Journal of Biomedical Science (2007) 14:255–263
DOI 10.1007/s11373-006-9124-4

Mechanism of bone induction by KUSA/A1 cells using atelocollagen honeycomb scaffold

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Received 26 March 2006; accepted 24 September 2006

Key words: atelocollagen honeycomb scaffold, bone formation, CD34, KUSA/A1 cells, PCNA, tissue engineering

Abstract

In order to induce new bone formation, mesenchymal stem cells were seeded onto atelocollagen honeycomb scaffold. We evaluated the mechanism of bone induction by KUSA/A1 cells combined with honeycomb atelocollagen scaffold. Scaffold alone, KUSA/A1 cells alone and with scaffold were implanted in the subcutaneous pockets of 4-week-old male SCID mice. The transplants were subjected to radiographical, histological and immunohistochemical examinations after 2 and 4 weeks of implantation. Radiographically, both KUSA/A1 cells alone and KUSA/A1-Scaffold showed some radiopaque areas formation but the latter disclosed a larger amount. Scaffold alone did not show any radiopacity. Histologically, Scaffold alone demonstrated only fibrous connective tissues in the periphery of the scaffold. KUSA/A1 cells alone showed few small islands of new bone formation surrounded by a thin layer of cellular proliferation. On the other hand, KUSA/A1-Scaffold revealed abundant new bone formation as well as cellular proliferation. We also determined the immunolocalization of type I collagen, CD34, Osteocalcin and PCNA in this newly formed bone. Our results indicated that less amount of stem cells are capable to induce the more amount of new bone in tissue engineering. This study support that atelocollagen honeycomb scaffold plays an important role in cellular anchorage and in vessel invasion, giving the precise shape and size for the new bone formation.

Introduction

Bone regeneration is a biological process to renew damaged bone tissue. When the bony defect is small, it will usually heal by regeneration. However, if the bony defect is large, tissue grafting is needed to allow bone repair [1]. Recent progress in tissue engineering offer the prospect of sophisticated physiological in vitro models with the aim of developing materials that will help the body to heal itself. Tissue engineering is defined as an interdisciplinary field where the engineering and life sciences principles are applied toward biologic substitute generation [2].

The general strategies in bone induction by tissue engineering are: (1) infusion or implantation of tissue-inducing substances such as BMP [3]; (2) implantation of cells on or within matrices [4]. In bone engineering, rhBMP combined with collagen is an effective material as biological onlay implant, showing osteoinductive properties and being completely replaced by new bone [5]. Mature KUSA/A1 osteoblasts, in combination with PLGA-colla-

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gen sponge, show promise for use in custom-shaped bone regeneration tool for basic research into osteogenesis and for development of therapeutic applications [6].

Until now, the best structure of the scaffold for stem cells and the precise shape and size of the new tissue have not been determined in bone engineering. However, we recently reported that honeycomb-like structure is a good candidate as scaffold for KUSA/A1 cells to induce the precise amount of new bone within a confined area [7].

Thus, to clarify the mechanism of bone induction by KUSA/A1 cell using atelocollagen honeycomb scaffold, we evaluated the mechanism of bone induction by KUSA/A1 cells combined with porous atelocollagen and the advantage of stem cells in bone engineering implanted in SCID mice subcutaneous tissues.

Materials and methods

Cell culture

In this study, KUSA/A1 cells (kindly provided by Dr. Umezawa from Keio University, Tokyo, Japan) were used [8]. These fibrocytic cells are marrow stromal cells obtained from primary bone culture of female C3H/He mouse. The cells were cultured in minimum essential medium alpha medium (α-MEM, GIBCO BRL, Inc., USA) supplemented with 10% fetal bovine serum (SIGMA, USA) and X1 antibiotic-antimycotic (GIBCO, USA). Then, they were seeded in 10 cm cell culture dishes (Falcon, Inc., USA) and incubated at 37 °C in humid air with 5% CO_2. The growth medium was changed every 3 days until the cells were nearly confluent.

Atelocollagen honeycomb scaffold

The atelocollagen honeycomb scaffold (KOKEN, Japan) $3 \times 3 \times 2$ mm^3 in size, composed by multiple collagen membranes (1 μm in thickness) with honeycomb-shape were used. The scaffold presents parallel pores extended from surface to surface [9].

