Bone Response of Gelatin Composite Including the Apatite Prepared from an Amino Acid Calcium Complex

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SYNOPSIS
The composite of apatite from calcium-asparatic acid (Asp) complex and gelatin was prepared and evaluated the bone response after the implantation into the cortical bone of rabbit tibiae. Four kinds of porous materials were employed in the present study. Namely, Gelatin: 3 wt % gelatin gel, Gelatin-Arg: 3 wt % gelatin-arginine (Arg) gel, CAP-Gelatin: L-Asparatic acid-Ca(OH)₂-H₃PO₄ and 3 wt % gelatin gel, CAP-Gelatin-Arg: L-Asparatic acid-Ca(OH)₂-H₃PO₄ and 3 wt % gelatin gel-Arg. These samples were freeze-dried and were sterilized with ethylene-oxide gas. Afterwards, four porous materials were implanted into the cortical bone defects of rabbit tibiae. After the 12 weeks of implantation, bone responses were evaluated by the observation of non-decalcified thin sections.

The histological appearances of Gelatin implantation promoted the limited bone formation and the space of bone defects was still present. Degradation residue of gelatin was observed close to the new bone. For Gelatin-Arg implantation, greater degree of bone formation was observed compared with Gelatin implantation. CAP-Gelatin and CAP-Gelatin-Arg implantation showed the partial new bone formation in the bone defects. Greater amount of degradation residue was observed in the space of bone defects rather than Gelatin and Gelatin-Arg implantation. Apatite particle and degraded gelatin could be observed inside the space of bone defects. It is suggested that degraded gelatin influenced the bone formation.

Key words: apatite, non-collagen protein, Aspartic acid, gelatin, cortical bone response
INTRODUCTION
Calcium phosphate such as hydroxyapatite or tricalcium phosphate (TCP) was widely used for bone substitutes or bone regeneration material due to its biocompatible and osteoconductive properties. Sometime these calcium phosphates were composited with biodegradable polymers such as poly(lactide-co-glycolide) (PLGA) or collagen to overcome the shortcomings of the brittleness of calcium phosphate.

Previously, we reported the preparation of porous composites of PLGA and apatite with different crystallinities and evaluation of in vivo and in vitro biocompatibility. Two types of apatite with a different crystallinity were prepared from calcium-ethylene diamine tetraacetic acid (Ca-EDTA) complex. PLGA/apatite porous composite with a lower crystallinity showed faster degradation in phosphate buffered saline solution and more rapid and greater amount of calcium phosphate precipitation in simulated body fluid. Moreover, porous PLGA/apatite composite with lower crystallinity produced a significantly greater amount of new bone after implantation into cortical bone defects of rabbits.

Mochizuki et al. reported the apatite preparation by varying the ligands of calcium complexes. Apatite with different crystallinity was prepared from calcium complexes of iminodiacetic acid (IDA) or aspartic acid (Asp). The stability constants of these complexes are smaller than that of EDTA. It was expected that the PLGA/apatite composite with lower crystallinity from Asp complex also showed the better bone response. However, no distinct differences were observed in the bone response between lower and higher crystallinity of apatite in PLGA/apatite composite. The degradation of PLGA in PLGA/apatite composite influenced new bone formation in addition to the release and dissolution of calcium from apatite.

Asp is one of the amino acids constituting non-collagenous protein. Non-collagen proteins played a significant role in the calcification of hard tissues such as bone or tooth. Recently, apatite preparation from calcium-Asp complex with arginine (Arg) has been developed. Arg is a basic amino acid and also a component of non-collagen proteins.

In the present study, the composite of apatite from calcium-Asp complex and gelatin was prepared and evaluated the bone response after the implantation into the cortical bone of rabbit tibiae.

MATERIALS AND METHODS
Preparation of implanted materials
Four kinds of materials were employed in the present study.
Gelatin: Gelatin (MediGelatin, Nippi. Inc, Tokyo, Japan) was dissolved in double-distilled water at a concentration of 3 wt %. After cooling in the refrigerator, the sample was frozen in liquid nitrogen and then dried in a freeze-dryer (EYELA FDU830, TOKYO RIKAKIKAI CO., LTD., Tokyo, Japan).
Gelatin-Arg: Arg solution was reacted at the solid phase of 3 wt % gelatin gel and then also freeze-dried.
CAP-Gelatin: L-Asp and Ca(OH)₂ were dissolved into double-distilled water and then 85 wt% H₃PO₄ solution was added. Afterwards, 3 wt % gelatin was added to the mixture solution. After cooling, the sample was also freeze-dried.
CAP-Gelatin-Arg: L-Asp and Ca(OH)₂ were dissolved into double-distilled water and then 85 wt% H₃PO₄ solution was added. Afterwards, 3 wt % gelatin was added to the mixture solution. After cooling, Arg solution was reacted at the solid phase of CAP-Gelatin gel and white crystals were deposited in the gel. The sample was also freeze-dried.
Implantation procedure
The animal study was conducted in accordance with the animal experimental ethical guidelines of Tsurumi University School of Dental Medicine (25A040). Six 3-month-old female Japanese White Rabbits weighing about 3.5-4kg were used. Four kinds of porous materials were inserted into the cortical bone of rabbits according to a previously reported technique\textsuperscript{9,10}. Each animal received two types of porous materials. Before surgery, all porous materials are sterilized with ethylene-oxide gas.

