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(Citation)
The Kobe journal of the medical sciences,46(6):265-282

(Issue Date)
2000-12

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/E0001064

KOBE UNIVERSITY

Kobe J. Med. Sci. 46, 265/282, December 2000

THE EFFECT OF MS-818, NEWLY SYNTHESIZED PYRIMIDINE COMPOUND, ON FRACTURE REPAIR

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KEY WORDS

MS-818; basic FGF; fracture repair; chondrogenesis; angiogenesis

ABSTRACT

Both periosteal cell proliferation and endochondral ossification accompanied are characteristic of the fracture healing process. This process is regulated by various kinds of soluble factors including prostanoids, cytokines and growth factors. In particularly, basic fibroblast growth factor (bFGF) stimulates the mesenchymal cell proliferation and differentiation in the periosteum and leads to the fracture healing. Recently, newly synthesized pyrimidine compound, 2-piperadino-6-methyl-5-oxo-5,6-dihydro (7H) pyrrolo [3,4-d] pyrimidine maleate (MS-818) has been reported to augment the biological effect of bFGF *in vitro*.

Received for publication : October 4, 2000

Therefore, we have studied the effect of MS-818 on fracture healing process in which bFGF has been reported to play an important role. In the rat fracture model, 5 mg/kg MS-818 which had been administered intraperitoneally for fourteen consecutive days enhanced the cartilage matrix formation. In the bone defect model, in which we can find only membranous ossification without chondrogenesis, cartilage matrix formation was observed in seven days after 1 μg of human bFGF containing polymer pellet was embedded in the defect site. Chondrogenesis induced by bFGF was enhanced significantly after 5 μg MS-818 containing pellet was implanted with bFGF pellet. These results suggest that MS-818 might promote the fracture healing process through enhancement of the effect of bFGF on endochondral ossification.

INTRODUCTION

Both periosteal cell proliferation and endochondral ossification accompanied with hyaline cartilage formation are characteristic of the fracture healing process. The mesenchymal cell recruitment and growth followed by differentiation to chondrocytes or osteoblasts proceeded step by step to complete this process.[13] Though detailed mechanisms of this process is still unknown, various kinds of soluble factors such as transforming growth factor-β (TGF-β), [5, 16, 22] bone morphogenetic proteins (BMPs), [27] platelet derived growth factor (PDGF) [20] and basic fibroblast growth factor (bFGF) [5, 17, 24] are reported to be involved in this process. Therefore, regulation of these factors would be one of the promising methods of the fracture treatment.

Basic FGF, which is abundant in the neural tissue, is a potent mitogen for a variety of cells of mesodermal and neuroectodermal origin and promote differentiation during embryonic growth and development.[12] During osteogenesis, bFGF is deposited and stored in bone extracellular matrix [11] and released during remodeling in skeletal growth or fracture healing. It is reported that the *in vivo* administration of both acidic and bFGF to fracture sites induces chondro-

genesis and promotes fracture healing.[14, 17] Thus, reported data suggest the direct role of bFGF in events leading to cartilagenous callus formation and administration of bFGF might give a therapeutical benefit for fracture repair. However, in order to obtain the biological augmentation of fracture healing by growth factors, continuous or intermittent application of factors is required for maintaining a local concentration which ensure the prolonged receptor occupancy at target site, which in turn sustained some of the effects of growth factors. Osmotic pump system,[8] sustained delivery using factor-impregnated carboxymethyl cellulose [1, 2] and multiple systemic injections [10] have been utilized for this purpose. Thus, significance of local or systemic administration of growth factors is reported for tissue remodeling such as articular cartilage repair and fracture healing. However, at present, as the cost of recombinant growth factors applied for clinical use is expensive, it is important to find and develop the chemicals capable of augmenting or enhancing the action of growth factors. [14]

Recently, 2-piperadino-6-methyl-5-oxo-5,6-dihydro (7H) pyrrolo [3,4-d] pyrimidine maleate (MS-818), which is a newly synthesized pyrimidine compound, was reported to promote the peripheral nerve regeneration *in vivo* [13, 14, ,23] and the skeletal muscle growth *in vitro*. It was also reported that MS-818 augmented the effect of bFGF on angiogenesis in chorioallantoic membrane (CAM) assay [28] and neurotrophic effects on mouse cortical tissue. Therefore, in order to clarify the role of MS-818 in fracture healing process in which bFGF plays an important role, we have examined the effect of MS-818 on both callus formation in rat fracture model and bFGF-induced chondrogenesis developed in bone defect remodeling model *in vivo*.

