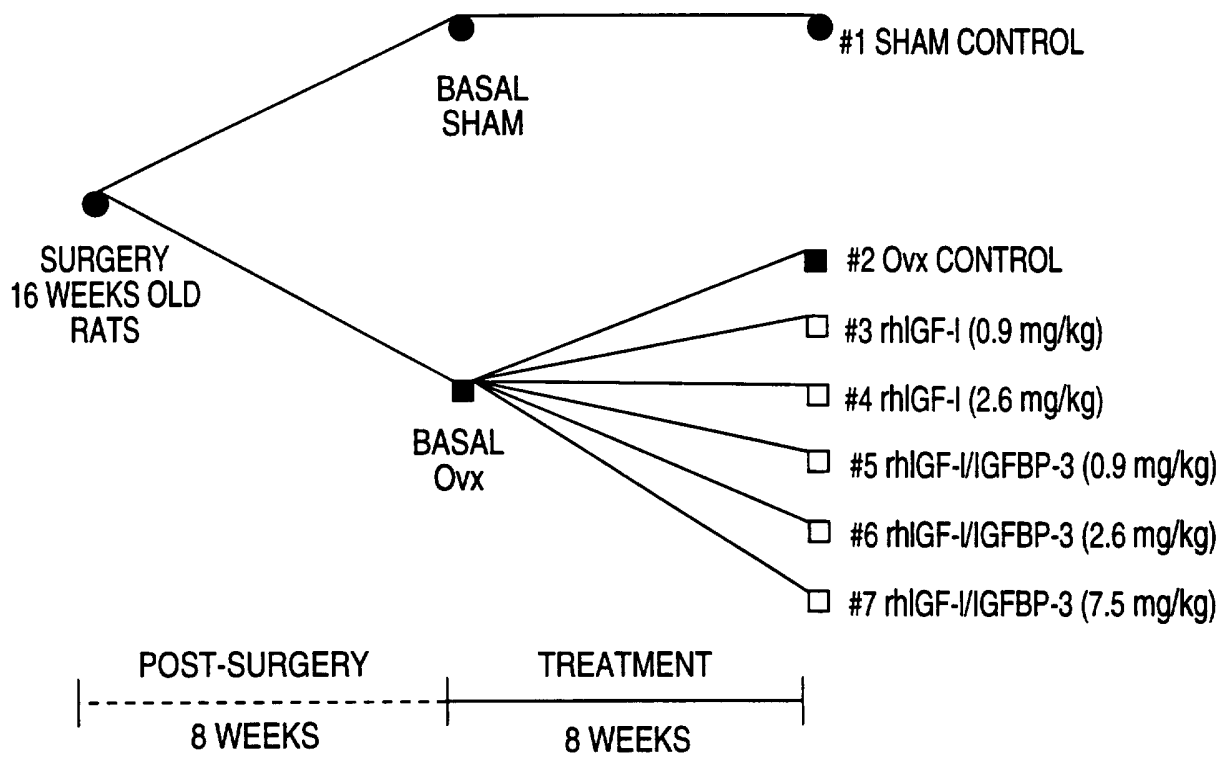
FIG. 1
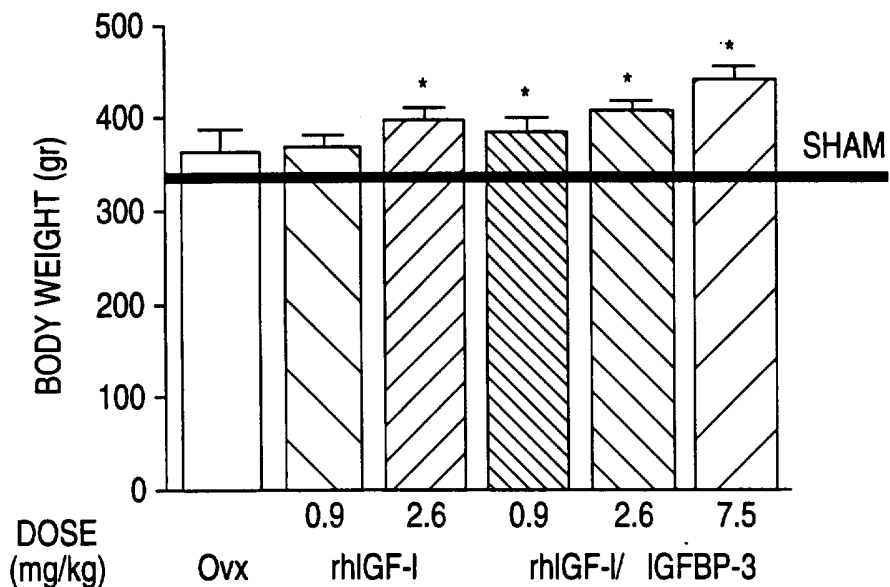
FIG. 2A　　*SIGNIFICANTLY DIFFERENT FROM Ovx GROUP
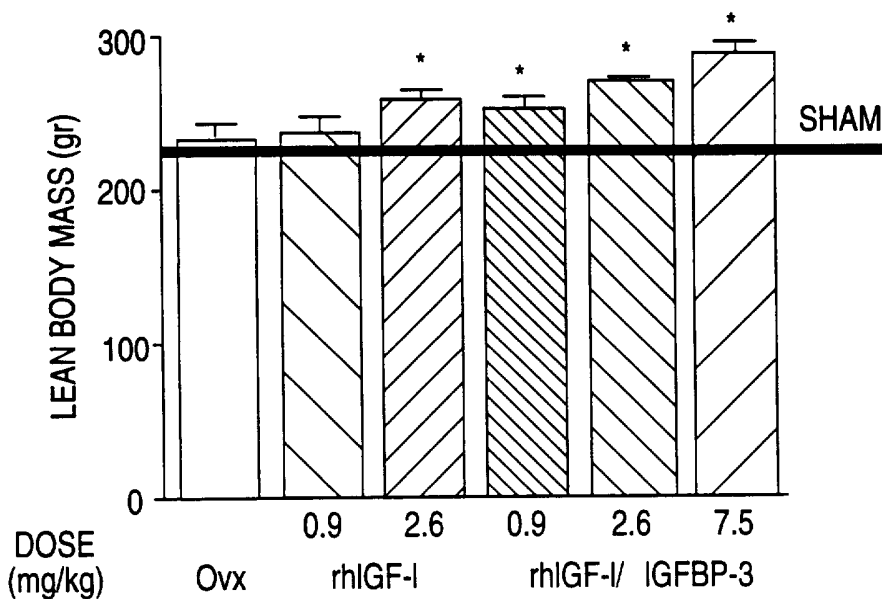
FIG. 2B　　*SIGNIFICANTLY DIFFERENT FROM Ovx GROUP

