Original Article

Effects of Matrices for Chondogenesis and Osteogenesis Induced by Bone Morphogenetic Protein-2

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Abstract

The effect of matrix on the chondrogenesis and osteogenesis induced by bone morphogenetic protein-2 (BMP-2) was examined to investigate the mechanism of hard tissue formation. Two types of membrane filter made with glass fiber, with fiber diameters of 0.6 and 1.0 m, were implanted subcutaneously in rats as a carrier of the BMP-2. The BMP-2 induced cartilage formation within the entire area of the membrane filter with fiber diameter of 1.0 m two weeks after implantation. Bone formation was induced at the surface of 1.0 m fiber diameter membrane, surrounding the cartilage tissue three weeks after implantation. Four weeks after implantation, bone formation was observed to progress from the surface area toward the subsurface area of the membrane filter while cartilage was persistent in the central area of the filter, indicating typical endochondral ossification. On the other hand, neither bone nor cartilage was formed in the membrane filter with 0.6 m fiber diameter even three weeks after implantation.

It was suggested that the fiber diameter of the membrane filter regulates cell-migration and vascularization into the membrane filter which triggers the transformation of cartilage to bone. It was also suggested that BMP-carrier is an important factor to control the cascade rate of endochondral ossification induced by BMP.

In this study, we concluded that the microenvironment produced by the BMP-carrier regulates BMP-induced chondrogenesis and osteogenesis.

Introduction

To understand the complex mechanism of hard tissue formation, it is important to classify the factors which are involved in the biological process of hard tissue formation. There are four factors required for hard tissue formation[1]. They are: (1) cells, (2) matrices, (3) mineral ions, and (4) regulators. It was proposed the importance of analysis of these four factors and of interaction among each factor for gaining a new knowledge on hard tissue formation.

Among the regulators, bone morphogenetic protein (BMP) has been known to induce chondrogenesis and osteogenesis *in vivo*[2-7]. Cloning and sequencing of the DNA has been shown that BMP is a member of the TGF-b supergene-family[8]. There are different types of BMPs, these are BMP-2 to BMP-15[9-11]. Gene-technology has produced recombinant BMP-2, -4, and -7 available, and they are known to induce ectopic and orthotopic bone formation[12-14].

To induce chondrogenesis and osteogenesis, BMP requires a carrier as it diffuses into the tissue without a carrier[1-5]. Furthermore, it is thought that the BMP-carrier has an important supporter for cell differentiation as well as a drug delivery system.

Since the discovery of BMP by Urist in 1965[15], insoluble bone matrix has conventionally been used as a BMP-carrier for bioassays of purified BMP[1-5], and BMP has been thought to induce undifferentiated mesenchymal cells to follow a process of endochondral ossification.

To elucidate the effect of BMP-carriers on BMP-induced cell differentiation, this study investigated differences in BMP-induced chondrogenesis and osteogenesis with membrane filter, made with glass fiber, with different fiber diameters used as carriers implanted subcutaneously in rats.

Materials And Methods

Preparation of BMP/Carrier Composites

The membrane filter of unwoven glass fibrils (Advantec, Tokyo) with two fiber diameters, 1.0 and 0.6

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mm (the fiber diameter is the same as exclusive size in the membrane) were cut into rectangular sizes, 10 x 5 x 1 mm (6 mg). Then, 1.1 mg of BMP-2 in 0.05ml of phosphate-buffered saline was absorbed to them (BMP-2/1.0 and BMP-2/0.6).

Implantation of BMP/Carrier Composites

Four-week-old wistar strain male rats (about 60 g body weight) were anesthetized intraperitoneally with pentobarbital sodium (4 mg/100 g body weight). The back skin was shaved, cleaned with 70% alcohol and iodine, and subcutaneous pockets were formed by using surgical knife and blunt instrument. The BMP/carrier composites were implanted with tweezers in the subcutaneous pockets and sutured. The animals were killed with an overdose of ether one, two, three and four weeks after implantation. The implants were removed and subjected to biochemical analysis and histological observation.

