Enhancement of bone ingrowth in a titanium fiber mesh implant by rhBMP-2 and hyaluronic acid

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The present study was designed to evaluate the efficacy of high molecular weight hyaluronic acid (HA) used as a carrier of recombinant human bone morphogenetic protein-2 (rhBMP-2) adsorbed to a titanium fiber mesh implant (TFMI) *in vivo*. The quantity of HA in the TFMI rapidly decreased during the initial 3-day period after implantation. BMP particles were trapped by the meshwork of HA as observed by scanning electron microscopy (SEM). TFMIs containing LF-6, HA, rhBMP-2, or HA combined with rhBMP-2 were implanted on the cranium of rats. Analysis of digitized SEM images of samples obtained six weeks post-implantation was performed to determine the area occupied by new bone. The area fraction of Ca relative to that of the pores of TFMI in the HA group was larger than that in the Ti group ($p < 0.05$). The area fraction of Ca in both the BMP and HA + BMP groups was larger than that in both the Ti and HA groups ($p < 0.01$), and that in the HA + BMP group was larger than that in the BMP group ($p < 0.05$). It is suggested that HA is not only an effective carrier of BMP, but also it may have a positive effect on the generation of new bone in the TFMI.

1. Introduction

Various types of titanium implants differing in morphology have been developed and used in clinical trials. Among them, the titanium fiber mesh implant (TFMI) has both excellent biocompatibility and excellent properties in terms of interbody fusion and weight bearing. Also, this type of TFMI has been used for replacement of intervertebral disks [1], mandibular reconstruction [2], surface coating on the stem of an artificial joint system [3], and as a percutaneous device [4, 5]. It has several merits as follows: (1) it is biocompatible and can afford immediate stability and weight bearing after operation, (2) the interface between the implant and bone allows bone ingrowth for long-term stability, and (3) it is resistant to fatigue fractures before bone ingrowth occurs. However, because ingrowth of bone into the TFMI in clinical applications is not always satisfactory, a need for acceleration of bone bondage remains.

To shorten the period until rigid bone union is completed, and to lessen the chance of failure of the implant after implantation, recombinant human bone morphogenetic proteins (rhBMPs) which are well known to induce bone formation in skeletal and non-skeletal sites [6, 7] are available. If rhBMPs are implanted without an appropriate carrier, however, they can not induce much bone formation because they are immediately dispersed. For this reason, biocompatible carriers are indispensable to introduce rhBMPs. The present study was designed to evaluate the efficacy of high molecular weight hyaluronic acid (HA), one of the major glycosaminoglycans, used as a carrier of BMP adsorbed to the TFMI *in vivo*.

2. Materials and methods

2.1. Manufacture of the TFMIs

Pure Ti fiber wires 250 mm in diameter were formed into sheets, compressed in a die-punch, and molded into a cylindrical shape 7 mm in diameter and 2 mm in thickness (Kyosera, Kyoto, Japan). The TFMIs prepared were cleaned in an ultrasonic sink using a neutral detergent, then rinsed with distilled water and heat-treated at 1300 °C in a vacuum chamber which provided an open pore structure, ensuring continuity between the pores. These TFMIs had a pore diameter of 50–450 mm, 40–60% porosity, and a Young's modulus of 4.2×10^3 MPa.

2.2. Changes in the concentration and distribution of HA after implantation

High molecular weight HA (9×10^5 MW) derived from cockscomb was obtained from Seikagaku Corporation (Tokyo, Japan). The pH of the HA preparation was adjusted to 6.8–7.8, and its osmotic pressure compared to saline was maintained at 1.0–1.2. The TFMIs were