3/21
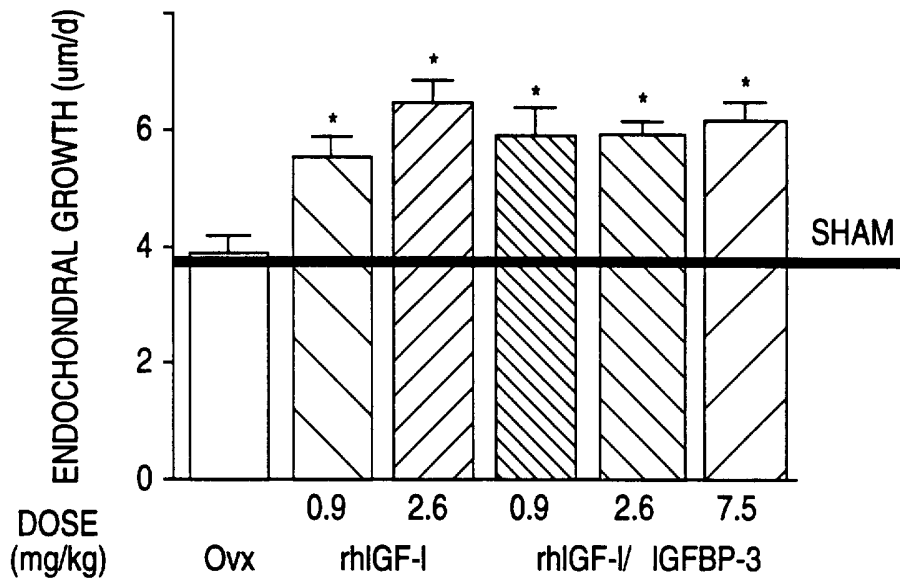
FIG. 3   *SIGNIFICANTLY DIFFERENT
                FROM Ovx GROUP
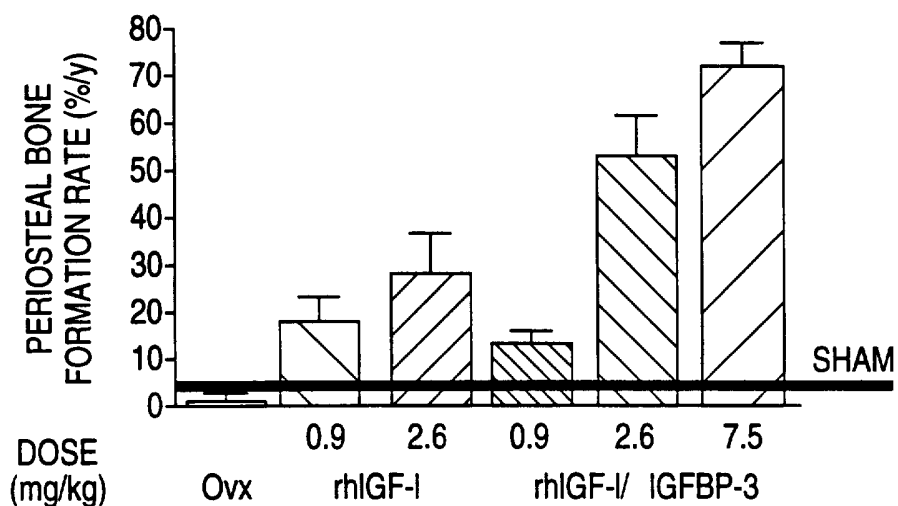
FIG. 4A
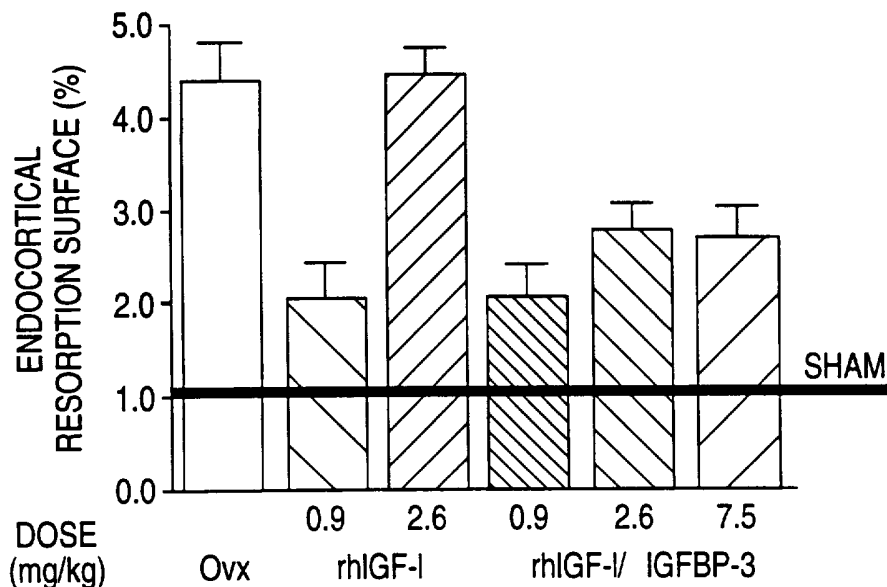
FIG. 4B

