行政院國家科學委員會專題研究計畫　成果報告

造骨細胞及骨肉瘤細胞形成 Fibronectin 基質微纖維之機轉研究及生長因子對其功能之調節(2/2)

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一、中文摘要

Fibronectin (Fn) 在調控細胞附著、移動及成骨細胞的成熟中扮演了一個非常重要的角色，而 Fn 的纖維固化也參與骨骼礦質化過程，Bone morphogenetic protein-4 (BMP-4)可從骨間質中抽取出來而促進骨質形成。在本次研究當中我們去研究 BMP-4 對老鼠成骨細胞 Fn 間質纖維聚合（fibrillogenesis）的調控作用。BMP-4 可以增加 Fn 合成及細胞外的纖維合成，BMP-4 也可以增加由外界直接加入 soluble fibronectin 的 fibrillogenesis。在之前的報告有指出 α5 and β1 integrins 與 Fn fibrillogenesis 有關，BMP-4 也可以增加α5 and β1 integrins 的合成，利用免疫螢光染色法也可看到促進α5 and β1 integrins 的聚合。BMP-4 可以增加成骨細胞黏著到 Fn 間質上，而蛇毒蛋白 triflavin 及 Gly-Arg-Gly-Asp-Ser (GRGDS)可以抑制其增加的作用。BMP-4 可以促進磷酸化 extracellular signal-regulated kinase (ERK) 及 focal adhesion kinase (FAK)。這個結果說明了 BMP-4 可以增加成骨細胞當中 Fn 纖維聚合是與增加 Fn 合成及α5 and β1 integrins 聚合有關。

關鍵詞：fibronectin、造骨細胞、bone morphogenetic protein-4、integrin、細胞間質.

Abstract

The skeletal extracellular matrix produced by osteoblasts contains the glycoprotein fibronectin (Fn), which regulates the adhesion, differentiation, and function of osteoblasts. Fn fibrillogenesis is involved in the process of bone mineralization. Bone morphogenetic proteins (BMPs) can be isolated from organic bone matrix and are able to initiate de novo cartilage and bone formation. In this study, the effect of BMP-4

on Fn fibrillogenesis in cultured rat osteoblasts was examined. BMP-4 enhanced Fn synthesis and extracellular Fn assembly in primary cultured osteoblasts. In addition, the extracellular assembly of Fn from exogenously applied soluble human Fn was also increased by BMP-4. It has been reported that α5β1 integrin is related to Fn fibrillogenesis. The synthesis of both α5 and β1 integrins was up-regulated by BMP-4. Immunocytochemistry showed that the clustering of α5 and β1 integrins was also increased by BMP-4. BMP-4 increased fibril formation of Fn and the adhesion of osteoblasts onto Fn matrix, which was inhibited by disintegrin triflavin and Gly-Arg-Gly-Asp-Ser (GRGDS) peptide. Phosphorylation of extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK) was increased by BMP-4. These results suggest that the enhancement of extracellular Fn fibrillogenesis by BMP-4 in cultured osteoblasts is related to the increase of the synthesis of Fn and clustering of α5 and β1 integrins.

Keywords: fibronectin、osteoblast、bone morphogenetic protein-4、 integrin、 extracellular matrix.

二、緣由與目的

Osteoblast differentiation is an essential part of bone formation, because active osteoblasts should be recruited at the site of osteoclastic bone resorption to compensate the continuous loss of bone matrix and maintain structural integrity of skeletal system. The biology of this process is also of considerable interest when applying therapies to promote bone repair after injury or during disease processes. Accumulating evidence indicates that several factors, such as hormones, cytokines, and growth factors, are involved in the osteoblastic differentiation.[1,2] Bone morphogenetic proteins (BMPs), with more than 20 members, belong to the TGF-β superfamily and were originally identified by their unique ability to induce ectopic cartilage and bone formation in vivo.[2,3] It has been shown that BMP-2 and BMP-4 are synthesized by osteoblasts.[4] BMPs play important roles in bone formation and bone cell differentiation by stimulating alkaline phosphatase activity and synthesis of proteoglycan, collagen, and osteocalcin.[2,5] The BMP-4 is a BMP-2 subfamily and induces apoptosis in different cells.[6,7] Mechanical stress induces osteoblast differentiation, which then leads to osteogenesis.[8,9] This osteoblast differentiation seemed to be accompanied by an increase in BMP-4 gene expression. Although BMP-4 expression is upregulated under tensile stress or during distraction osteogenesis,[8,9] the exact role of BMP-4 in these systems is still unclear.

