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Enhancement of Albumin Expression in Bone Tissues With Healing Rat Fractures

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Abstract The characterization of 66 kDa protein molecule, a major protein component which is produced from femoral-diaphyseal tissues with fracture healing (Igarashi and Yamaguchi [2002] Int. J. Mol. Med. 9:503–508), was investigated. Weaning rats were killed at 7 and 14 days after femoral fracture. When the femoral-diaphyseal tissues with fracture healing were cultured for 48 h in a serum-free medium, many proteins in the bone tissues were released into the medium. Analysis with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showed that a protein molecule of approximately 66 kDa was markedly increased in culture medium from bone tissues with fracture healing. N-terminal sequencing of 66 kDa protein indicated that its N-terminus was identical to that of rat albumin. Western blot analysis of medium 66 kDa protein showed expression of albumin. This expression was significantly enhanced by fracture healing. The expression of albumin was seen in the diaphyseal (cortical bone) and metaphyseal (trabecular bone) tissues of rat femur. When the femoral-diaphyseal tissues obtained at 7 days after femoral fracture were cultured in a serum-free medium containing either vehicle, parathyroid hormone (1–34) (10^{-7} M), insulin-like growth factor-I (10^{-8} M) or zinc acexamate (10^{-4} M), medium albumin was significantly increased in the presence of those bone-stimulating factors. The addition of albumin (0.5 or 1.0 mg/ml of medium) caused a significant increase in calcium and deoxyribonucleic acid contents in the femoral-diaphyseal and -metaphyseal tissues obtained from normal rats in vitro. The present study demonstrates that fracture healing induces a remarkable production of albumin which is a major protein component produced from femoral-diaphyseal tissues of rats, and that albumin has an anabolic effect on bone components. J. Cell. Biochem. 89: 356–363, 2003.

Key words: albumin; fracture healing; bone formation; rat femur

Osteoporosis is widely recognized as a major problem of public health. The most dramatic expression of this disease is represented by fractures of the proximal femur [Bonjour et al., 1996]. Fracture healing is physiologic cascade in which bone heals for the purpose of transferring mechanical loads [Brighton and Hunt, 1991; Einhorn, 1998; Barnes et al., 1999]. The regulatory mechanism in fracture repair is complex. It has been shown that many growth factors, cytokines, and their cognate receptors are present at elevated levels in and around the fracture site during fracture healing [Einhorn et al., 1995; Bolander, 1998]. Many of those proteins are normally expressed in skeletal tissue, and others are released from inflammatory cells at the site of injury. The induction of these proteins may be regulated, both spatially and temporally, in promoting fracture repair. The molecular and cellular mechanism by which bone with fracture heals, however, has not been fully clarified.

We showed previously that many protein molecules were produced in rat femoral-diaphyseal tissues with fracture healing [Igarashi and Yamaguchi, 1999a, 2001]. Fracture healing increased IGF-I, TGF-β1, osteocalcin and ~66 kDa protein molecule in the femoral-diaphyseal tissues [Igarashi and Yamaguchi, 2001, 2003]. Especially, a ~66-kDa protein was present in the bone tissues as a major bone protein and it was greatly released in the medium by culture of femoral-diaphyseal tissues with

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fracture healing [Igarashi and Yamaguchi, 2001]. A ~66-kDa protein molecule, which is released greatly to culture medium from bone tissues with fracture healing, may be inducible [Igarashi and Yamaguchi, 2002]. The role of ~66 kDa protein molecule in the femoral-diaphyseal tissues which are greatly increased with fracture healing is unknown at present. Presumably, this protein plays an important role in the promotion of fracture healing.