FIG. 5A

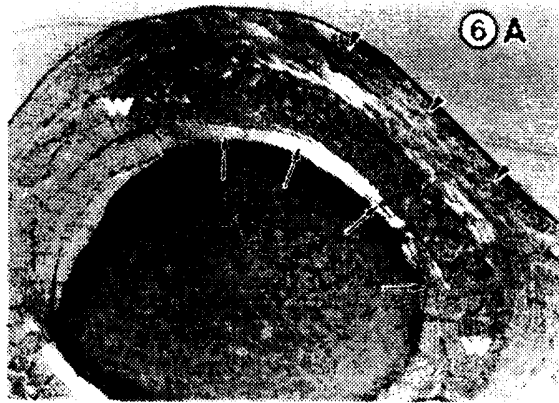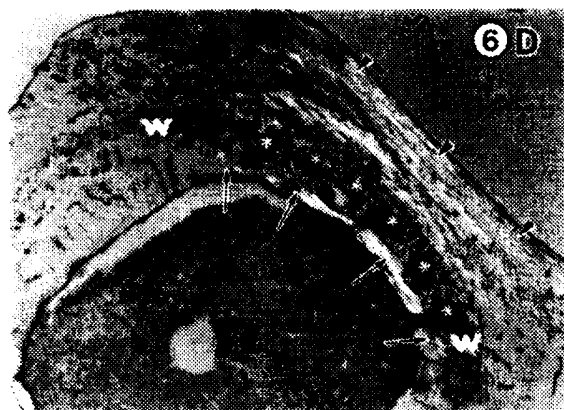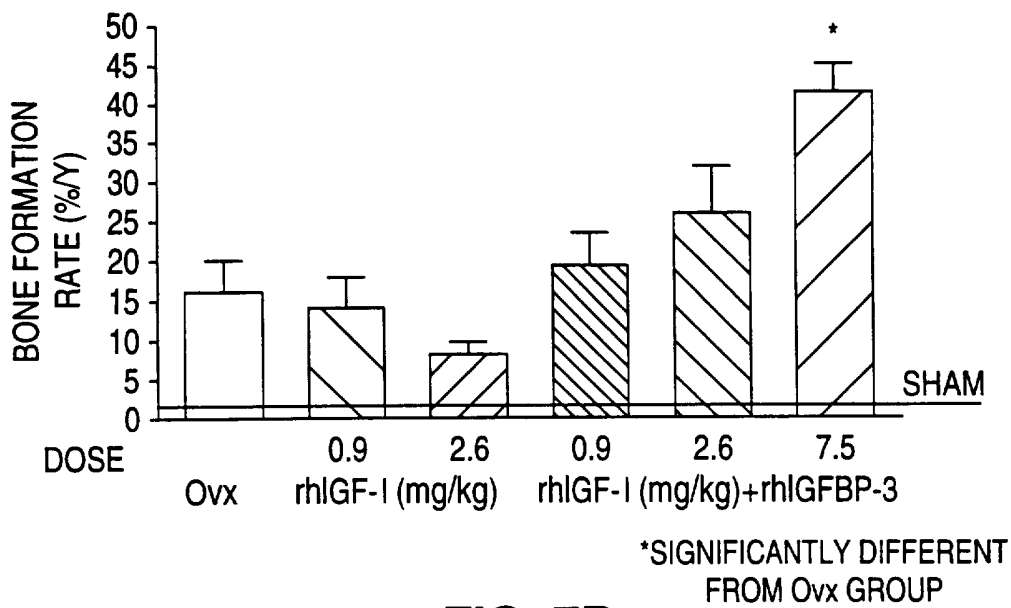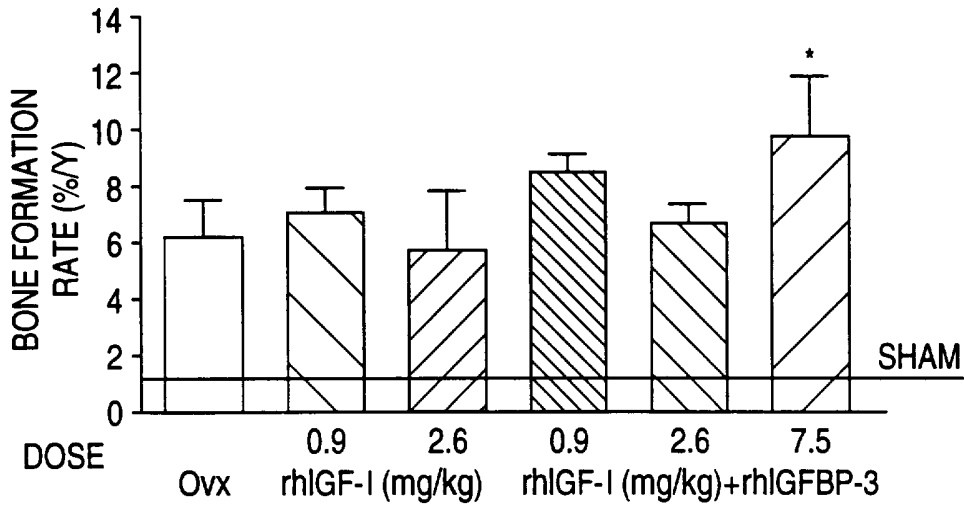FIG. 5B