ECMs and growth factors are likely to act cooperatively to stimulate osteoblast differentiation, as has been shown for other cell types.[10] There is strong evidence to suggest a role for Fn in the early stages of osteogenesis. The distribution of Fn in areas of skeletogenesis suggests that it may be involved in early stages of bone formation.[11,12] However, the effect of BMPs on Fn fibrillogenesis in osteoblasts have been mostly unknown. We here found that BMP-4 enhanced Fn fibrillogenesis of osteoblasts by increasing the synthesis and assembly of Fn. Furthermore, the increase of synthesis and clustering of α5 and β1 integrins is involved in the mechanism of action of BMP-4.

三、結果與討論

The fibrillogenesis from the endogenously released Fn by the primary cultured rat osteoblasts was studied using immunocytochemistry. Day 3 to day 5 osteoblasts were changed to serum-free medium and incubated with BMP-4 (20 ng/ml) for 12 h. The mean immunofluorescence intensity underneath a cell group of 10–15 cells was measured using confocal microscope. As shown in Fig. 1A, osteoblasts are able to form Fn network underneath the cell using endogenously released Fn. Fn fibril formation increased in response to the treatment of BMP-4 for 12 h (Fig. 1B). The mean fluorescence intensity underneath 10–15 cells was 36.4 ± 2.5 and 63.3 ± 4.1 (n =23–28) for control and

BMP-4-treated cells, respectively. We also examined the effect of other species of BMP on the Fn fibrillogenesis. BMP-2 and BMP-7 (20 ng/ml each) also increased Fn fibrillogenesis, whereas TGF-β1 dose dependently decreased Fn assembly. These results suggest that the action of BMPs is different from TGF-β1 on the assembly of Fn. We investigated the action mechanism of BMP-4 on Fn fibrillogenesis in the following experiments.

Western blotting was used to examine the effect of BMP-4 on the protein levels of Fn. Day 3 to day 5 osteoblasts were changed to serum-free cultured medium and treated with BMP-4 for 12 h. The cultures were then washed with cold PBS and protein samples were collected by the addition of lysis buffer. The result from Western blotting may contain both soluble cytosolic Fn and extracellular immobilized Fn. As shown in Fig. 2A, BMP-4 dose-dependently increased protein levels of Fn, α5 and β1 integrins after incubation with cells for 12 h. In addition, BMP-4 also time dependently enhanced protein levels of Fn, α5 and β1 integrins when examined at a concentration of 20 ng/ml (Fig. 2B). We further quantified cytosolic soluble Fn levels by the digestion of the cells with trypsin-EDTA (0.25% trypsin, 1 mM EDTA) for 2 minutes to get cytosolic fraction of the cell suspension after treatment with BMP-4 for 12 h. As shown in Fig. 2C, BMP-4 treatment (20 ng/ml) also increased cytosolic Fn. These results indicate that BMP-4 may increase Fn fibrillogenesis by upregulating both Fn synthesis and extracellular Fn assembly.

We then used flow cytometry to investigate the effect of BMP-4 on the cell surface expression integrins. As shown in Fig. 3, incubation with BMP-4 (20 ng/ml) significantly enhanced the fluorescence intensity α5 or β1 integrin after 12 h incubation. The cell surface expression of integrins increased in a time-dependent manner in response to the administration of BMP-4. In contrast, TGF-β1 dose-dependently decreased the surface expression α5 or β1 integrins after 12 h incubation (α5: the fluorescence intensity was 136.1 ± 19.1, 100.3 ±13, 86.6 ± 15.1, and 66.3 ± 9.8 for control, TGF-β1 1 ng/ml, ng/ml, and 100 ng/ml, respectively; β1: the fluorescence intensity was 61.9 ± 3.9, 50.3 ± 1.9, 41.2 ± 2.8, and 29.1 ± 4.3 for control, TGF-β1 1 ng/ml, 10 ng/ml, and 100 ng/ml, respectively). On the other hand, effects of BMP-4 the expression of different subunits of integrin is rather selective. BMP-4 treatment only slightly increased the fluorescence intensity of surface expression of α2 (control, 79.7 ± 6.2; BMP-4, 119.5 ± 9.4, $n = 4$) but not those of α3 and α4.