Biochemical Analysis

The extracted pellets were lyophilized and stored frozen at -80 °C until analysis. The samples were cut with scissors in a conical tube into fine powders of about 0.1 mm in particle size. An aliquot of sample was shaken with a vortex in 0.2% Nonidet P-40, 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5 at 4 °C for 24 hours. After centrifugation of the suspension, the ALP activity in the supernatant was measured by the Kind-King phenylphosphate method[16].

A further aliquot of sample was hydrolyzed with 6N hydrochloric acid. The calcium content in the hydrolyzed sample was measured by the orthocresolphtalein complexon method[17].

For determination of type II collagen, the remaining minced pellets were treated with 0.1% pepsin, 0.5 M acetic acid followed by 20 mM dithiothreitol [18], 1 M NaCl, 50 mM Tris-HCl, pH 7.4 including protease inhibitors (50 mM aminocaproic acid, 5 mM benzamidinehydrochloride, 1 mM benzylsulfonylfluoride) at 4 °C. The digests were purified by 0.8 M salt precipitation at acidic pH. The precipitation was solved with 0.5 M acetic acid and used for the assay. Type II collagen was identified and quantified by enzyme-linked immunosorbent assays using anti-bovine type II collagen antibody (Life Science, Tokyo) with bovine type II collagen as the standard.

Histological Observation

The extracted pellets were fixed in 10% neutral formalin buffer, demineralized in 10% formic acid, embedded in paraffin, sectioned at 4 mm, and stained with hematoxylin and eosin for light microscopy observation.

Results

Biochemical Analysis

The dry weight, ALP activity, calcium and type II collagen contents induced were compared between BMP-2/1.0 and BMP-2/0.6, two weeks after implantation (Fig. 1). The BMP-2/1.0 showed higher values in dry weight, ALP activity, calcium and type II collagen contents than the BMP-2/0.6. Particularly, the BMP-2/0.6 showed very low values in ALP activity, calcium and type II collagen contents.

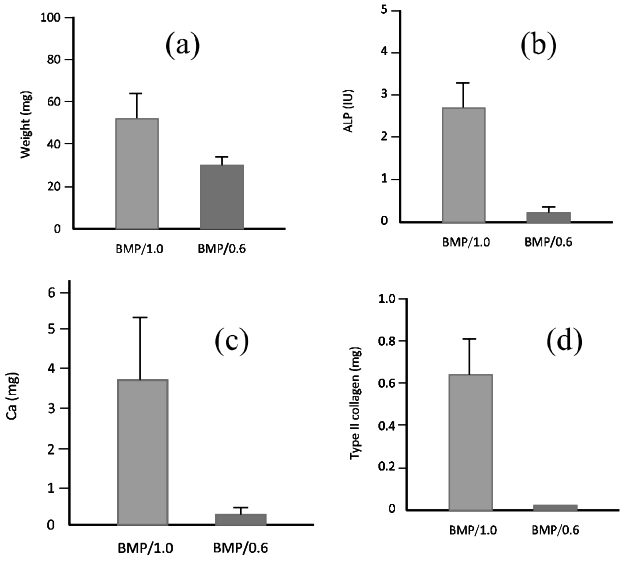

Figure 1. Dry weights (a), alkaline phosphatase activities (b), calcium contents (c), and type II collagen contents (d) of BMP-2/1.0 and BMP-2/0.6 harvested two weeks after implantation. Vertical bars indicate standard deviations (n=3).

Histological Observation

In BMP-2/1.0, one week after implantation, cell migration was observed at only surface area of the membrane filter (Fig. 2a). Two weeks after implantation, cartilage was observed in the filter (Fig. 2b). Most of the volume of the filter was filled with cartilageous matrix and chondrocyte-like cells. Bone formation surrounding the cartilage was observed on the surface of the membrane three weeks after implantation (Fig. 2c). Bone formation was observed to progress from the surface area toward the subsurface area of the membrane filter while cartilage was persistent in the central area of the filter (Fig. 2d).