FIG. 5C

FIG. 5D

FIG. 6A
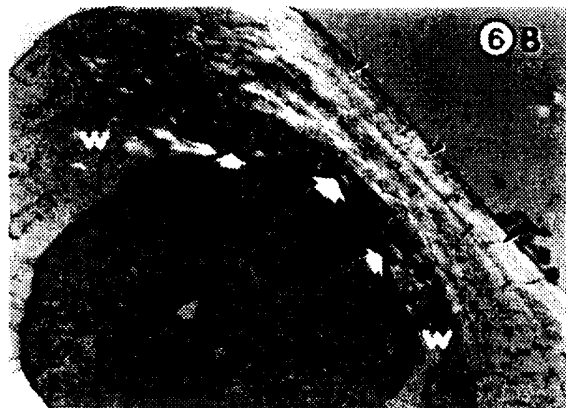
FIG. 6B
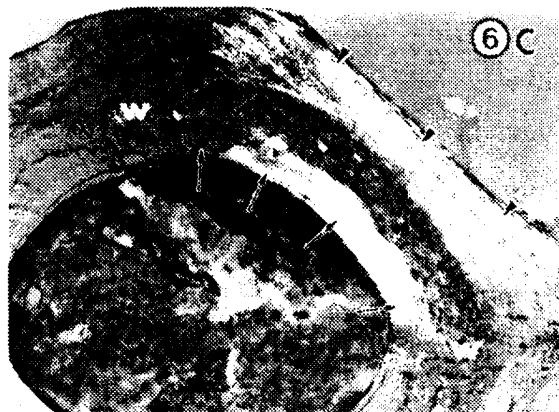
FIG. 6C