BMPs signal through heteromeric complexes of transmembrane type I and type II Ser/Thr kinase receptors that then propagate signals to the Smad or other signaling pathway.[13] Treatment with BMP-4 for 10–15 minutes induced phosphorylation of FAK and ERK (Figs. 4A and 4B). We then examined the involvement of ERK activation in the enhancement of Fn fibrillogenesis by BMP. Treatment with MEK inhibitor PD 98059 (30 μM) inhibited the potentiating action of BMP-4 (20 ng/ml) on the extracellular Fn assembly. We thus examined the effect of BMP-4 on the expression of β1 integrin mRNA. As shown in Fig. 4C, amplification was accomplished with 17 cycles, which was within the linear range of PCR product. Treatment of BMP-4 for 1 h increased the mRNA expression of β1 integrin, which was antagonized by concomitant treatment with PD 98059 (Figs. 4C and 4D). These results suggest that ERK activation may be involved in the action of BMP-4 on Fn fibrillogenesis.

BMPs belong to a group of proteins that act to induce the differentiation of mesenchymal-type cells into chondrocytes and osteoblasts before initiating bone formation.[14] The results from this study provide evidence that BMP also regulates Fn fibrillogenesis in cultured rat osteoblasts. The dynamic nature of these Fn fibrils is related to the differentiation and functional regulation of osteoblasts. It has been reported that osteoblasts become increasingly dependent on Fn for survival when they differentiate and form nodules.[15]

In conclusion, our results show that BMP-4 increases Fn fibrillogenesis from both endogenously released or exogenously applied soluble Fn. BMP-4 upregulates the

synthesis and clustering of α5 and β1 integrins. ERK activation is involved in the action of BMP-4 in the dynamic regulation of Fn and bone tissue remodeling.

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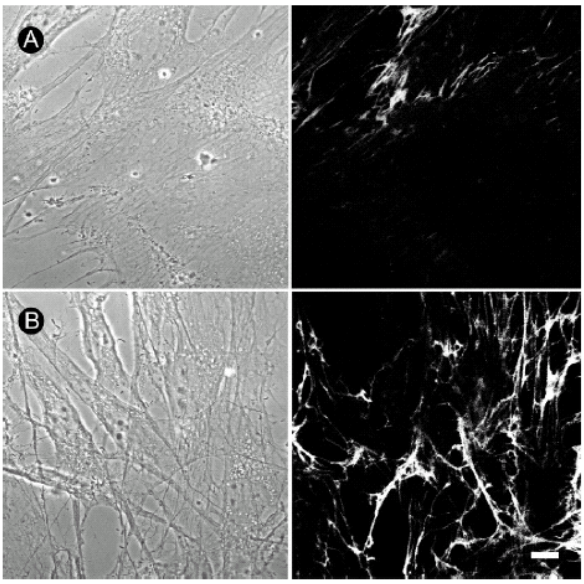

FIG. 1. Increase of Fn fibrillogenesis by BMP-4 in cultured rat osteoblasts. Fn network, which was shown by immunofluorescence, formed underneath the cultured osteoblasts. Compared with (A) control, (B) treatment with BMP-4 (20 ng/ml) for 12 h increased Fn fibrillogenesis in cultured osteoblasts.

Phase-contrast images are shown in the left panels. Bar = 10μm.