The present study was undertaken to determine the characterization of ~66 kDa protein molecule which is a major component produced from femoral-diaphyseal tissues with rat healing fracture. We found that a ~66-kDa protein is identical to albumin which is produced by femoral tissues in rats, and that albumin has an anabolic effect on bone components in the femoral-diaphyseal and -metaphyseal tissues in vitro. This finding is the first one in our knowledge.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (high glucose, 4.5 g/dl) and a penicillin–streptomycin solution (5,000 U/mg penicillin; 5,000 μg/ml streptomycin solution) were obtained from Gibco Laboratories (Grand Island, NY). Bovine serum albumin (fraction V), parathyroid hormone (1–34) (PTH human), and insulin-like growth factor-I (IGF-I) were obtained from Sigma Chemicals (St. Louis, MO). Rabbit anti-rat albumin antibody (IgG fraction) was obtained from Inter-Cell Technologies, Inc. (Hopewell, NJ). Zinc acexamate [zinc 6-(actyl-amino) hexanoate] was obtained from Nissho Co. (Osaka, Japan). Other chemicals were reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water was glass distilled.

Bone Fracture

Male Wistar rats (4-weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The five animals in each group were fed commercial laboratory chow (solid) containing 57.4% carbohydrate, 1.1% calcium, and 1.1% phosphorus at a room temperature of 25°C, and were given distilled water freely. Rats were given a bone fracture under ether anesthesia; the right femoral-diaphyseal section was surgically fractured with scissors after cutting open the adherent muscle around the femur [Igarashi and Yamaguchi, 1999a]. In the sham-operated animals, the femur was handled, but not fractures. All animals were fed matched amounts of the chow described above for 7 or 14 days.

Bone Culture

Rats were bled by cardiac puncture under light anesthesia with ether. The femurs were removed aseptically after bleeding and soaked in ice-cold 0.25 M sucrose solutions. The femur was cleaned of soft tissue, and the diaphysis and metaphysis (containing epiphyseal tissue) were separated. Marrow cells were completely removed by washing of and cut into small pieces. Femoral-diaphyseal and -metaphyseal fragments were cultured for 48 h in a 35-mm dish in 2.0 ml medium consisting of Dulbecco's modified Eagle's medium (high glucose, 4.5 g/dl) supplemented with antibiotics (100 U penicillin and 100 μg streptomycin/ml of medium) [Yamaguchi et al., 1987]. In separate experiments, the culture medium contained either vehicle or bone-stimulating factors. The medium used in the experiments did not contain albumin, serum,and zinc. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air.

Gel Electrophoresis

The characterization of protein components in the culture medium with bone tissues was assayed by using SDS–PAGE. SDS–PAGE was performed by the method of Laemmli [1970] with minor modification. The electrophoresis was carried out using 12% polyacrylamide resolving gel and the discontinuous Tris-glycine buffer system. Twenty-five microliters (containing 15 or 60 μg of protein) of bone protein samples were dissolved in SDS gel-loading buffer containing 4% SDS, 10% β-merecaptoethanol and bromophenol blue (BPB) marker. The protein mixture was denatured by heating at 90°C for 2 min and applied to individual wells. Electrophoresis was applied to the gel at 35 mA for 4 h at room temperature. After separation, proteins were simultaneously fixed with methanol-acetic acid and stored in water containing 20% glycerol.

N-Terminal Amino Acid Sequencing

Proteins secreted in culture medium were subjected to electrophoresis on a 12% SDS–polyacrylamide gel and transferred to NT-32 polyvinylidene difluoride membrane (Nihon

Eido, Tokyo). Protein bands (~66 kDa) were visualized by staining with Coomassie blue, excised from a polyvinylidene difluoride membrane, and the amino acid sequencing was performed with a Procise® cLC Protein Sequencing System (Applied Biosystems, Foster City, CA).