MATERIALS AND METHODS

1. Rat fracture model

Twenty adult Wistar rats weighing 260-330g were anaesthetized by an

intraperitoneal injection of sodium pentobarbital (50 mg/kg). The right legs were shaved and sterilized prior to a mid line longitudinal skin incision over the anterior aspect of tibia. Each tibia was exposed without stripping the periosteum and broken manually before nailing with 22-gauge needle inserted from the anteromedial aspect of the proximal tibia midway between the joint and the tubercle to the ankle for stabilizing the fracture site. The wound was closed by a routine procedure and no external fixation was applied. Movement and weight loading onto the right leg were allowed without limitation after the operation.

These rats were divided into two groups as follows. As a group of control, physiological saline was injected intraperitoneally for fourteen consecutive days after the operation. On the other hand, 5mg/kg of MS-818 were administered to the experimental group by the same manner. On14th experimental day, animals were sacrificed under the euthanasia using an overdose of sodium pentbarbital and the right tibiae were excised. After extracting the Intramedullary nail from the tibia, the soft tissue surrounding the tibia was gently removed. Then, the tibiae were fixed in phosphate-buffered 10 % formaldehyde, decalcified in 4 % ethylenediamine tetraacetic acid (EDTA; pH 7.3), embedded in paraffin following dehydration with graded ethanol, and sectioned longitudinally for histological analysis.

2. Rat bone defect remodeling model

In order to investigate the effect of MS-818 on chondrogenesis induced by bFGF, bone defect animal model was established and used for the experiment. Twenty adult Wistar rats weighing 260-330g were anaesthetized using sodium pentobarbital and both legs were shaved and sterilized. A mid line longitudinal skin incision was made on the anterior aspect of tibia. Then, each tibia was exposed subperiosteally and 2.6 mm in diameter hole was made on the medial cortex of the tibia.

Pellets containing MS-818 or bFGF were prepared as described previ-

ously with some modifications.[19] Equal volume of hydroxyethylmethacrylate (Polyscience, Warrington, MA) were dissolved in 100 % of ethanol at room temperature (polymer casting solution). One μg of recombinant human bFGF (R &D SYSTEMS INC., Minneapolis. MN) or 5 μg of MS-818 was placed in 10 mg of polymer casting solution. This solution was dried under a mild vacuum at 4 °C to leave the bFGF or MS-818 trapped within the polymer matrix. This small pellet was placed in the bone marrow of tibia through 2.6 mm in diameter hole.

The rats were divided into two experimental groups. First 10 rats were implanted with pellet containing MS-818 (MS-818 pellet) in the bone defect of left tibiae, while pellet containing saline vehicle (saline pellet) only were embedded in the hole of right tibiae. Remaining 10 rats were implanted with bFGF contained pellet (bFGF pellet) in the bone defect of both tibiae. In the left tibiae, MS-818 pellet was placed in combination with bFGF pellet simultaneously, while leaving the bone defect of right tibiae implanted with bFGF pellet alone. Animals were sacrificed on 7th experimental day using an overdose of sodium pentbarbital same as stated above. The excised tibiae were then fixed in phosphate-buffered 10 % formaldehyde and decalcified in 4 % EDTA (pH 7.3). The tibiae embedded in paraffin were sectioned longitudinally in order to evaluate the extent of membranous ossification and cartilage matrix formation. Based on the histological findings, extent of cartilage matrix formation was classified into four groups in bone defect sites as shown in Table 1. According to this grading system, the number of animals classified into each grade was compared and statistically analyzed with use of χ^2 -test.

3. Preparation of specimen for histological examination and evaluation of cartilage matrix formation

Specimens obtained from the fracture model were sliced as thin serial sections through the long axis of tibiae along the sagittal plane. Sections (6 μm) in the midsaggital plane were processed in hematoxylin-eosin (H-E) staining and

examined by light microscope. Both the extents of periosteal cell proliferation and chondrogenesis around the fracture site were compared between the two experimental groups.

Specimens from bone defect model were also processed and multiple sections were stained with H-E for histological analysis, and immunostained using anti-bFGF antibody (Promega, Madison, WI) in order to evaluate the distribution of bFGF in the bone defect area.