Surgery was performed under general inhalation anesthesia with a 4% isoflurane and oxygen mixture, which was reduced to 2% isoflurane during surgical manipulation. Local anesthesia was administered by xylocain injection. To reduce the perioperative infection risk, a prophylactic antibiotic, Shiomalin\textsuperscript{5} (equivalent to Latamoxef Sodium, Shionogi & Co., Ltd, Japan), was administered postoperatively by subcutaneous injection.

Porous materials (4.5 mm x 3.5 mm x 2 mm) were inserted into the left and right tibial diaphyses of the rabbits. The hind legs of the rabbits were shaved, washed, and disinfected with iodine tincture. Cortical bone defects measuring 2 x 4 mm were created through the medial cortex and the medulla. These defects were prepared with a very gentle surgical technique using a low rotational drilling speed (500 rpm) and continuous internal cooling. After press-fit insertion, the soft tissues were closed in separate layers using restorable Vicryl 3-0 sutures.

Postoperatively, the animals were placed in a standard cage. They were fed with water and rabbit diet \textit{ad libitum} and were allowed to move unrestricted at all times. The rabbits were sacrificed by peritoneal injection of an overdose of thiamyril sodium (Isozol\textsuperscript{6}) after 12 weeks of implantation.

Histological observation
Each porous material surrounding bone was excised immediately after sacrifice. Excess tissue of the excised specimen was then removed and fixed in 10% buffered formalin solution. Afterwards, tissue block containing porous material was dehydrated through a graded series of ethanol and embedded in methylmethacrylate. After polymerization at 37°C, non-decalcified thin sections were prepared using a cutting-grinding technique (EXAKT-Cutting Grinding System, BS-300CP band system & 400 CS microgrinding system, EXAKT)\textsuperscript{15}. Sections with a final thickness of approximately 50-100 μm were made in a direction parallel to the long axis of the implanted composites and were stained with toluidine blue. The bone response towards the composite materials was evaluated using a light microscope (BX51, Olympus Corp., magnification x 100).

RESULTS
During the test period, the experimental animals remained in good health. At sacrifice, no clinical signs of inflammation or adverse tissue reactions were seen.

The histological appearances of porous materials after 12 weeks of implantation are shown in Figures 1-4. No inflammatory reactions were seen in any samples, and no clear appearances of macrophages or foreign-body giant cells were recognized in any specimen.

As shown in Fig.1, Gelatin implantation promoted the limited bone formation and the space of bone defects was still present. Degradation residue of gelatin was observed close to the new bone. For Gelatin-Arg implantation, greater degree of bone formation was observed compared with Gelatin implantation as shown in Fig.2. The presence of degraded residue was observed in the newly formed bone.
Fig. 1  Histological appearance after 12 weeks of implantation of porous Gelatin

Fig. 2  Histological appearance after 12 weeks of implantation of porous Gelatin-Arg

Fig. 3  Histological appearance after 12 weeks of implantation of porous CAP-Gelatin

Fig. 4  Histological appearance after 12 weeks of implantation of porous CAP-Gelatin-Arg
Figs. 3 and 4 show the histological appearances of CAP-Gelatin and CAP-Gelatin-Arg implantation, respectively. Both showed partial new bone formation in the bone defects. Greater amount of degradation residue was observed in the space of bone defects rather than gelatin and gelatin-Arg implantation. Blue stained particles recognized in the degradation residue is presumed to be calcium phosphate material.

DISCUSSION
In the present study, we evaluated the cortical bone response towards 4 types of gelatin porous composites.

Asp and Arg are the components of non-collagenous protein. It is well known that non-collagenous protein such as osteocalcin or osteopontin is involved in mineralization process.

In the present study, apatite was prepared form calcium-Asp complex with Arg. The advantage of this method is easy control of crystallinity of apatite and the incorporation of Asp and Arg into the synthesized apatite. It is expected that incorporated Asp and Arg will enhance the bone formation after the implantation of bone defects of rabbits. However, bone responses towards present porous materials were complicated.

Implantation of porous gelatin without apatite implantation promoted the new bone formation, although new bone formation was insufficient. Various kinds of gelatin could be available. For example, acidic or basic gelatin could be obtained by the different treatment method. Molecular weight and distribution of molecular weight will be also variable. The origin of gelatin was different. Gelatin was made from bovine skin, bovine bone or pig skin. Kanda et al. reported that pore size of porous gelatin influenced the cell proliferation. The detailed study for bone formation by gelatin should be further investigated.

CAP-Gelatin and CAP-Gelatin-Arg did not enhance bone formation. Apatite particle and degraded gelatin could be observed inside the space of bone defects. Our previous study suggested that degradation of PLGA in PLGA/apatite composite influenced new bone formation and that the remnant of PLGA might have inhibit new bone formation after the implantation of PLGA/apatite composite into the tibia bone defects of rabbits. In the present study, degraded gelatin also influenced the bone formation as mentioned above. Although the degradation behavior of present CAP-Gelatin and CAP-Gelatin-Arg composite is not clear, control of gelatin degradation and apatite dissolution is presumed to influence the bone formation. The degradation behavior of gelatin will be influenced by the presence of apatite.

In conclusion, porous gelatin composite could be easily prepared and it is suggested that degraded gelatin influenced the bone formation. More detailed study for gelatin decomposition or apatite dissolution will contribute the development of bone substitute or scaffold materials.

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