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sterilized in an autoclave and soaked in the HA preparation under reduced pressure for 30 min. They were lyophilized and stored at 80 °C until use. Under anesthesia induced by intraperitoneal administration of Nembutal (sodium pentobarbital, 50 mg/kg body weight), TFMIs containing HA were implanted in the back of 18 male Wistar rats (180–200g). The implants were put on the fascia between the scapula. Three rats each day were sacrificed by intraperitoneal injection of a high dose of Nembutal at 1, 3, 5, 7, 14 and 21 days after transplantation, and the quantity of HA in the implants was measured. As a control, the quantity of HA in three implants was measured before operation. The approval of the review board of our university was obtained before use of the animals, and all procedures employed in this study were in accordance with the standards of the guidelines for the care and use of laboratory animals of our university. The TFMIs were dipped into 1–2 ml of 0.25% hyaluronidase, and the HA in the implants was digested for 12 h at 55 °C. The extracts obtained were filtered through a membrane microfilter with a molecular weight cut-off of 1×10^4 MW. The quantity of HA in the filtrate was measured by high-performance liquid chromatography (HPLC).

Furthermore, TFMIs containing fluorescence-labeled HA (9×10^5 MW) were implanted in the back of 18 male Wistar rats by the same procedures, and the implants were recovered with the surrounding soft tissue from 3 rats each day on day 1, 3, 5, 7 and 14 after operation. The fresh specimens were observed under a fluorescence microscope (Olympus AX-URBL; Tokyo, Japan).

2.3. Morphology of rhBMP-2 in HA

The morphology of the complex formed between rhBMP-2 and HA was observed by scanning electron microscopy (SEM, Hitachi S-900; Tokyo, Japan). A mica surface was covered with droplets of 0.1% poly-L-lysine hydrobromide solution for 10 min, and dried at room temperature after washing. A 5 µl drop of high molecular weight HA (9×10^5 MW) with or without rhBMP-2 (100 µg/ml, Genetic Institute, Cambridge, MA, USA) was placed on the mica for 10 min, immersed in 2.5% glutaraldehyde for 2 h, post-fixed in 2.0% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated through a graded ethanol series and critical-point dried using liquid CO_2. The specimens were ion-sputter-coated with platinum and observed by SEM.

2.4. Evaluation of the efficacy of high molecular weight HA used as a carrier of BMP

Under anesthesia induced by intraperitoneal administration of Nembutal, TFMIs containing HA (9×10^5 MW, HA group), TFMIs containing rhBMP-2 (100 µg/ml, BMP group) and TFMIs containing HA combined with rhBMP-2 (100 µg/ml, HA + BMP group) were implanted on the cranium of male Wistar rats (180–200g). The periosteum was elevated and the implant was grafted between the periosteum and the cortical bone of the cranium. As a control group, rats received grafted TFMIs containing LF-6 (a solvent of rhBMP-2, pH 4.5) only (Ti group). The HA and Ti groups each consisted of eleven rats, and the BMP and HA + BMP groups each consisted of sixteen rats.

Four specimens were collected together with the surrounding tissue including bone at three weeks post-surgery in the case of each group. Seven specimens from rats in the HA and Ti groups, and twelve specimens from rats in the BMP and HA + BMP groups, were collected at six weeks. Each week, two specimens were fixed first in 2.5% glutaraldehyde and then in 2.0% osmium tetroxide; thereafter, they were embedded in Epon812 in the standard manner. Thin sections of the tissue between the TFMI and the cranium were prepared and stained with toluidine blue for light microscopic observation. Ultrathin sections of that region were double-stained in uranyl acetate and lead citrate for transmission electron microscopy (TEM, Hitachi H-600; Hitachi, Tokyo, Japan). Two and five specimens from rats in the HA and Ti groups, respectively, and two and ten specimens from rats in the BMP and HA + BMP groups, respectively, were harvested at three and six weeks after implantation. Each of the specimens was dehydrated by sequential immersion in a graded series of alcohol (70–100%), immersed in styrene monomer and then placed in polyester resin. Polymerized and cured samples were cut into two parts at the center using a diamond disk and the stump of the TFMI was observed by SEM. And further, SEM images of five samples from rats in the HA and Ti groups, ten samples from rats in the BMP group, and nine samples from rats in the HA + BMP group collected six weeks after implantation were examined to determine the area occupied by new bone. These SEM images obtained at a magnification of 30 × were digitized by an EMAX-7000 analysis system (Horiba, Ltd, Tokyo, Japan). Gray level thresholding of Ca and Ti was controlled by the operator in order to define the Ti fiber and mineralized bone area within the stump of the TFMIs. Analysis of digitized images of Ca and Ti was performed on a Macintosh model computer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health). The area fraction of Ca of newly generated bone relative to that of the pores of TFMI was calculated $(S_{Ca}/(S - S_{Ti}) \times 100\ (\%)$; S_{Ca}: area of Ca, S_{Ti}: area of Ti fiber, S: total area of TFMI calculated) (Fig. 1 (a)–(c)). The differences between the experimental and control groups were determined and evaluated for statistical significance by means of one-way analysis of variance (one-way ANOVA) and assessed by Mann-Whitney's U test. The level of significance was $p < 0.05$.