Three-dimensional culture

When the cells became nearly confluent, they were harvested with trypsin–EDTA and placed at a concentration of 2×10^5 cell/ml onto atelocollagen honeycomb scaffold in suspension culture treated dishes. The cells were grown for 10 days at 37 °C in humid air with 5% CO_2. The medium was changed every 3 days.

Animals

In this study, we used twelve 4-week-old male Severe Combined Immunodeficient (SCID) mice. This study was performed in accordance with the Guidelines for Animal Experiments at Okayama University Medical School, Japanese Government Animal Protection and Management Law (No.105) and Japanese Government Notification on Feeding and Safekeeping of Animals (No.6).

Subcutaneous implantation and explantation

SCID mice were subjected to intramuscular anesthesia with Ketamine (Fuji Chemical Industry Co., Ltd. Japan) and Dormitol (Meiji Seika Kaisha LTD. Japan). Honeycomb scaffold alone, KUSA/A1 cells alone and with scaffold were implanted in the subcutaneous pockets of four SCID mice in each group. On the left side of the back, 10 atelocollagen honeycomb scaffold combined with 1×10^6 KUSA/A1 cells were implanted in the subcutaneous pockets and sutured. On the right side of the back, 5×10^6 of KUSA/A1 cells alone and Scaffold alone, as control, were implanted in subcutaneous tissues. The animals were sacrificed with an overdose of ether at 2 and 4 weeks after implantation. All specimens and surrounding tissues were removed and fixed by 4% paraformaldehyde and subjected to soft X-ray (SOFTEX). Then, they were decalcified with 10% EDTA, embedded in paraffin, sectioned at 4 μm in thickness, and stained by hematoxylin–eosin (H&E). Moreover, the implants were subjected to immunohistochemical studies (Figure 1).

Immunohistochemical staining of CD 34, PCNA, and Osteopontin (OP)

The sections were immunostained with monoclonal antibodies against CD 34 (abcam, UK) using Vectastain ABC (Adivin–Biotin–peroxidase Complex, USA) Rat Kit method, Osteopontin (IBL, Japan) and PCNA (Novocastra, UK) using Vectastain ABC Mouse Kit method (USA). The main steps were as follows: (1) incubation by primary antibody at 4 °C overnight. The primary antibody

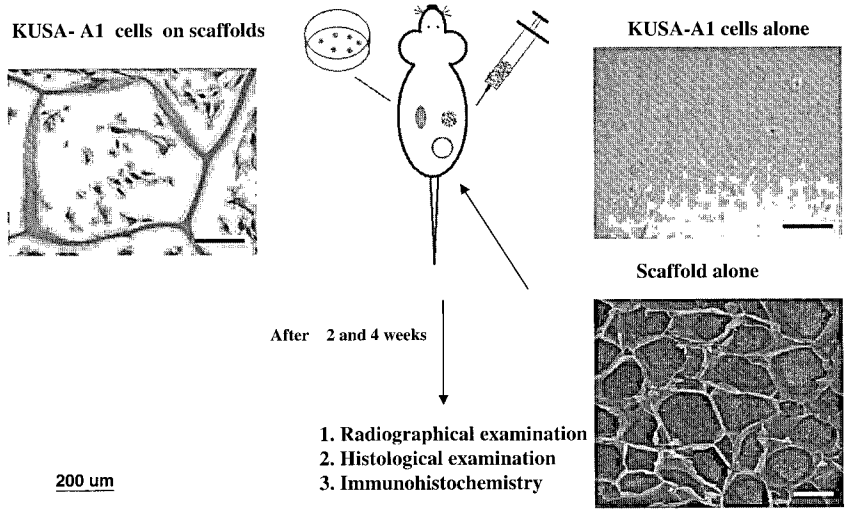

Figure 1. Scheme of Experimental Design. Atelocollagen honeycomb scaffolds combined with KUSA/A1 cells were implanted on the left side of subcutaneous pockets. Scaffold alone and KUSA/A1 alone were implanted on the right side of subcutaneous tissues. The animals were sacrificed at 2 and 4 weeks after implantation and subjected to radiographical examination, histological examination and immunohistochemical examination.

optimal dilutions were performed as follows: (CD-34) 1:100, (OP) 1:50 and (PCNA) 1:100; (2) incubation by anti-rat IgG (1:200) and anti-mouse IgG (1:200) respectively for 30 min; (3) incubation by ABC at a dilution of 1:50 for 30 min; (4) treatment with DAB color development and counterstaining with Mayer's hematoxylin.