FIG. 6D
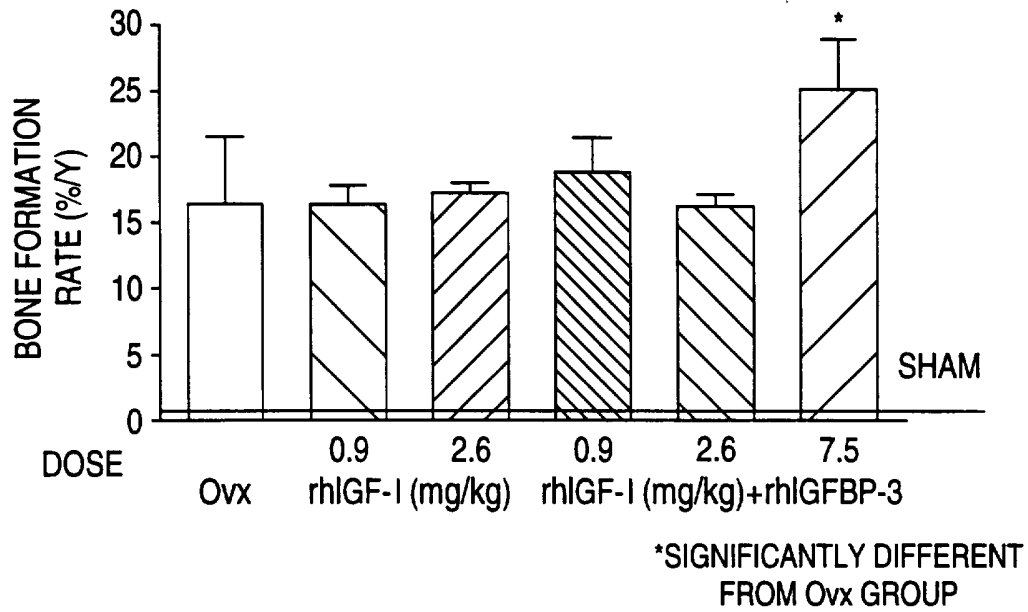
FIG. 7A

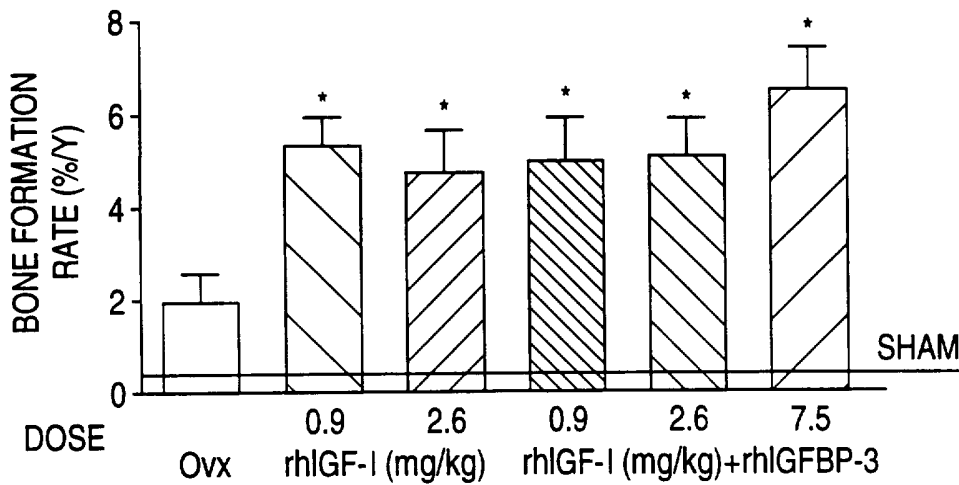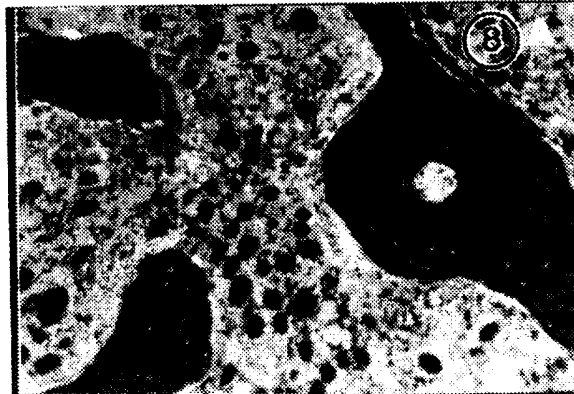**FIG. 7B**