3. Results

3.1. The concentration and distribution of HA

The quantity of HA in the TFMI rapidly decreased during the initial 3-day period after implantation, and only a little quantity of it remained in the TFMI after four days (Fig. 2(a)).

Although the intensity of the fluorescence signal from the surface of TFMIs did not change throughout the observation period, that from the soft tissue covering the TFMIs increased as time passed (Fig. 2(b)).

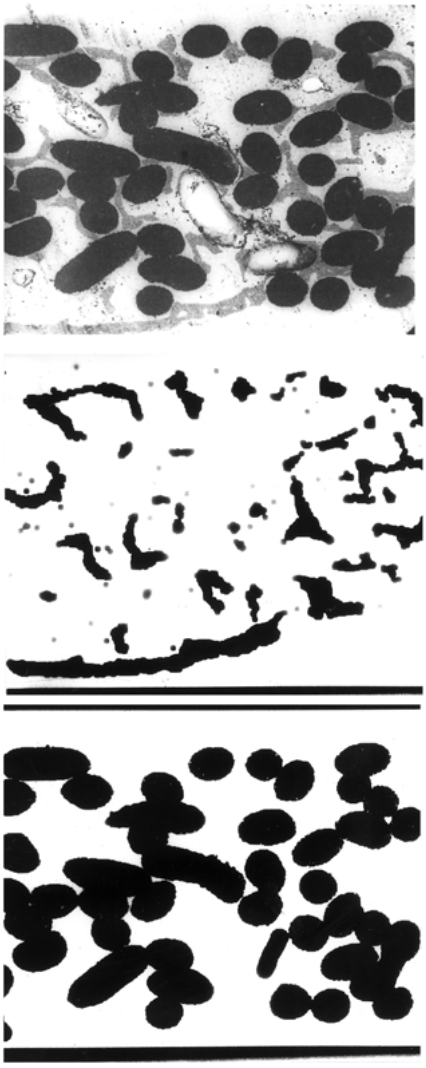


Figure 1 The procedure to calculate the area fraction of Ca of newly generated bone relative to that of the pores of TFMI. (a) The SEM image of the stump of the TFMI in the BMP group. (b) The area of Ca of newly generated bone (S_{Ca}). (c) The area of Ti fiber of the TFMI (S_{Ti}). The area fraction of Ca is calculated as follows: $S_{Ca}/(S - S_{Ti}) \times 100$ (%). S: total area of TFMI calculated.

3.2. The SEM images of HA and rhBMP-2

Two types of HA structures were observed on SEM: (1) a highly porous and sheet-like surface structure and (2) a closely woven mesh-like structure (Fig. 3(a)). Each large pore had a continuous polygonal structure. The wall of it was formed by accumulation of fine meshwork structures of HA (Fig. 3(b)). In the HA with BMP added, some particles of BMP with a diameter of about 100 nm and clumps of these particles were trapped by the meshwork of HA (Fig. 3(b)).