Immunohistochemical staining of type I collagen (Col I) and Osteocalcin (OC)

The sections were also immunostained with polyclonal antibodies against Collagen type I (LSL, Japan) and Osteocalcin (LSL, Japan) using PAP method (DAKO, Denmark). The main steps were as follows: (1) incubation with primary antibody Col I (1:500) and osteocalcin (1:100) containing 1% bovine serum albumin at 4 °C overnight; (2) incubation by anti-rabbit IgG (DAKO) (1:40) 30 min; (3) incubation by PAP (1:40) 30 min; (4) treatment with DAB and counterstaining with Mayer's hematoxylin.

Results

Radiologic examination

At 2 weeks, in Scaffold alone, the scaffold did not show any radiopacity (Figure 2A). KUSA/A1 alone implants revealed weak and small radiopacity with indistinct border (Figure 2C). In KUSA/A1-Scaffold, the scaffolds were partially filled with a weak, wide and diffuse radiopacity (Figure 2E).

At 4 weeks, in Scaffold alone, the scaffold did not show any radiopacity (Figure 2B). KUSA/A1 alone at 4 weeks showed irregular and small islands of radiopacities. The radiopacities were homogeneous and dense with well defined border (Figure 2D). In KUSA/A1-Scaffold, the scaffolds were filled with radiopaque areas of different degrees of calcification and diffuse border (Figure 2F). KUSA/A1-Scaffold showed larger but less dense radiopacity than KUSA/A1alone at 2 and 4 weeks.

Histologic examination

1. Scaffold alone at 2 and 4 weeks: They presented fibrous connective tissue with scanty inflammatory infiltration in the periphery of the scaffold. New bone formed neither was not detected (Figure 3A, B).

2. KUSA/A1 alone at 2 weeks: They showed few small islands of new bone formation surrounded by areas of cellular proliferation and areas having a thin layer of KUSA/A1 cell. The newly hard tissue consisted of immature bone with evidence of osteocytes within their lacunae and vessel formation (Figure 3C).

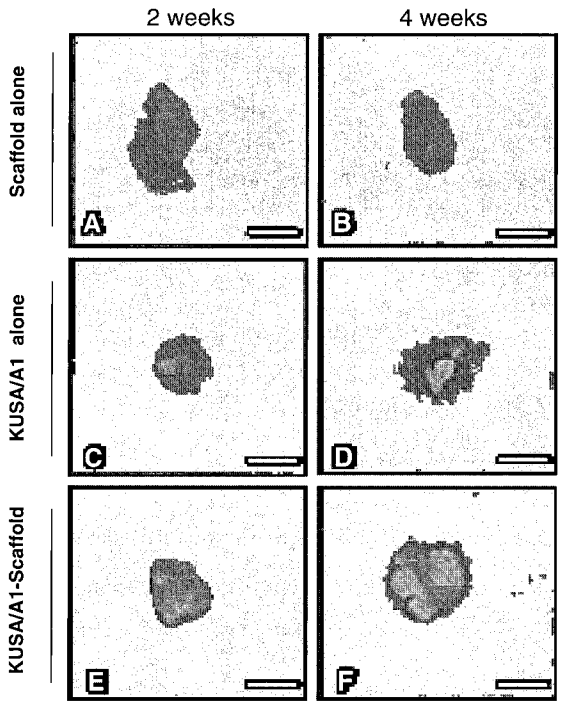

Figure 2. Soft X-ray of Scaffold alone, KUSA/A1 alone and KUSA/A1-Scaffold after implantation. Scaffold alone did not show any radiopaque area at 2 weeks (A) and 4 weeks (B). KUSA/A1-Scaffold at 2 weeks (E) and 4 weeks (F) showed larger radiopacity compared to KUSA/A1 alone at 2 weeks (C) and 4 weeks (D).