Western Blot Analysis

The medium protein cultured with the femoral-diaphyseal tissues of normal or fracture-healing rats were used for Western blot analysis [Wessendorf et al., 1993]. Aliquots of culture medium (10 μg of protein) or albumin (1 μg) were mixed with 5× Lemmli sample buffer, boiled for 5 min, and SDS–PAGE was performed by the method of Laemmli [1970] using 9% polyacrylamide resolving gel. After SDS-PAGE, the proteins were then transferred onto a plyvinylidene difluoride membrane at 100 mA for 4 h. The membranes were incubated with a polyclonal rabbit anti-rat albumin antibody, which was diluted 1:2,000 in 10 mM Tris-HCl, pH 8, containing 150 mM NaCl, 0.1% w/v Tween-20 (washing buffer), and 5% (w/v) skim milk for 1 h. The membranes incubated with antibody were washed four times with washing buffer. Then membranes were incubated for 1 h with horse-radish peroxidase linked anti-rabbit IgG, which was diluted 1:5,000 with washing buffer containing 5% (w/v) skim milk, and again they were washed. Detection of the protein bands was performed using a enhanced chemiluminescent kit (Amersham, Buckinghamshire, UK) following the manufacture's instructions. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel. The membranes obtained by using culture medium were exposed for 10 s on the film, respectively.

Assay of Culture Medium Protein

To determine the concentration of protein released in the culture medium, the medium was collected after the culture of 48 h with the femoral-diaphyseal or -metaphyseal tissues obtained from normal or fracture-healing rats. The medium used in this experiment did not contain serum and albumin. Protein concentration in culture medium was measured by the method of Lowry et al. [1951]. Also, albumin concentration in culture medium was determined by the procedure of Dumas et al. [1997]. Protein or albumin content was expressed as milligram (mg) per mg of DNA in the diaphyseal or metaphyseal tissues.

Bone DNA Determination

To measure bone DNA content, the femoral-diaphyseal or -metaphyseal tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 h after the homogenization of bone tissue [Flangan and Nichols, 1962]. After alkali extraction, the samples were centrifuged at 10,000 g for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti [1955] and expressed as the amount of DNA (mg) per gram (g) wet weight of bone tissues.

Bone Calcium Determination

The femoral-diaphyseal and -metaphyseal tissues were dried for 18 h at 120°C, weighed, and then digested with nitric acid overnight. Calcium was determined by atomic absorption spectrophotometry [Yamaguchi et al., 1987]. Bone calcium content was expressed as milligram of calcium per gram dry bone tissues.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's t-test. A P-value < 0.05 was considered to indicate statistically significant difference.

RESULTS

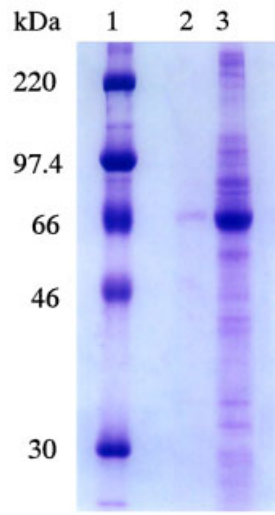
Characterization of Bone Protein Components Increased With Fracture Healing

Rat femoral-diaphyseal tissues with fracture healing have been shown to produce great protein components [Igarashi and Yamaguchi, 1999b]. When diaphyseal tissues with fracture healing were cultured, bone proteins were markedly released into culture medium [Igarashi and Yamaguchi, 2001]. Bone ~66 kDa protein component is greatly increased with healing rat fracture [Igarashi and Yamaguchi, 2002]. The characterization of bone ~66 kDa protein with fracture healing is shown in Figure 1. Femoral-diaphyseal tissues were cultured for 48 h in a serum-free medium in vitro. Bone protein components were released into culture medium. This release was markedly enhanced in the femoral-diaphyseal tissues obtained at 7 days after femoral fracture (Fig. 1A). A great increase in 66 kDa protein was shown to be released into culture medium with SDS–PAGE analysis.

The N-terminal amino acid sequence of 66 kDa protein in culture medium was identical to that of the mature rat serum albumin (Fig. 1B) [Sargent et al., 1981]. The N-terminal amino acid sequences of the mature rat serum albumin is compared with the reported sequences of vertebrate albumin (Fig. 1C).