FIG. 7C

*SIGNIFICANTLY DIFFERENT
FROM Ovx GROUP

FIG. 7D
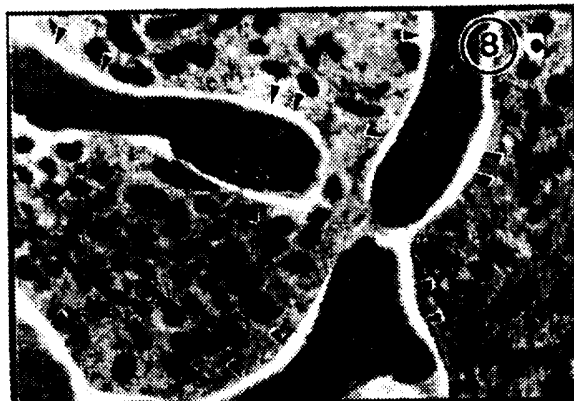
*SIGNIFICANTLY DIFFERENT
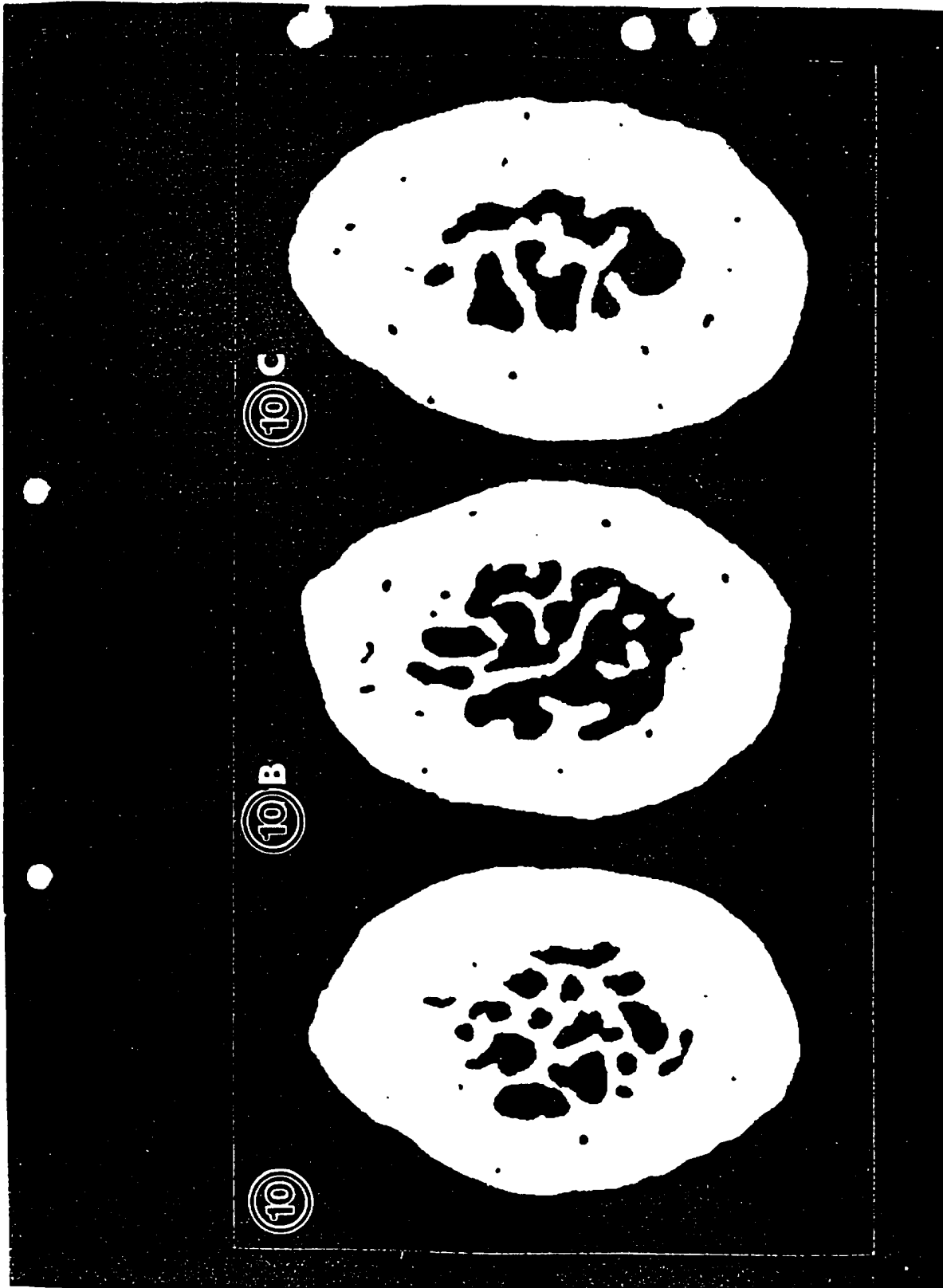FROM Ovx GROUP

10/21

FIG. 8A

FIG. 8B

FIG. 8C

FIG. 9A

FIG. 9B

FIG. 9C

FIG. 10A through 10C

GPETLCGAELVDALQFVCGDRGFYFNK
PTGYGSSSRRAPQTGIVDECCFRSCDL
RRLEMYCAPLKPAKSA

FIG. 11

*

```
GASSAGLGPVVRCEPCDARALAQCAPPPAVCAELVRE
PGCGCCLTCALSEGQPCGIYTERCGSGLRCQPSPDEA
RPLQALLDGRGLCVNASAVSRLRAYLLPAPPAPGNAS
ESEEDRSAGSVESPSVSSTHRVSDPKFHPLHSKIIII
KKGHAKDSQRYKVDYESQSTDTQNFSSESKRETEYGP
CRREMEDTLNHLKFLNVLSPRGVHIPNCDKKGFYKKK
QCRPSKGRKRGFCWCVDKYGQPLPGYTTKGKEDVHCY
SMQSK
```

* There is a natural heterogeneity at position 5; glycine can also occur at this position.

FIG. 12

15/21

```
156  ATG GGT GCA TCT TCT GCA GGT TTA GGT CCA GTT GTT CGT TGT GAA CCA
     Met Gly Ala Ser Ser Ala Gly Leu Gly Pro Val Val Arg Cys Glu Pro
      1               5                  10                  15

204  TGT GAT GCT CGT GCT CTT GCT CAA TGT GCT CCA CCA GCT GTT TGT
     Cys Asp Ala Arg Ala Leu Ala Gln Cys Ala Pro Pro Ala Val Cys
                        20                  25                  30

252  GCT GAA CTT GTT CGT GAA CCG GGT TGT TGT CTG ACT TGC GCA
     Ala Glu Leu Val Arg Glu Pro Gly Cys Cys Leu Thr Cys Ala
                        35                  40                  45

300  CTT TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT TGT GGT TCT
     Leu Ser Glu Gly Gln Pro Cys Gly Ile Tyr Thr Glu Arg Cys Gly Ser
                        50                  55                  60

348  GGT CTG CGT TGT CAA CCA TCT CCA GAT GAA GCT CGT CCT CTG CAG GCT
     Gly Leu Arg Cys Gln Pro Ser Pro Asp Glu Ala Arg Pro Leu Gln Ala
                        65                  70                  75                  80
```