3. KUSA/A1-Scaffold at 2 weeks: The specimens showed areas with cellular proliferation and vessel formation, and areas of immature bone not attached the collagen within the porous of the scaffold. Some spaces of collagen membrane of the scaffold revealed many vessels formation with few cells. Moreover, the whole scaffolds were surrounded by proliferating KUSA/A1 cells (Figure 3E).

4. KUSA/A1 alone at 4 weeks: The specimens showed islands of immature bone surrounded by a thin layer of cells. The new bone formed was composed of lamellae irregular rings. Moreover, these bones are more mature compared to the bone formed in KUSA/A1-Scaffold (Figure 3D).

5. KUSA/A1-Scaffold at 4 weeks: The specimens showed that the scaffolds were completely filled with new bone, excessive cellular proliferation and vessel formation. The new hard tissue was formed at the periphery of the scaffold and the center was filled with high cellularity. The new hard tissue was formed of irregular trabeculae surrounded in the periphery by a thin layer of KUSA/A1 cells (Figure 3F).

Immunohistochemical examination

1. KUSA/A1 alone at 2 weeks: Few and small vessels were positive for CD34. Some cells among the woven bone and the layer of cells surrounding the hard tissue were positive for PCNA. Osteoblasts and young osteocytes were positive for Col I and OC. Bone matrix was weakly positive for Col I (data not shown).

2. KUSA/A1-Scaffold at 2 weeks: Many endothelial cells were clearly positive for CD34 in woven bone and in low cellular areas (Figure 4A, Stage I). In areas with high cellular proliferation, many vessels strongly positive for CD34 and excessive amount of nuclei strongly positive for PCNA were shown (Figure 4A, Stage II). However, Col I and OC were negative (Figure 4A, Stage II).

3. KUSA/A1 alone at 4 weeks: Small vessel formation was positive for CD34. Some layer of cells surrounding the hard tissue and only few nuclei among the woven bone were positive for PCNA. Osteoblasts in the woven bone and young osteocytes were positive for type I collagen and OC. Bone matrix was weakly positive for Col I (data not shown).

4. KUSA/A1-Scaffold at 4 weeks: The bone matrix was positive for Col I (Figure 4B, Stage III). In areas of newly bone, differentiated osteoblasts and young osteocytes were positive for Col I and OC (Figure 4B, Stage IV). Many endothelial cells in developing vessels were clearly positive for CD34 in the whole scaffold. In areas with high cellular proliferation, some cells within the woven bone were positive for PCNA, but fewer than that of 2 weeks.

Discussion

The basic principle of tissue engineering is to use seeded cells and biocompatible and biodegradable scaffold to generate a certain type of tissue either *in vitro* or *in vivo*. Most studies of bone tissue engineering focus on searching ideal osteogenic seeded cells and optimal scaffolds bone engineering.