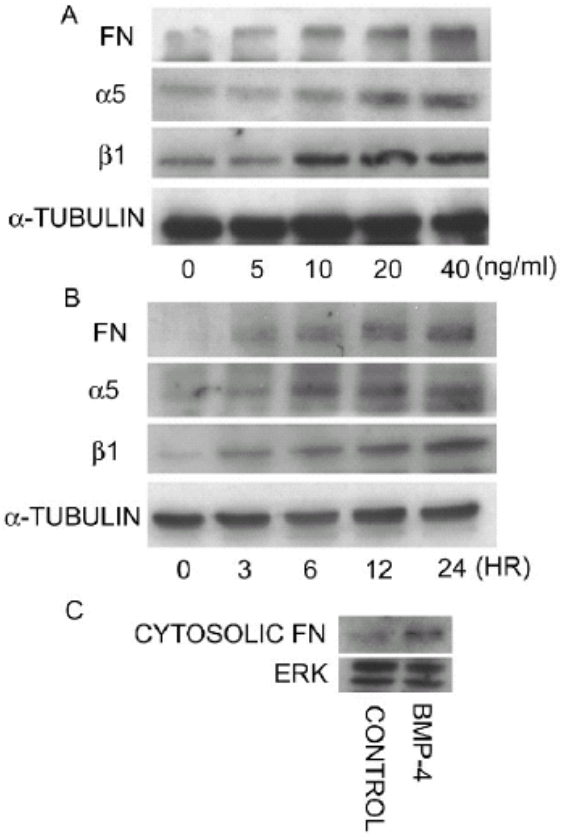

FIG. 2. Upregulation of protein levels of Fn, α5 and β1 integrins by BMP-4. (A) Osteoblast cultures were treated with different concentrations of BMP-4 for 12 h. The cultures were washed with cold PBS, and protein samples for Western blotting analysis were collected by the direct addition of lysis buffer to cultures without trypsin digestion. Compared with control, BMP-4 dose-dependently increased the protein levels of Fn, α5 and β1 integrins. (B) Compared with control, BMP-4 time-dependently increased the protein levels of Fn, α5 and β1 integrins at a concentration of 20 ng/ml. (C) Cytosolic soluble Fn levels were measured by obtaining cytosolic fraction of the cultured cells after treatment with BMP-4 for 12 h. Note that BMP-4 also increased cytosolic Fn.

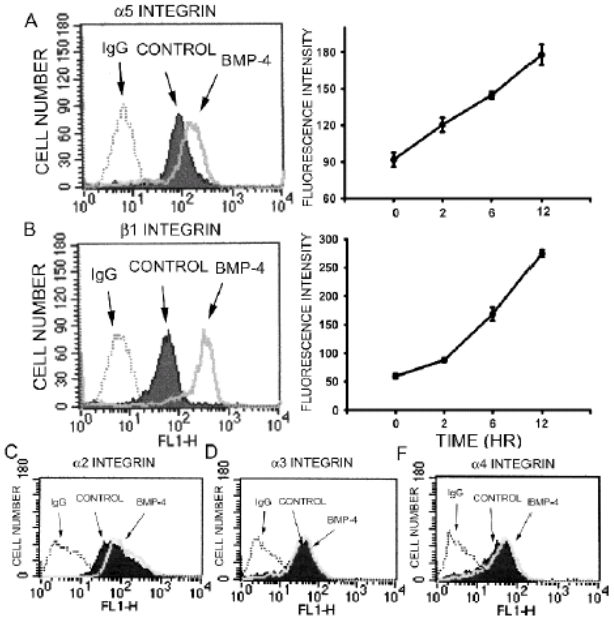

FIG. 3. Increase of the cell surface expression of α5 and β1 integrins by BMP-4 using flow cytometric analysis. Compared with control, treatment with BMP-4 (20 ng/ml) for 12 h enhanced the fluorescence intensity of (A) α5 and (B) β1integrins, respectively. The quantitative data are shown in the right panels. The cell surface expression of α5 and β1 integrins increased in response to BMP-4 in a time-dependent manner. BMP-4 treatment slightly increased (C) α2, but not (D) α3 or (E) α4 integrin. Data are presented as mean ±SE (n = 3).

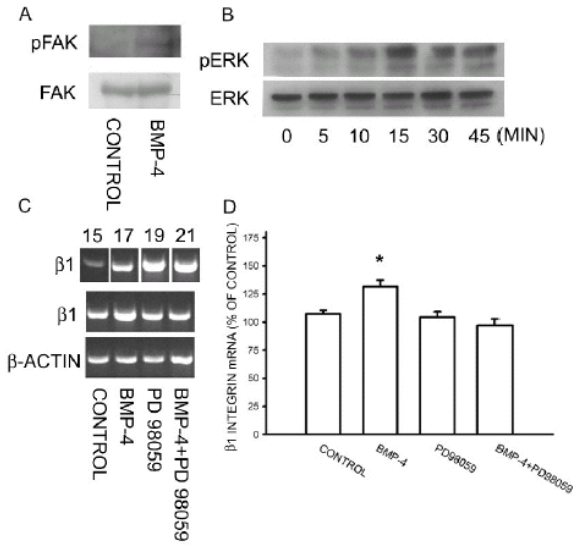

FIG. 4. Activation of FAK and ERK by BMP-4. (A) Osteoblasts were treated with BMP-4 (20 ng/ml) for 10 minutes, and FAK was then immunoprecipitated from cell lysate. PFAK was detected by Western blotting. Note that BMP-4 treatment increased the phosphorylation of FAK. (B) ERK was detected from cell lysate after osteoblasts were incubated with BMP-4 for different time intervals. Note that BMP-4 treatment increased the phosphorylation of ERK and reached a peak level at 15 minutes. (C) mRNA level of β1 integrin was measured using RTPCR and

amplification was accomplished with 17 cycles, which was within the linear range of PCR product (cycles of 15-21 were shown in the upper panel). Note that BMP-4 treatment enhanced the mRNA expression of β1 integrin, which was antagonized by MEK inhibitor PD98059. The quantitative data were shown in D. (mean± SE, $n = 3$). *$p < 0.05$ compared with control.

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