3.3. Histological findings of the tissue between the TFMI and the cranium

Although diffuse deposits of hydroxyapatite (HAp) on collagen fibrils were observed by TEM in the Ti and HA groups at three weeks after implantation, the area occupied by soft tissue was large. There was no infiltration of inflammatory cells. In the HA group, prominent neovascularization was observed (Fig. 4(a)). Osteoblast-like cells containing many swollen mitochondria and rough endoplasmic reticulum (RER) which cavities were irregularly expanded were present (Fig. 4(b)). The high magnification view of the extracellular matrix revealed the presence of needle-shaped crystals among the collagen fibrils. In the Ti group, there was no obvious calcifying structure between the TFMIs and the cranium. However, in both the BMP and HA + BMP groups, newly formed trabecular bone with spindle cells scattered, and numerous infiltrating inflammatory cells were observed (Fig. 4(c)). Giant cells were distributed extensively along the newly formed bone. These giant cells were confirmed to be osteoclasts on TEM (Fig. 4(d)).

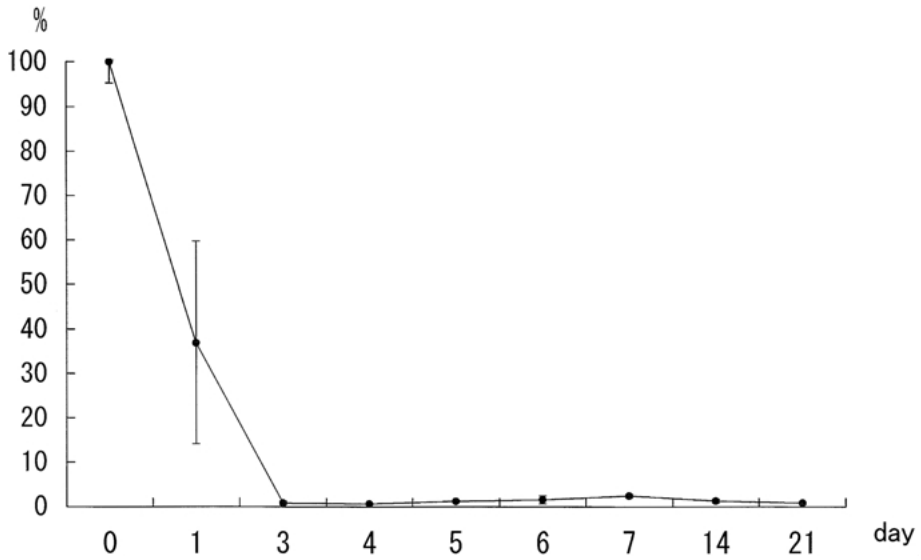
Even after six weeks, the area of soft tissue remained large in the Ti and HA groups. In the Ti group, direct deposits of HAp on collagen fibrils suggesting epitaxy [8] was observed. In the HA group debris of organella released from necrotized cells was observed extensively, on which HAp deposited. This is a finding suggesting the formation of matrix vesicles. In both the BMP and HA + BMP groups, new bone with lamellae-like structure was formed (Fig. 4(e)). Cell infiltration had already disappeared after six weeks.