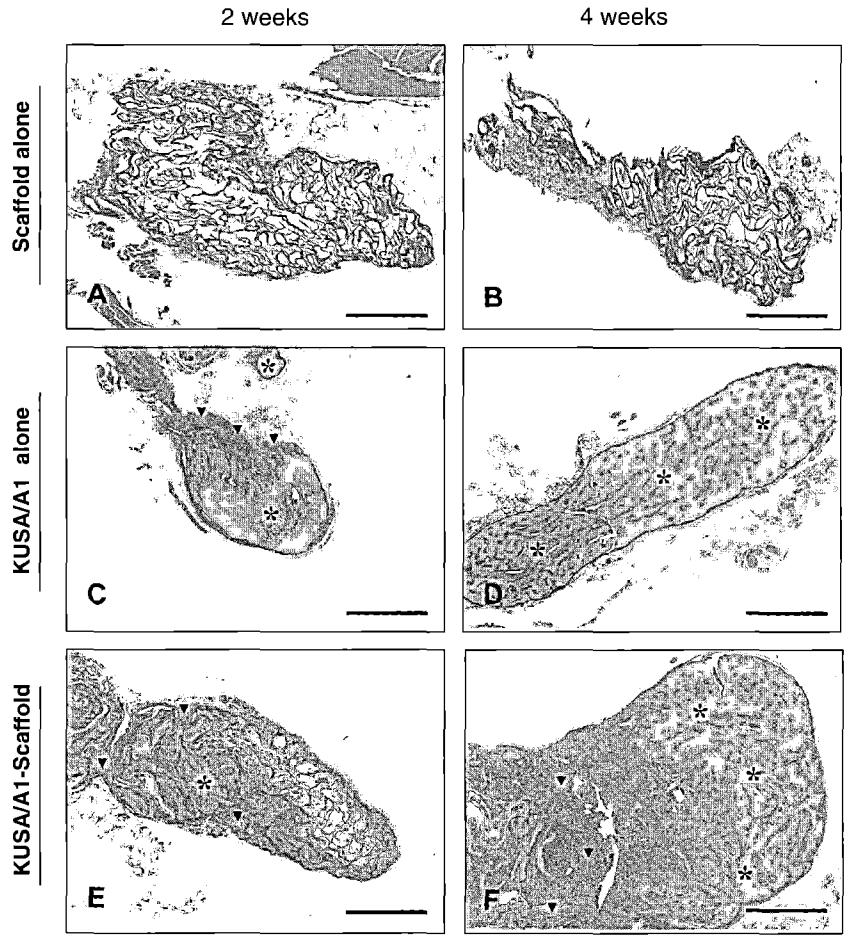

Figure 3. Histological examination of Scaffold alone at 2 and 4 weeks (A,B). They presented fibrous connective tissue with scanty inflammatory infiltration in the periphery of the scaffold. New bone formed neither was not detected. Histological examination of KUSA/A1-Scaffold at 2 weeks. (A) Areas of new bone and cellular proliferation within the scaffold were observed. Histological examination of KUSA/A1 alone at 2 weeks. (B) Islands of new bone and cellular proliferation were clearly seen. Histological examination of KUSA/A1-Scaffold at 4 weeks. (C) All scaffolds were filled of new bone and cellular proliferation. Histological examination of KUSA/A1 alone at 4 weeks. (D) Small islands of new bone surrounded by few cells were seen. Areas of new bone (*), areas of cellular proliferation (arrow). Bar = 0.5 mm.

Intramembranous ossification is characterized by capillary ingrowths into a mesenchymal region of embryonic connective tissue, followed by mesenchymal cell differentiation into osteogenic cells that secrete collagen and mineral to produce woven bone. This woven bone is later remodeled into mature lamellar bone. In this study, the process of bone formation within a pore of the scaffold demonstrated that, first, there was excessive vessel formation strongly positive for CD 34 and few cells (Figure 4A, Stage I). After vessel invasion within the pore of the scaffold, there was high cellular proliferation, strongly positive for PCNA (Figure 4A, Stage II), was followed by cellular differentiation with osteoid matrix formation, which were positive for Col I (Figure 4B, Stage III). The bone matrix starts in the center of the micromass and also from the collagen membrane. Finally, there was bone formation positive for OC (Figure 4B, Stage IV). This result shows that the mechanism of bone induction is similar to intramembranous bone formation.

It is well known that vascularization is an important pre-requisite for osteogenesis [10, 11]. Atelocollagen honeycomb scaffold contains parallel tubes easily invaded by vessels *in vivo*. The number of the vessels, which were strongly positive to CD34, increased in KUSA/A1-Scaffold at 2 and