The result of Western blot analysis for albumin in the medium cultured with the femoral-diaphyseal tissues of normal and fracture-healing rats is shown in Figure 2. Femoral-diaphyseal tissues were obtained at 7 days after femoral fracture, and the bone tissues were cultured for 48 h in a serum-free medium in vitro.

A



B

Mature rat serum albumin:	EAHKSEIAHRFKDLGEQH···
Culture medium 66 kDa protein:	EAHKSEIAHRFKDLG···

C

Rat	EAHKSE IAHRFKDLG
Mouse	EAHKSE IAHRYNDLG
Human	DAHKSEVAHRFKDLG
Bovine	DTHKSEIAHRFKDLG
Cat	EAHQSEIAHRFNDLG
Rabbit	EAHKSEIAHRFNDVG
Dog	EAYKSEIAHRYNDLG
Horse	DTHKSEIAHRFNDLG
Pig	DTYKSEIAHRFKDLG
Chicken	AEHKSEIAHRYNDLK

Fig. 1. Characterization of protein components in the medium released by culture with the femoral-diaphyseal tissues of rats. Femoral-diaphyseal tissues were obtained at 7 days after femoral fracture. The bone tissues from normal or fracture healing rats were cultured for 48 h. **A**: Samples of culture medium with bone tissues of normal (**lane 2**; 15 μg protein/μg DNA in bone tissues) or fracture healing (**lane 3**; 60 μg protein/μg DNA) for SDS–PAGE were pretreated with SDS in the presence of β-mercaptoethanol. The protein bands were visualized by a Coomassie blue staining method. The result shows one of four experiments with separate rats. **Lane 1** indicates protein marker. **B**: The result shows the N-terminal amino acid sequence of 66 kDa protein in culture medium. About 160 pmol of 66 kDa protein (lane 3 from figure part A), were analyzed by N-terminal sequencing, and its sequence was compared with the reported sequence of mature rat serum albumin. **C**: The result shows the N-terminal amino acid sequence of the mature rat serum albumin and other vertebrate albumins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

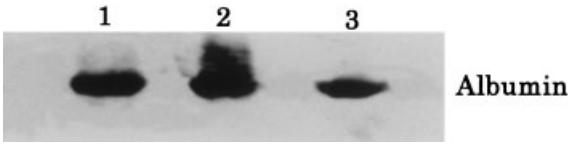


Fig. 2. Western blot analysis for albumin in the medium cultured with the femoral-diaphyseal tissues of normal and fracture healing rats. Bone tissues were obtained at 7 days after femoral fracture. Diaphyseal tissues were cultured for 48 h. Western blot analysis was carried out on the medium proteins (10 μg of proteins). The figure shows one of four experiments. The densitometric data showed 192 ± 5.6 (% of normal rats; mean ± SEM of four rats). **Lane 1**, normal rats; **lane 2**, fracture-healing rat; **lane 3**, albumin (1 μg) as the standard.

Albumin was found in culture medium with femoral-diaphyseal tissues. The bone tissues with fracture healing induced a significant ($P < 0.01$) increase in albumin production in culture medium.

Characterization of Bone Albumin Production With Fracture Healing

The production of albumin in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) of normal rats is shown in Figure 3. Bone tissues were cultured for 48 h in a serum-free medium in vitro. The results indicated that albumin was released from the diaphyseal and metaphyseal tissues. The release of bone total protein (Fig. 3A) and albumin (Fig. 3B) was greater in the metaphyseal tissues than those of the diaphyseal tissues. Albumin release from diaphyseal tissues was about 35% of total protein. In metaphyseal tissues, it was about 25%.