3.4. Histological analysis of bone ingrowth

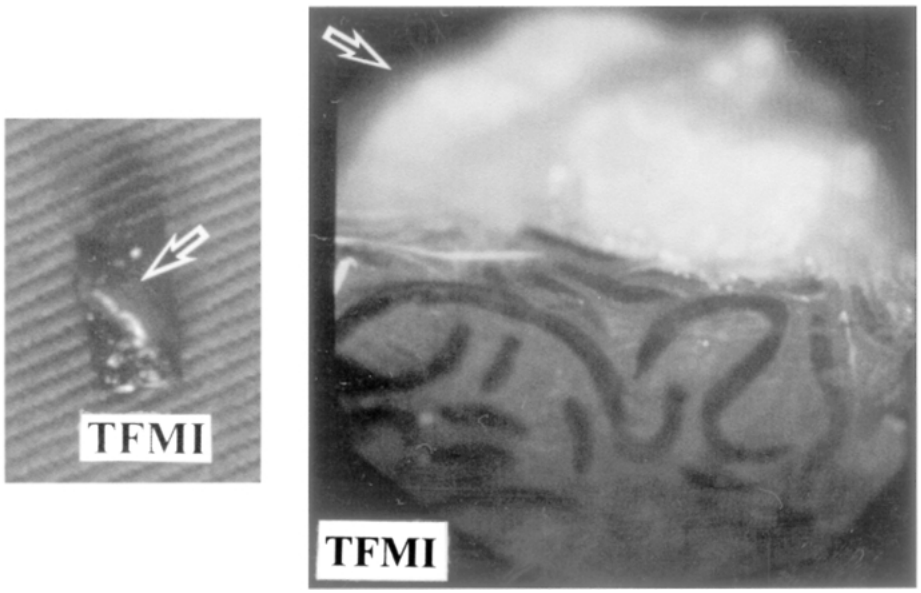
The area fraction of Ca of newly generated bone relative to that of the pores of TFMI in the HA group was larger than that in the Ti group $(p < 0.05)$ (Fig. 5). The area fraction of Ca in both the BMP and HA + BMP groups was larger than that in both the Ti and HA groups $(p < 0.01)$, and that in the HA + BMP group was larger than that in the BMP group $(p < 0.05)$ (Fig. 5).

4. Discussion

If rhBMPs are expected to induce much bone formation, appropriate carrier is indispensable to prevent immediate dispersing. For this reason, many biocompatible carriers have been reported: B-tricalcium phosphate (B-TCP) [9], insoluble bone matrix (IBM) [6, 7, 10], collagen [11], polylactic acid-polyglycolic acid copolymer [12], titanium (Ti) [13], ceramics [14–20] and biphasic calcium phosphate (BCP) [21]. Kawai *et al.* [13] made a BMP-Ti composite. To bind Ti and BMP, BMP was suspended in gelatin solution, which infiltrated into the surface of Ti and the product was lyophilized. In the quantitative analysis of new bone formation, they reported that there was no statistically significant difference between BMP-Ti composites and BMP only, but that the average extent of new bone formation in the case of the composite was slightly less than that in the case of BMP only. This result indicates that some other carrier of BMP is needed to facilitate new bone formation when Ti implants are used. Collagen may be available for TFMI, but it has antigenicity and it is a weak carrier for use in promotion of bone formation [11]. While, hyaluronic acid (HA) has been reported to increase osteoblastic bone formation through increased mesenchymal cell differentiation and migration both *in vitro* and *in vivo* [22, 23]. In a recent study reported by Toole and Trelstad [24], it is suggested that HA binds strongly to extracellular matrix materials such as collagen and proteoglycan, and the higher its molecular weight the stronger its interaction with the cell