RESULTS

1. Effect of MS-818 on fracture healing process in rat

As reported previously, periosteal cells proliferated and accumulated adjacent to the fracture site within a several days after fracture.[13] The growth of undifferentiated mesenchymal cells originated from both bone marrow and periosteum were also observed in combination with inflammatory cell infiltration in the fracture hematoma. At 14 days after fracture, membranous ossification occurred on the surface of cortical bone adjacent to the fracture site and small amount of newly developed chondrocytes and formation of hyaline cartilage matrix were observed in fracture hematoma. Therefore, we have compared the histological findings of the fracture site at 14 days after fracture between control and MS-818 administered group.

As shown in Figure 1-a, the moderate amount of hyaline cartilage matrix was observed in the proliferated periosteal cell layer beside the fracture site. Membranous ossification was also seen in this proliferated periosteal cell layer. These observations were detected in all ten rats without MS-818 administration. When 5mg/kg of MS-818 was administered to the experimental group for fourteen consecutive days after fracture, larger amount of cartilage matrix accompanied by membranous ossification beside the fracture site was observed in the experimental group of rat than that in control group (Fig.1-b). Remarkable his-

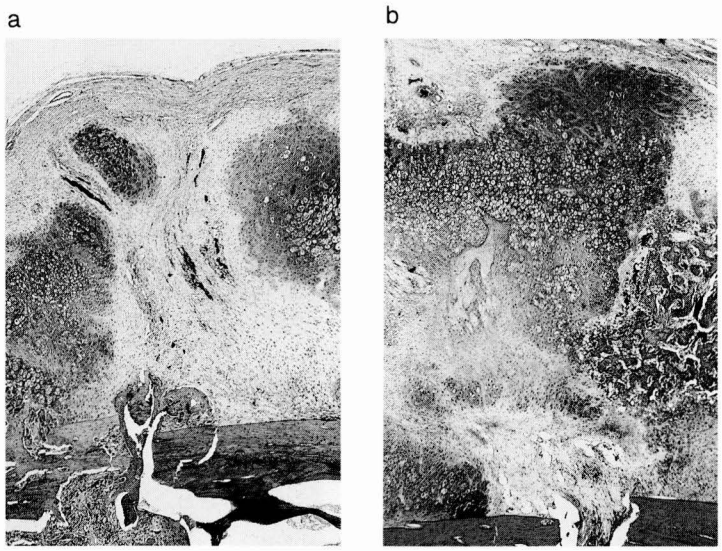

Fig. 1. Histological findings of the rat fracture model. (a) Moderate amount of hyaline cartilage was observed in the proliferated periosteal cell layer beside the fracture site of control rats. Membranous ossification was also seen in this layer. (b) Larger amount of cartilage mass accompanied by membranous ossification was observed just beside the fracture site in MS-818 administered rat.

tological differences were detected between all of the experimental and the control groups. Thus, these results suggest that MS-818 may enhance the formation of hyaline cartilage matrix in fracture healing process.

2. Effect of MS-818 on bFGF induced chondrogenesis in rat bone defect remodeling model.

When the cortical bone of rat was partly resected, this bone defect was fulfilled with only the woven bone by membranous ossification for up to 14 days.