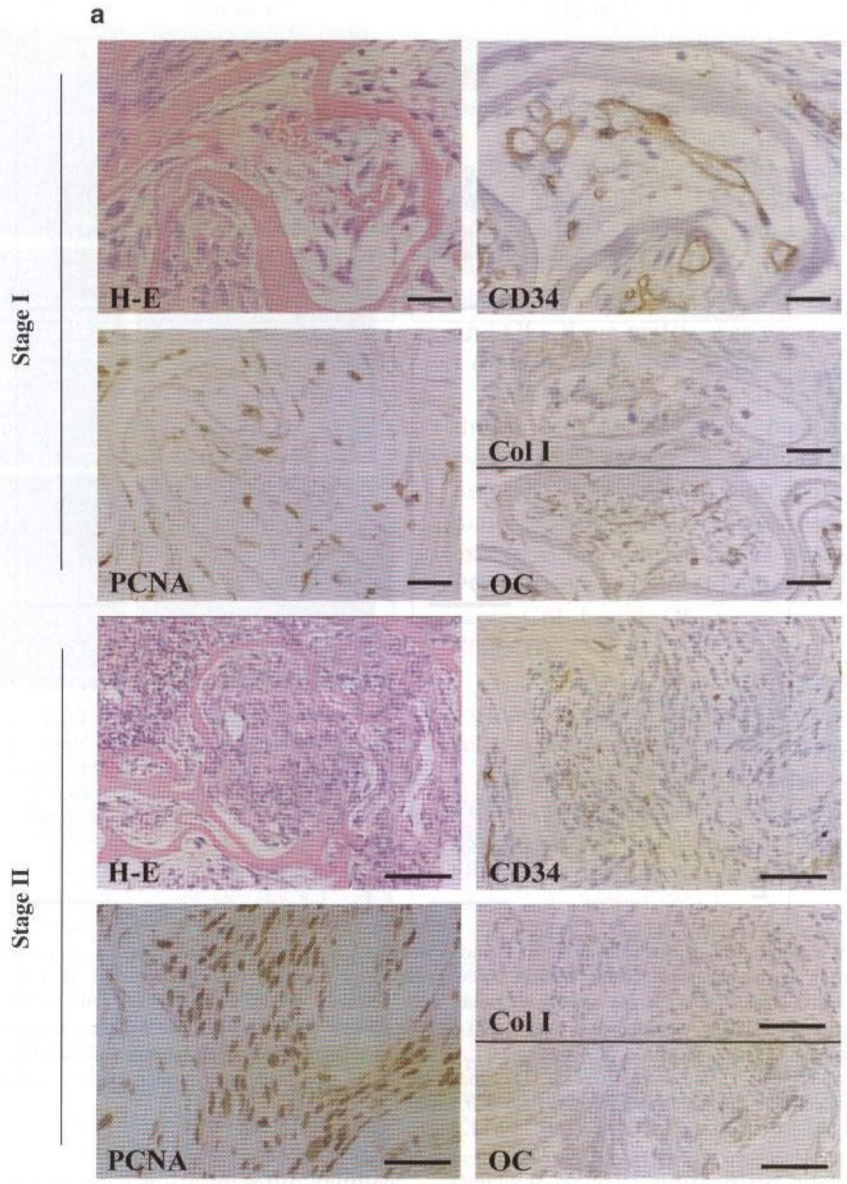

Figure 4. (A) Mechanism of bone induction by KUSA/A1 cells within a pore of the scaffold. *I – Vessel formation stage*: excessive developing vessels within the pore of the scaffold (H-E), which are strongly positive for CD34. Evidence of few cells positive for PCNA protein is observed. Note that Col I and OC expressions are negative. *II – Cellular proliferation stage*: presence of areas with high cellularity of KUSA/A1 cells (H-E) with many small vessel formations strongly positive for CD34. These proliferating KUSA/A1 cells demonstrated strongly positive reaction for PCNA within their nucleus, which were negative for Col I and weak or negative for OC. Bar = 50 μm. (4) Mechanism of bone induction by KUSA/A1 cells within a pore of the scaffold. *III – Matrix formation stage*: evidence of bone matrix production (H-E) and vessels with evident lumen formation positive for CD34. The cells surrounding the bone matrix are positive for PCNA protein. However, the young osteoblast-like cells within the bone matrix were negative for PCNA and positive for osteogenic markers such as Col I and OC. *IV – Bone formation stage*: presence of new bone formation (H-E) with large new vessels positive for CD34. Within the newly formed bone, few proliferating cells, which are negative for PCNA, and excessive osteoblast-like and osteocyte-like cells, which are positive for Col I and OC are observed. Bar = 50 μm.