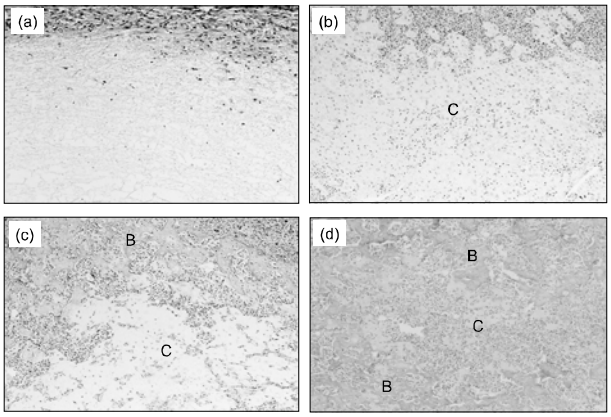

Figure 2. Histological observations of BMP-2/1.0 harvested one (a), two (b), three (c) and four (d) weeks after implantation. C: cartilage. B: bone. Original magnification x 100.

On the other hand, neither bone nor cartilage was formed in the membrane filter in BMP-2/0.6 even three weeks after implantation (Fig. 3).

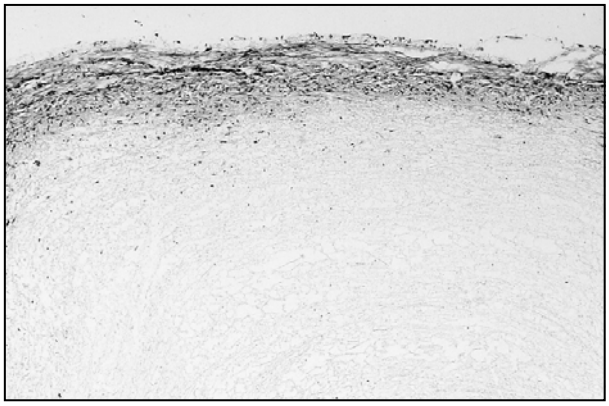

Figure 3. Histological observations of BMP-2/0.6 harvested two weeks after implantation. Original magnification x 100.

Discussion

This study showed the effect of BMP-carrier on the processes of osteogenesis and chondrogenesis induced by BMP-2. The biochemical and histological analysis showed cartilage formation in the BMP-2/1.0 two weeks after implantation. Only a small number of undifferentiated mesenchymal cells may be able to invade into the membrane with 0.6 mm diameter of fibers because the mesh structure is tight while a larger number of cells may invade the membrane with a looser mesh structure. Thus, the BMP-2/0.6 induced either bone formation or cartilage formation. Bone formation was observed at the surface of the 1.0mm filter surrounding the cartilage which was induced at the central area of the membrane filter three weeks after implantation. Moreover, bone formation progressed from the surface area toward the subsurface area of the membrane filter while cartilage was persistent in the central area of the filter four weeks after implantation, showing the slow cascade of endochondral bone formation.

The effect of the cell-substratum on cell differentiation was first described by Reddi and Huggins[23]. They implanted decalcified bone powders containing intact BMP with two distinct particle sizes under rat skin, and found that the coarser powder, with particle sizes of 420-850 mm, induced more bone formation than the finer powder, with particle sizes of 44-74 mm. This indicated that BMP-induced bone formation depends on the carrier geometry.

The membrane filter with the 1 mm fiber diameter combined with BMP induced endochondral ossification which cartilage is persistent for a long period. We attributed this phenomenon in the filter to the delay of vascularization and blood supply into the carrier. Bassett reported that a low oxygen concentration plays a role in the induction of chondrogenesis, while a high oxygen concentration was necessary to induce bone formation[24]. We hypothesized that the membrane filter, with its fine solid fibrillar structure, prevents blood vessels from invasion into the membrane in the early stage of implantation with BMP. Therefore, it was suggested that the membrane filter creates a lacking in blood supply and oxygen supply, which is favorable for chondrogenesis. This microenvironment seemed to facilitate the differentiation of immature mesenchymal cells, which had invaded the membrane filter by chemotaxis induced by BMP, into chondrocytes, and sustain cartilage in the membrane filter for a long time.

This study suggested that the local oxygen pressure supplied by invasion of blood vessels into the BMP-carrier is very important for cell-differentiation. It was also indicated that the microenvironment induced by the geometry of matrices on which undifferentiated mesenchymal cells attach is involved in regulating the direction of BMP-derived cell differentiation. Further, this study suggested the possibility of individual induction of bone or cartilage formation utilizing BMP by regulation of the microenvironment surrounding the cells according to the purpose.

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