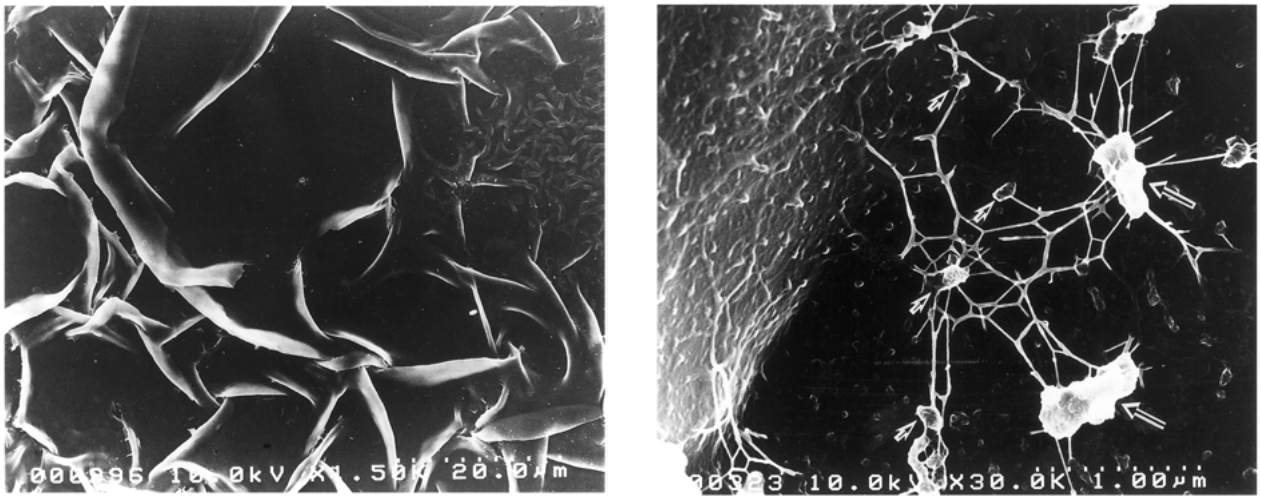


(a)



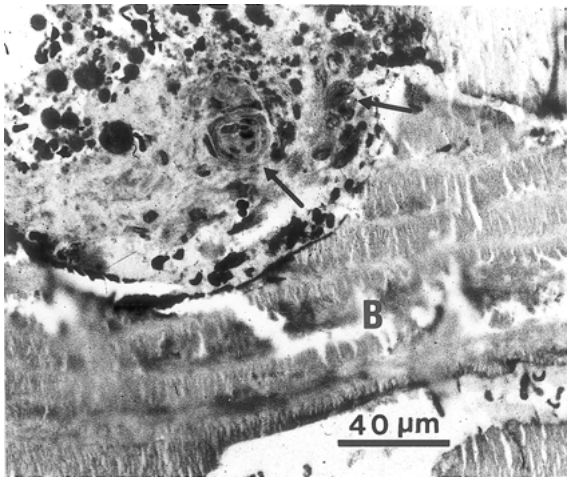
(b)

Figure 2 (a) Transient of the quantity of HA in the TFMI. (b) A fluorescence microscope image of the TFMI harvested at 14 days after implantation. Strong fluorescence signal from the soft tissue covering a TFMI is observed.

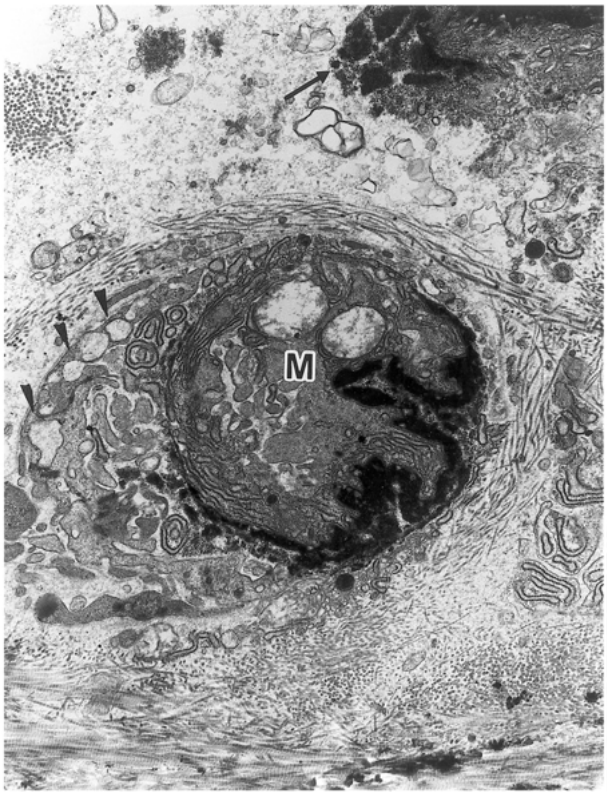


(a) (b)