The change in albumin production in the femoral-diaphyseal tissues with healing rat-fracture is shown in Figure 4. Femoral-diaphyseal tissues obtained at 7 and 14 days after femoral fracture, and the bone tissues were cultured for 48 h in a serum-free medium in vitro. The production of total proteins (Fig. 4A) and albumin (Fig. 4B) was markedly enhanced in the medium cultured with the diaphyseal tissues obtained at 7 and 14 days after femoral fracture. Their production was of the same extent at 7 and 14 days after femoral fracture.

The effect of bone-stimulating factors on albumin production in the femoral-diaphyseal tissues with healing rat-fracture is shown in Figure 5. The diaphyseal tissues obtained at 7 days after femoral fracture were cultured for 48 h in a serum-free medium containing either vehicle, PTH (10^{-7} M), IGF-I (10^{-8} M) or zinc acexamate (10^{-4} M) in vitro. The presence of these bone-stimulating factors caused a significant increase in total protein (Fig. 5A) and albumin (Fig. 5B) productions into the culture medium.

Effect of Albumin Addition on Bone Components

The effect of albumin addition on calcium and DNA contents in the femoral-diaphyseal and -metaphyseal tissues of normal rats is shown

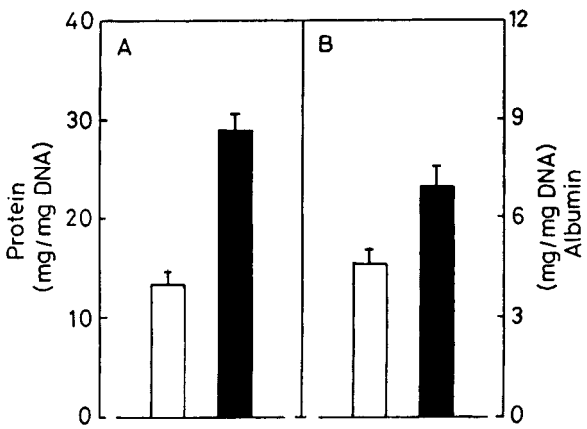


Fig. 3. Protein and albumin concentrations in the culture medium with the femoral-diaphyseal and -metaphyseal tissues obtained from normal rats. Bone tissues were cultured for 48 h, and protein (**A**) or albumin (**B**) in the culture medium was measured. Each value is the mean ± SEM of five rats. White bars; diaphysis; black bars, metaphysis.

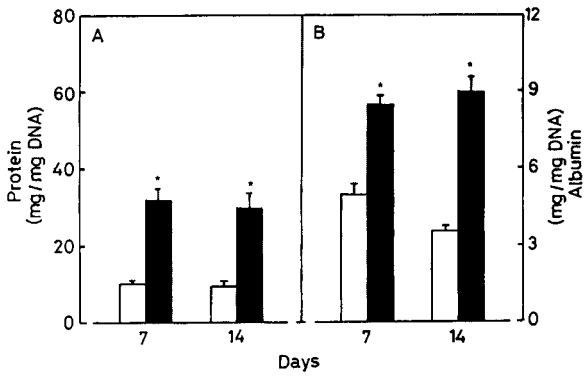


Fig. 4. Change in protein and albumin concentration in the culture medium with the femoral-diaphyseal tissues obtained from fracture-healing rats. Femoral-diaphyseal tissues were obtained at 7 or 14 days after femoral fracture. Bone tissues were cultured for 48 h, and protein (**A**) or albumin (**B**) in the culture medium was measured. Each value is the mean ± SEM of five rats. *$P < 0.01$, compared with the value obtained from normal rats. White bars, normal rats; black bars, fracture-healing rats.