FIG. 13A

```
396   CTG CTG GAC GGT CGT GGT CTG TGC GTT AAC GCT TCC GCT GTT TCC CGT
      Leu Leu Asp Gly Arg Gly Leu Cys Val Asn Ala Ser Ala Val Ser Arg
                      85                  90                  95

444   CTG CGC GCC TAC CTG CTG CCA GCG CCG CCA GCT GGA AAT GCT AGT
      Leu Arg Ala Tyr Leu Leu Pro Ala Pro Pro Ala Gly Asn Ala Ser
                  100                 105                 110

492   GAG TCG GAG GAA GAC CGC CGC AGC GCC GGC AGT GTG GAG AGC CCG TCC GTC
      Glu Ser Glu Glu Asp Arg Arg Ser Ala Gly Ser Val Glu Ser Pro Ser Val
                      115                 120                 125

540   TCC AGC ACG CAC CGG GTG TCT GAT CCC AAG TTC CAC CCC CTC CAT TCA
      Ser Ser Thr His Arg Val Ser Asp Pro Lys Phe His Pro Leu His Ser
                      130                 135                 140

588   AAG ATA ATC ATC AAG AAA GGG CAT GCT AAA GAC AGC CAG CGC TAC
      Lys Ile Ile Ile Lys Lys Gly His Ala Lys Asp Ser Gln Arg Tyr
                  145                 150                 155     160
```

FIG. 13B

636 AAA GTT GAC TAC GAG TCT CAG AGC ACA GAT ACC CAG AAC TTC TCC TCC
    Lys Val Asp Tyr Glu Ser Gln Ser Thr Asp Thr Gln Asn Phe Ser Ser
                    165   170   175

684 GAG TCC AAG CGG GAG ACA GAA TAT GGT CCC TGC CGT AGA GAA ATG GAA
    Glu Ser Lys Arg Glu Thr Glu Tyr Gly Pro Cys Arg Arg Glu Met Glu
            180   185   190

732 GAC ACA CTG AAT CAC CTG AAG TTC CTC AAT GTG CTG AGT CCC AGG GGT
    Asp Thr Leu Asn His Leu Lys Phe Leu Asn Val Leu Ser Pro Arg Gly
                195   200   205

780 GTA CAC ATT CCC AAC TGT GAC AAG AAG TTT TAT AAG AAA AAG CAG
    Val His Ile Pro Asn Cys Asp Lys Lys Gly Phe Tyr Lys Lys Gln
                210   215   220

828 TGT CGC CCT TCC AAA GGC AGG AAG CGG GGC TTC TGC TGG TGT GTG GAT
    Cys Arg Pro Ser Lys Gly Arg Lys Arg Gly Phe Cys Trp Cys Val Asp
                225   230   235   240

FIG. 13C

876 AAG TAT GGG CAG CCT CTG CCA GGC TAC ACC ACC AAG GGG AAG GAG GAC
     Lys Tyr Gly Gln Pro Leu Pro Gly Tyr Thr Thr Lys Gly Lys Glu Asp
                      245   250   255