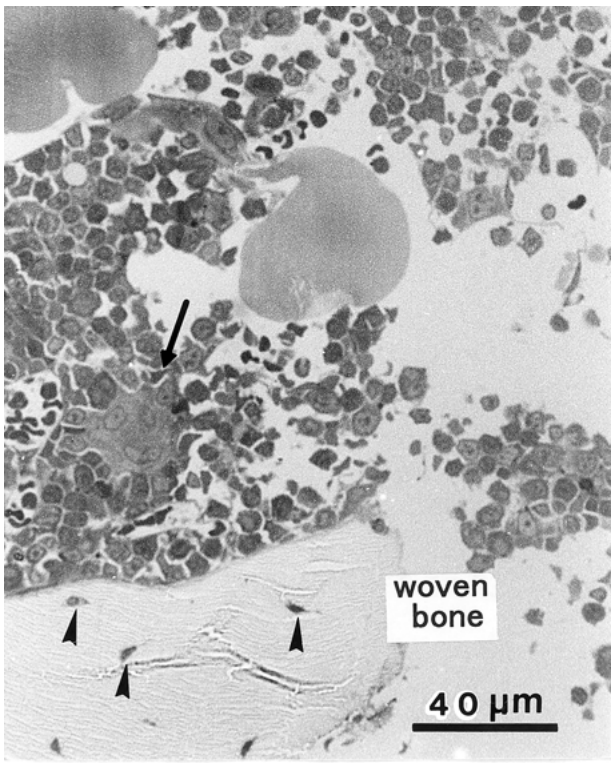
Figure 3 The SEM images of HA and BMP particles in HA. (a) A highly porous and sheet-like surface structure (central part), and a closely woven mesh-like structure (upper right) observed in HA. Original magnification is × 1500. (b) The meshwork structures composing a wall of pore (left side). Some particles of BMP (small arrow) and clumps of these particles (large arrow) are trapped by the meshwork of HA. Original magnification is × 30 000.



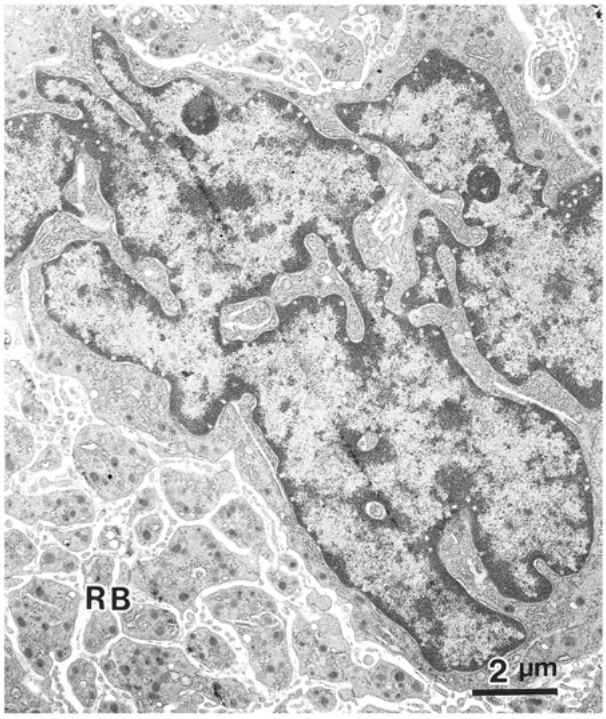
(a)



(b)



(c)



(d)

(e)

Figure 4 Histological findings of the tissue between the TFMI and the cranium. (a–d) 3 weeks after implantation, (e) 6 weeks after implantation. (a) Neovascularization (arrow) in the soft tissue between the TFMI and cranium (B) in the HA group. Original magnification is × 500, toluidine blue. (b) Osteoblast-like cells containing many swollen mitochondria (M) and RER (arrowhead) observed in the HA group. Diffuse deposits of HAp on the collagen fibrils (arrow) are also observed. (c) Woven bone with spindle cells scattered (arrowhead), and numerous infiltrating inflammatory cells in the BMP group. Giant cells (arrow) distribute along the newly formed bone. Toluidine blue. (d) The high magnification view of the same region as the Fig. 4(c) reveals the giant cell to be osteoclast with ruffled border (RB). (e) Lamellae-like structure with living cells (arrowhead) is formed in the BMP group. Toluidine blue.