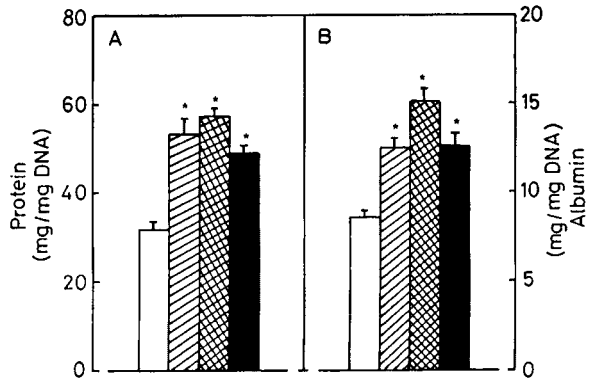


Fig. 5. Effect of bone-stimulating factors on protein and albumin concentrations in the culture medium with the femoral-diaphyseal tissues obtained from fracture-healing rats. Femoral-diaphyseal tissues were obtained at 7 days after femoral fracture. Bone tissues were cultured for 48 h in a medium containing either vehicle, parathyroid hormone (PTH; 10^{-7} M), insulin-like growth factor (IGF-I; 10^{-8} M) or zinc acexamate (10^{-4} M). Protein (**A**) or albumin (**B**) in the culture medium was measured. Each value is the mean ± SEM of five rats. $^*P < 0.01$, compared with the control (none) value. White bars, control (none); hatched bars, PTH; double hatched bars, IGF-I; black bars, zinc acexamate.

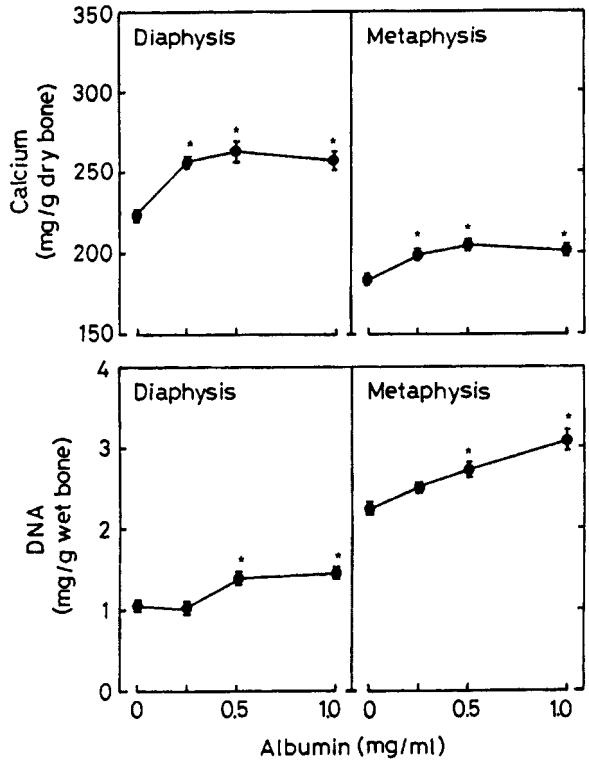


Fig. 6. Effect of albumin addition on calcium and DNA contents in the femoral-diaphyseal and -metaphyseal tissues of normal rats in vitro. Bone tissues were cultured for 48 h in a medium containing either vehicle or albumin (0.25, 0.5, or 1.0 mg/ml of medium). Each value is the mean ± SEM of five rats. $^*P < 0.01$, compared with the control (none) value.

in Figure 6. Femoral-diaphyseal and -metaphyseal tissues obtained from normal rats were cultured for 48 h in a serum-free medium containing either vehicle or albumin (0.25, 0.5, or 1.0 mg/ml of medium) in vitro. The addition of albumin (0.25–1.0 mg/ml) caused a significant increase in calcium content in the femoral-diaphyseal and -metaphyseal tissues. The effect of albumin addition in increasing bone calcium content was saturated at 0.25 mg/ml of albumin addition.

DNA contents in the femoral-diaphyseal and -metaphyseal tissues were significantly increased by the addition of albumin (0.5 or 1.0 mg/ml).