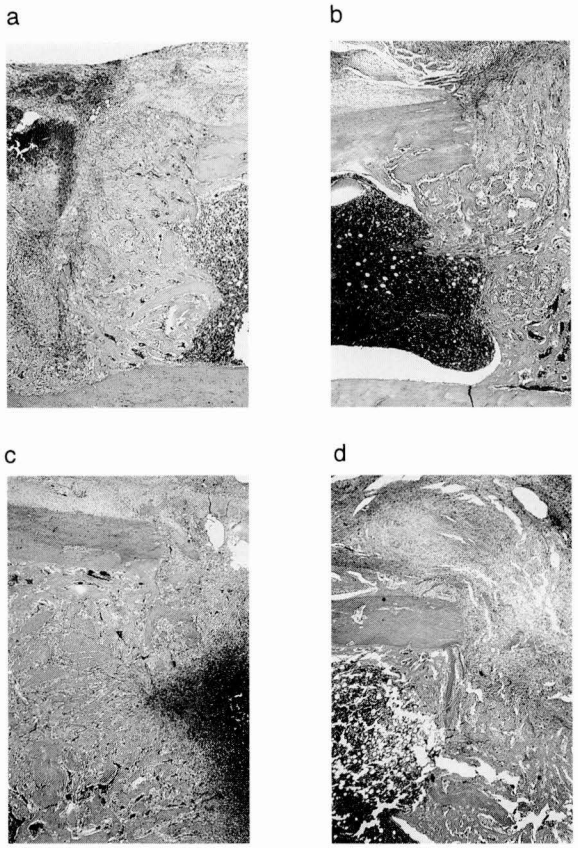

Fig.2. Histological findings of the rat bone defect-remodeling model. When the cortical bone of rat was partly resected, this bone defect was fulfilled with only the woven bone by membranous ossification for up to 14 days. (a) No endochondral ossification was accompanied with this remodeling process. (b) Small amount of chondrogenesis was observed around the bone defect site in some of bFGF pellet implanted rats. (c) When MS-818 pellet was implanted, endochondral ossification was observed in few rats and only the membranous ossification was seen at the bone defect site. (d) MS-818 pellet in combination with bFGF enhanced the cartilage matrix formation at the bone defect site in almost number of the experimental rats.

No endochondral ossification was accompanied with this remodeling process. No hyaline cartilage was detected in the bone defect of rats implanted with saline pellet (Fig.2-a), while small amount of chondrogenesis was observed around the bone defect site in some rats with bFGF pellet(Fig.2-b). Therefore, we have examined the effect of MS-818 in combination with bFGF on the remodeling process of this bone defect remodeling model in order to investigate whether MS-818 might enhance bFGF action in fracture healing process.

When MS-818 pellet was implanted alone in the bone defect, endochondral ossification was observed in few rats and only the membranous ossification was seen at the bone defect site in the remaining rats (Fig.2-c). However, MS-818 pellet in combination with bFGF enhanced the cartilage matrix formation at the bone defect site in all of the experimental rats (Fig.2-d). According to the histological grading, no chondrogenesis was observed in the rats that were implanted with saline pellet. MS-818 pellet alone induced the chondrogenesis in fracture site of a few rats. In the three tenth of experimental rats, small to moderate amount of hyaline cartilage matrix was detected in the fracture site. On the other hand, small to moderate amount of chondrogenesis was observed in nine of ten tibiae implanted with bFGF pellet. One tibia implanted with bFGF pellet has shown a large amount of cartilage matrix formation. When MS-818 pellet was implanted with bFGF pellet, a large amount of cartilage matrix was observed in seven of ten tibiae. Remaining three tibiae showed a moderate amount of matrix formation at the fracture site.

When the extent of cartilage matrix formation was compared according to the histological grading, bFGF pellet induced the chondrogenesis in fracture site more extensively than MS-818 pellet. In the tibiae where MS-818 pellet was implanted with bFGF pellet, larger extent of cartilage matrix formation was induced than the tibiae of bFGF pellet, whereas there was no statistical difference in the extent of matrix formation between saline pellet and MS-818 pellet implanted tibiae.

Table I. Effect of MS-818 on bFGF induced cartilage matrix formation in bone defect remodeling model.

Based on the histological findings, extent of cartilage matrix formation was classified and compared between saline pellet and bFGF with or without containing MS-818 pellet implanted animals. The number in each graded group was analyzed statistically with use of χ^2 -test. Grading of the amount of cartilage matrix formation as follows. (-): no cartilage matrix formation, (±): small, (+): moderate, (++): large amount of cartilage matrix formation.

	Pellets embedded in the bone defect			
Grade	saline [a)]	MS-818 [b)]	bFGF [c)]	bFGF+MS-818 [d)]
-	10	7	0	0
±	0	1	1	0
+	0	2	8	3
++	0	0	1	7
No. of rats	10	10	10	10

a) vs. b): p = Not significant, a) vs. c): $p < 0.05$, a) vs. d): $p < 0.01$

3. Distribution of bFGF in remodeling site of bone defect model

In immunohistochemistry, localization of bFGF was detected in the periosteal cells around the bone defect site and osteoblasts lined along the new bone in which saline or MS-818 containing pellet embedded group (Fig.3-a, b). There is no difference of the number and types of cells positively stained by anti-bFGF antibody among these groups. When bFGF pellet was embedded alone or in combination with MS-818 pellet, large amount of hyaline cartilage matrix was observed and the chondrocytes in this area were strongly stained with anti-bFGF antibody (Fig.3-c, d). However, there is no difference of the number of periosteal cells, osteoblasts and chondrocytes positively stained by anti-

bFGF antibody among the groups of rat implanted with bFGF alone and with bFGF in combination of MS-818.