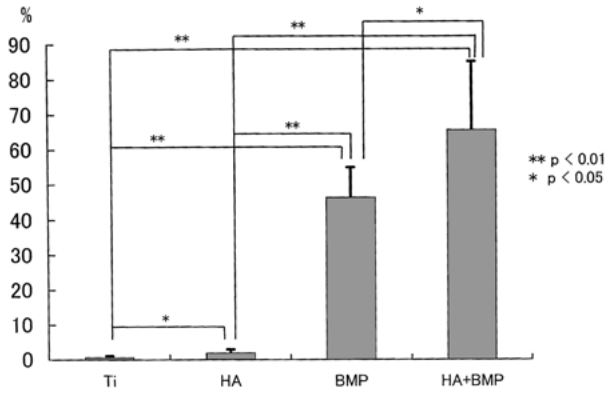


Figure 5 The area fraction of Ca of newly generated bone.

surface becomes. It is also suggested that HA may serve as a suitable substratum for the migration of influenced mesenchymal cells, and that the enormously extended molecular configuration of HA and its highly negative charge are presumed to be important factors. Based on these results, HA is expected to be a good carrier of BMP. In fact, the SEM image of the HA containing BMP showed that the particles of BMP were trapped by the meshwork of HA, though the binding force between the HA and BMP remains to be elucidated.

Although the quantity of HA in the TFMI rapidly decreased during the initial 3-day period after implantation, the intensity of the fluorescence signal from the soft tissue covering the TFMIs increased as time passed. These results suggest that although HA in TFMIs is released rapidly after implantation, it remains in the surrounding granulation tissue, instead of being absorbed. Because the SEM image showed that the particles of BMP in HA were trapped by the meshwork of HA, and the results of analysis of digitized images of Ca and Ti showed that the area fraction of Ca in the HA + BMP group was larger than that in the BMP group, it is suggested that the BMP particles bound to HA may facilitate new bone formation locally after being released from the TFMI. Furthermore, Nakase and Nukamura [25] reported that BMP-4 mRNA signals were detectable in the rib during the initial 12-hour period after fracture and three days after fracture, but disappeared after day five. This report suggests that because HA with BMP is released rapidly from TFMI during the initial 3-day period after implantation, this delivering system can supply a high dose of BMP at the time when BMP works most effectively.

From the results of analysis of digitized images of Ca and Ti, although the TFMI itself has little ability to promote new bone formation, HA has stronger such effects.

Furthermore, although TFMI itself was a good carrier of BMP and TFMIs soaked in BMP showed good conduction of new bone formation, when it was soaked in HA combined with BMP, facilitation of new bone formation was observed. While, stimulation of angiogenesis by partially degraded HA was reported to be due to the direct action of HA on endothelial cells, possibly via a receptor-mediated mechanism [26]. In this study, too, hypervascularity in the HA group was confirmed by histological study. It is suggested that increase of blood-vessel invasion into the TFMI enhanced by HA may serve to facilitate new bone formation. Further, studies by Sasaki and Watanabe [23] in which the effects of elastoviscous high molecular weight HA on bone wound healing after bone marrow ablation were examined, suggested that HA was capable of accelerating new bone formation through mesenchymal cell differentiation in bone wounds. In this study, osteoblast-like cells perhaps differentiated from mesenchymal cells were also observed In the HA group. Thus, high molecular weight HA is not only an effective carrier of BMP, but also may have a positive effect on the generation of new bone in the TFMI.

Acknowledgments

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