DISCUSSION

A pathophysiological mechanism by which bone fracture heals is complex. During fracture repair, a number of growth factors, cytokines, and their cognate receptors are present at elevated levels in and around the fracture site [Barnes et al., 1999]. Many of these proteins are normally expressed in skeletal tissue, and others are released from associated inflammatory cells at the site of injury. We demonstrated previously that many protein molecules were produced in rat femoral-diaphyseal tissues with fracture healing [Igarashi and Yamaguchi, 1999b, 2001]. Especially, fracture healing increased production of a ~66-kDa protein molecule in the femoral-diaphyseal tissues [Igarashi and Yamaguchi, 2001]. This protein component was present in the bone tissue as a major bone protein and it was greatly released into the medium by culture of femoral-diaphyseal tissues with fracture healing [Igarashi and Yamaguchi, 2001, 2002]. The 66 kDa protein component may play an important role in fracture repair.

N-terminal sequencing of 66 kDa protein indicated that its N-terminus was identical to that of rat albumin. Western blot analysis with anti-rat albumin antibody (IgG fraction) for 66 kDa protein showed a remarkable expression of albumin in the medium cultured with rat femoral-diaphyseal tissues. The expression was markedly enhanced by fracture healing. The colorimetric measurement of albumin with bromcresol green [Dumas et al., 1997] indicated that albumin was expressed in the diaphyseal and metaphyseal tissues of normal rat femur.

These results demonstrate that albumin is produced from rat femoral tissues.

Bone cells (including osteoblast, osteoclast, and osteocyte), which express albumin in bone tissues, are unknown at present. It has been reported that hepatocyte growth factor induced differentiation of rat bone marrow cells into a hepatocyte lineage in vitro [Oh et al., 2000; Miyazaki et al., 2002]. Multipotent progenitor cells from bone marrow differentiate into functional hepatocyte-like cells which expresses albumin [Schwartz et al., 2002]. Which bone cells can express albumin remains to be elucidated? However, it is speculated that albumin is expressed in osteoblasts, since albumin production was stimulated by PTH or IGF-I which the receptors are located on osteoblasts.

Albumin was markedly produced in the medium cultured with the femoral-diaphyseal and -metaphyseal tissues obtained at 7 and 14 days after femoral fracture. A remarkable production of albumin was seen at the earlier and later stages of fracture healing. The production of albumin in the femoral-diaphyseal tissues with fracture healing was significantly stimulated in the presence of PTH, IGF-I, or zinc acexamate [Yamaguchi and Gao, 1998], which are bone formation-stimulating factors. Zinc acexamate has been shown to increase the expression of IGF-I in the femoral-diaphyseal tissues [Igarashi and Yamaguchi, 2001]. These observations suggest that the expression of albumin in the femoral tissues is stimulated by PTH, IGF-I, or zinc acexamate. Especially, the increase in IGF-I with fracture healing may induce the production of albumin in the femoral-diaphyseal tissues with fracture healing. Presumably, albumin plays a pathophysiologic role in fracture repair in collaboration with IGF-I.

Albumin was found to have an anabolic effect on bone components. When the femoral-diaphyseal and -metaphyseal tissues obtained from normal rats were cultured with the addition of albumin (0.5 or 1.0 mg/ml of medium), calcium and DNA contents in the femoral-diaphyseal and -metaphyseal tissues were significantly increased. It appears that albumin stimulates bone formation and osteoblastic proliferation in bone tissues. The cellular mechanism by which albumin stimulates anabolic effect on bone metabolism remains to be elucidated. The present finding that albumin has a stimulatory effect on bone calcium and DNA content, may support the view that albumin plays an important role in the promoting of fracture repair.

In conclusion, it has been demonstrated that the 66-kDa protein molecule, which is a major component produced from femoral-diaphyseal tissues of rats with fracture healing, is identical to albumin. Also, albumin has been found to have an anabolic effect on bone components in the femoral-diaphyseal and -metaphyseal tissues in vitro. The present finding is the first one in our knowledge.

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