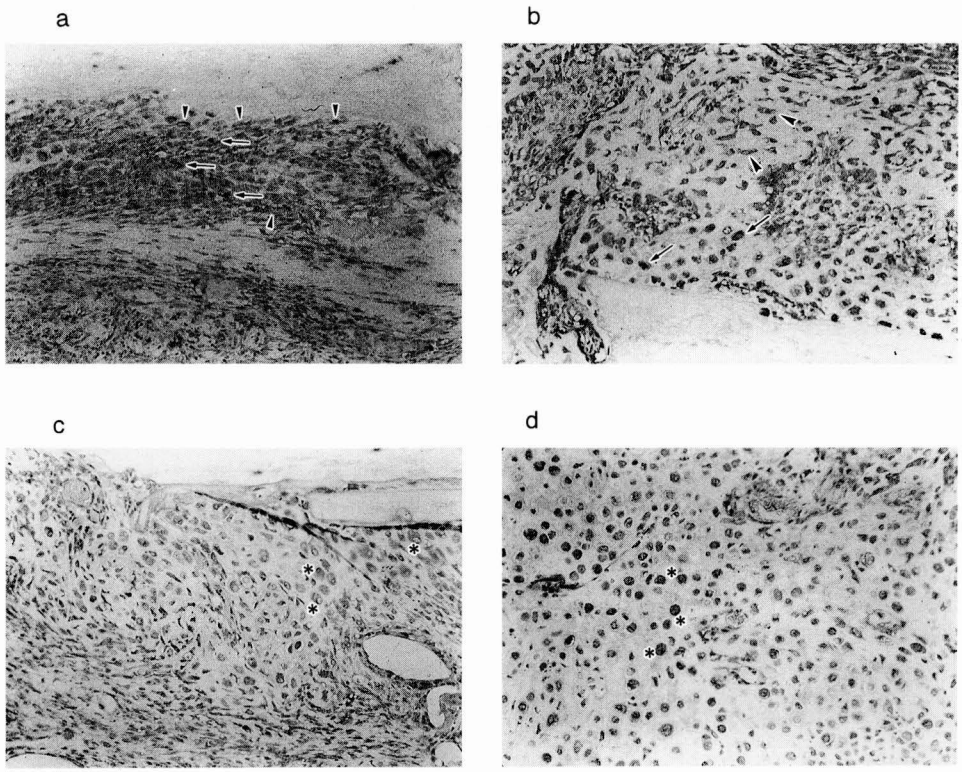

Fig. 3. Immunohistological findings of the bone defect site with b-FGF antibody. Localization of bFGF was detected in the periosteal cells around the bone defect site and osteoblasts lined along the new bone in (a) saline or (b) MS-818 pellet embedded group. When bFGF pellet was embedded (c) alone or (d) in combination with MS-818 pellet, moderate to large amount of hyaline cartilage matrix was observed and the chondrocytes in this area were strongly stained with bFGF. There is no difference of the number and types of cells positively stained by anti-bFGF antibody in all animals.

DISCUSSION

The fracture healing process is regulated by various kinds of soluble factors including BMPs, PDGF, TGF-β, acidic and basic FGF. Basic FGF was shown to stimulate an increase in preosteoblast number after intravenous injection of rats and the essential effect of bFGF on callus formation seemed to be the stimulation of mesenchymal cell proliferation in the periosteum. [20] It was reported that preosteoblasts produced intrinsic TGF-β and induced alkaline phosphatase activity within experimental day one following repeated injections of bFGF into rats [20]. Basic FGF is synthesized by chondrocytes and osteoblasts, and present in extracellular matrix in association with matrix components. [5, 6, 11] The release of bFGF from both cells and matrix at the time of injury may act locally on available receptors and permit them to promote mitogenesis and angiogenesis for bone healing. [26]

Basic FGF is shown to be present in the fracture callus at all stages of fracture healing process, [5] which presumably exerts a continuous effect on the various cell populations of the proliferating and differentiating fracture callus. Basic FGF is also regarded as the most potent mitogen for chondrocyte both *in vitro* and *in vivo* .[17] It was reported that bFGF might regulate endochondral ossification through stimulation of both gene expression of syndecan-3 that is a cell surface receptor for bFGF and chondrocyte proliferation in chick embryo.[24]