924 GTG CAC TGC TAC AGC ATG CAG AGC AAG TAG
     Val His Cys Tyr Ser Met Gln Ser Lys
                  260   265

FIG. 13D

```
          10        20        30        40        50        60
123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890

GGT GCT TCT GCT GGT CTT GGA CCA GTT GTT CGT TGT GAA CCA TGT GAT GCA CGA GCT        60

TTA GCT CAA TGT GCT CCA CCA GCT GTT GTT TGT GCT GAA TTA GTT CGA GAA CCA GGT TGT   120

GGT TGT TGT TTA ACT TGT GCT TTA TCT GAA GGT CAA CCA TGT GGT ATT TAT ACT GAA CGT   180

TGC GGT AGT GGT TTG CGT TGT CAA CCA AGC CCA GAT GAA GCT AGG CCT TTA CAA GCA TTA   240

TTA GAT GGT CGA GGT CTG TGT GTT TTG CGT TCC GCT GTT TCT CGA TTG CGC GCT TAT TTA   300

TTA CCT GCC CCA CCG GCA CCG GGT AAC GCC TCC GAA GAG GAT CGT TCT GCG GGT           360

TCC GTT GAA TCT CCA AGT GTG AGT TCT ACC CAT CGA GTT AGC GAC CCG AAA TTT CAT CCG   420

TTG CAC TCT AAA ATC ATT ATT AAA AAG GGT CAC GCA AAG GAT TCT CAA CGT TAT AAG       480

GTG GAT TAT GAA AGC CAA TCT ACC GAC ACT CAA AAT TTT AGT AGT GAA AGT AAA CGT GAA   540

ACC GAG TAC GGC CCG TGT CGA CGT GAG ATG GAG GAT ACC TTA AAC CAT TTA AAA TTT TTG   600

AAC GTT TTA TCC CCG CGT GTT CAT ATC CCG AAT TGC GAT AAA AAA GGC TTC TAC AAA       660

AAG AAA CAA TGC CGT CCG AGT AAG GGT CGT AAA CGA GGT TTT TGT TGG TGC GTT GAC AAA   720

TAC GGT CAA CCG TTG CCG GGT TAT ACT ACT AAA GGC AAA GAA GAT GTT CAT TGT TAT TCT   780

ATG CAA TCT AAA TAA TGC ATC TCG AGA ATT C                                        811
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FIG. 14

ATGCAGCGGGCGGGACCCACGCTCTCTGCTGGCCGCTGCGCTGGTGCTGCTCCCGGGGCCGCGTGGCGCGGGGCTGGC
MetGlnArgAlaGlyProThrLeuTrpAlaAlaAlaLeuThrLeuLeuValLeuLeuArgGlyProProValAlaArgAlaGly
                                                                    -1 +1

GCGAGCTCGGGCGGGGCTTGGGGTCCGTGGTGGCGCTGCGGAGCCGGTGCACTGGCCCGCGCCTCCGCCCGTGTGCGCGGGAGCTG
AlaSerSerAlaGlyLeuGlyProValValArgCysGlyProGluProProAlaArgAlaAlaLeuAlaGlnCysAlaProProAlaAlaValCysAlaGluLeu

GTGGCGCGAGCCGGGCTGCCGGCTGCTGCCTGACGTGCGCGGCCACTGAGCGGGCCAGCCGTGCGGCATCTACACCGAGCGCTGTGGCTCCGGCCTTCGCTGC
ValArgGlyLeuProGlyCysGlyCysGlyLysThrCysAlaLeuSerGluGlyGlnProCysGlyIleIleTyrThrGluArgCysGlyLeuArgCys

CAGCCGTCGCCCGACGAGGCGCGACCGCTGCAGGCGGGCTCTGCTGGACGGCCGCCGGGCTCCGGCCTGCGCGCCTACCTG
GlnProSerProAspGluAlaArgProLeuGlnAlaLeuLeuAspGlyArgGlyLeuCysValAsnAlaSerAlaValSerArgLeuArgAlaTyrLeu

CTGCCAGCGCCGCCAGCTCCCAGGAAATGCTAGTGAGTCGGAGGAGGAAGACCGCAGCGCCGGCAGTGTGGAGAGCCCGTCCGTCTCCAGCACGCACCGGGTG
LeuProAlaProProAlaProGlyAsnAlaSerGluGluGluAspArgSerAlaGlySerGluProSerValSerSerThrHisArgVal

TCTGATCCCAAGTTCCACCCCCTCCATTCAAAGATAATCATCATCAAGAAAGGGCATGCTAAAGACAGCCAGGCGCTACAAAGTTGACTACGAGTCTCAG
SerAspProLysPheHisProLeuHisSerLysIleIleIleIleLysLysGlyHisAlaLysAspSerGlnAlaLeuGlnArgTyrLysValAspTyrGluSerGln

FIG. 15A

AGCACAGATACCCAGAACTTCTCCTCCGAGTCCGAGTCCCTGCCGTAGAGAAATATGGTCCCTGCCGTAGAGAAATGGAAGACACACTGAATCACCTGAAGTTC
SerThrAspThrGlnAsnPheSerSerGluSerLysArgGluThrGluTyrGlyProCysArgArgGluMetGluAspThrLeuAsnHisLeuLysPhe

CTCAATGTGCTGAGTCCCAGGGGTGTACACATTCCCAACTGTGACAAGAAGGGATTTTATAAGAAAAAGCAGTGTCGCCCTTCCAAAGGGCAGGAAGCGG
LeuAsnValLeuSerProArgGlyValHisIleProAsnCysAspLysLysGlyPheTyrLysLysLysGlnCysArgProSerLysGlyArgLysArg

GGCTTCTGCTGTGTGTGGATAAGTATGGGCAGCCTCTCCCAGGCTACACCACCAAGGGGGAAGGAGGACGTGCACTGCTACAGCATGCAGAGCAAGTAG
GlyPheCysTrpCysValAspLysTyrGlyGlnProLeuProGlyTyrThrThrLysGlyLysGlyGlyLysGluAspValHisCysTyrSerMetGlnSerLys.

FIG. 15B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/08925

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :Please See Extra Sheet.
US CL : 514/12, 21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Spencer, "Modern Concepts of Insulin-like Growth Factors," published 1991 by Elsevier Science Publishing Co., Inc. (New York, NY), pages 381-383, see entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 SEPTEMBER 1995	17 OCT 1995

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A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 14/00, 14/65; A61K 38/00, 38/28