A novel synthesized heterocyclic pyrimidine compound, MS-818, was reported to promote the neurite outgrowth of neuroblastoma cell line and potentiate the nerve growth factor-induced neurite sprouting of rat pheochromocytema cells [3]. It is also found that MS-818 regenerated the peroneal nerve of mice after crush injury and enhanced the angiogenesis induced by bFGF. [13, 23, 28]

In terms of the augmentation of bFGF action by MS-818, MS-818 promoted the angiogenesis induced by 10 ng/ml (probably at optimal doses), but not by 1 and 100 ng/ml of bFGF in chick-embryo CAM assay. To date, however, no studies have addressed the effect of MS-818 on the development of callus

formation in fracture healing process. We have, therefore, investigated the role of MS-818 in fracture healing process and its effect of on bFGF induced chondrogenesis *in vivo*.

In the present study, general administration of MS-818 was found to enhance the chondrogenesis in fracture healing process in rat. Fracture site morphology showed that numerous mesenchymal cells were recruited and began to differentiate into not only osteoblasts but also chondrocytes accompanied with hyaline cartilage. Both membranous ossification and cartilage matrix formation were more prominent in MS-818 treated group than those in physiological saline administered group in rat fracture model. These findings suggest that MS-818 may augment the fracture healing process through enhancing the formation of cartilage matrix.

The effects of *in vivo* administration of bFGF on fracture repair have been explored in rats and rabbits, respectively by two laboratories, but differences have been concluded regarding the stimulation of cartilage formation in fracture callus. Kawaguchi et al. observed that bFGF stimulation has increased in callus volume, bone mineral content, and bone strength,[18] whereas Bland et al. reported that bFGF did not significantly affect the size or amounts of bone or cartilage formed at the fracture site.[4]

When the cortical bone of rat was partly resected, this bone defect was fulfilled with only the woven bone by membranous ossification for up to 14 days. No endochondral ossification was accompanied with this remodeling process. When bFGF pellet was implanted in the bone defect, small amount of chondrogenesis was observed around the bone defect. These findings are somewhat consistent with the report that bFGF stimulation increased callus volume. Therefore, the bone defect model described in the present study was shown to be useful for the assessment of MS-818 effect on bFGF induced chondrogenesis *in vivo*.

Basic FGF was observed to enhance the chondrogenesis in bone remodeling process and local administration MS-818 augmented this bFGF in-

duced chondrogenesis in rats. According to histological grading analysis, MS-818 in combination with bFGF induced the cartilage matrix formation in bone defect site more extensively than MS-818 pellet or bFGF pellet alone with statistical significance. In immunohistological findings, the large number of bFGF positive mesenchymal cells were observed around the bone defect site. Numerous chondrocytes positively stained by anti-bFGF antibody were also observed when bFGF pellet was implanted with MS-818 pellet. However, we could not find any obvious increase in the number of bFGF positive cell in control group, and bFGF or MS-818 pellet embedded group. These results indicate that MS-818 might promote the fracture healing process through augmentation of bFGF action.

It is still unknown how MS-818 stimulates the chondrogenesis *in vivo*. We have investigated the effect of MS-818 on aggrecan synthesis of articular chondrocyte in combination with bFGF *in vitro*. There was no remarkable enhancement of aggrecan synthesis by MS-818 (data not shown). It has been reported that periosteum plays an important role in chondrogenesis in fractures healing process. The hypothesis was examined whether MS-818 might stimulate the periosteal cell proliferation in combination of bFGF. As expected, bFGF has stimulated the cell proliferation derived from rabbit periosteum, whereas MS-818 had no effect on bFGF induced cell proliferation *in vitro* (data not shown).

Angiogenesis is necessary for supplying not only the nutrition but also the oxygen to fracture site and abrupt change in oxygen availability have been suggested to have a regulatory role in bone growth and remodeling of fracture healing. [9] MS-818 was reported to promote the angiogenesis induced by bFGF. It is, therefore, supposed that MS-818 might augment the angiogenesis in fracture site induced by bFGF. Another possibility is that MS-818 may act on macrophages and/or mast cells, which do play roles in angiogenesis though it does not have an angiogenic effect by itself. MS-818 may exert an effect on mononuclear cells infiltrated in fracture site in the presence of bFGF. [7]

It has not been fully investigated in this study that the detailed mecha-

nisms of the induction of the endochondral ossification by bFGF in combination with MS-818 and whether MS-818 may augment bFGF action on chondrocyte directly. The results of the present study provide the promising possibility of clinical application of MS-818 on the treatment of fracture.

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