4 weeks. On the other hand, KUSA/A1 cells alone consisted of few vessel formations. These results suggest that this scaffold is an efficient conductor for vessel invasion and enhances the vessel formation. The angiogenesis might be stimulated by hypoxic microenvironment in a large scaffold. A

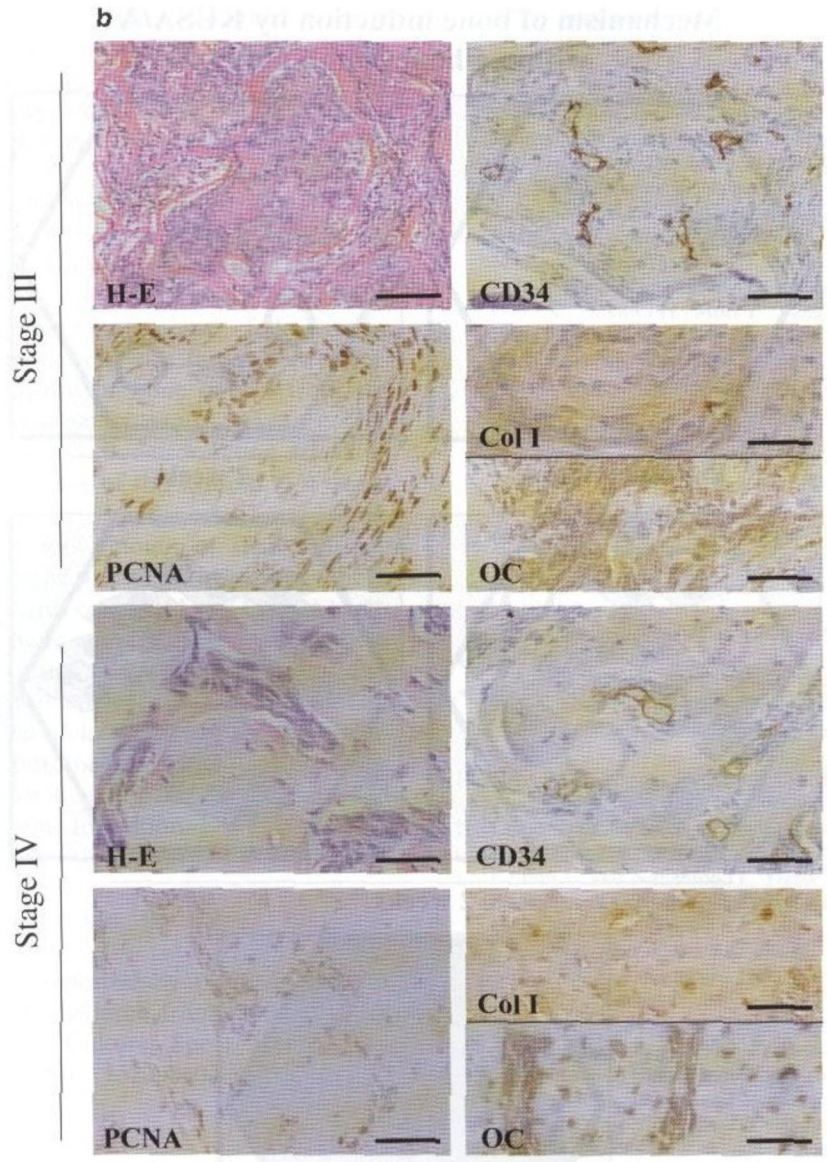

Figure 4. Continued

shortage of oxygen causes an increase in KUSA/A1 cells of angiogenic growth factors, which diffuses through the host tissue, and acts on nearby endothelial cells [12, 13].

As it has been previously reported, proliferating cell nuclear antigen (PCNA) is immunopositive in cellular proliferation of osteo/chondrogenic cells during the process of bone formation [14]. KUSA/A1-Scaffold at 2 weeks showed excessive cellular proliferation being strongly positive for PCNA marker (Figure 4A, Stage II). In contrast, KUSA-A1 cells alone revealed only few nuclei stained with PCNA. These results showed that this scaffold enhanced cellular proliferation being easily invaded with vessels in the whole carrier offering oxygen supply for the cells, which permitted KUSA/A1 cells to proliferate within porous of the scaffold during the first 2 weeks.

Interestingly in this study, although the cells were cultured without osteogenic medium and

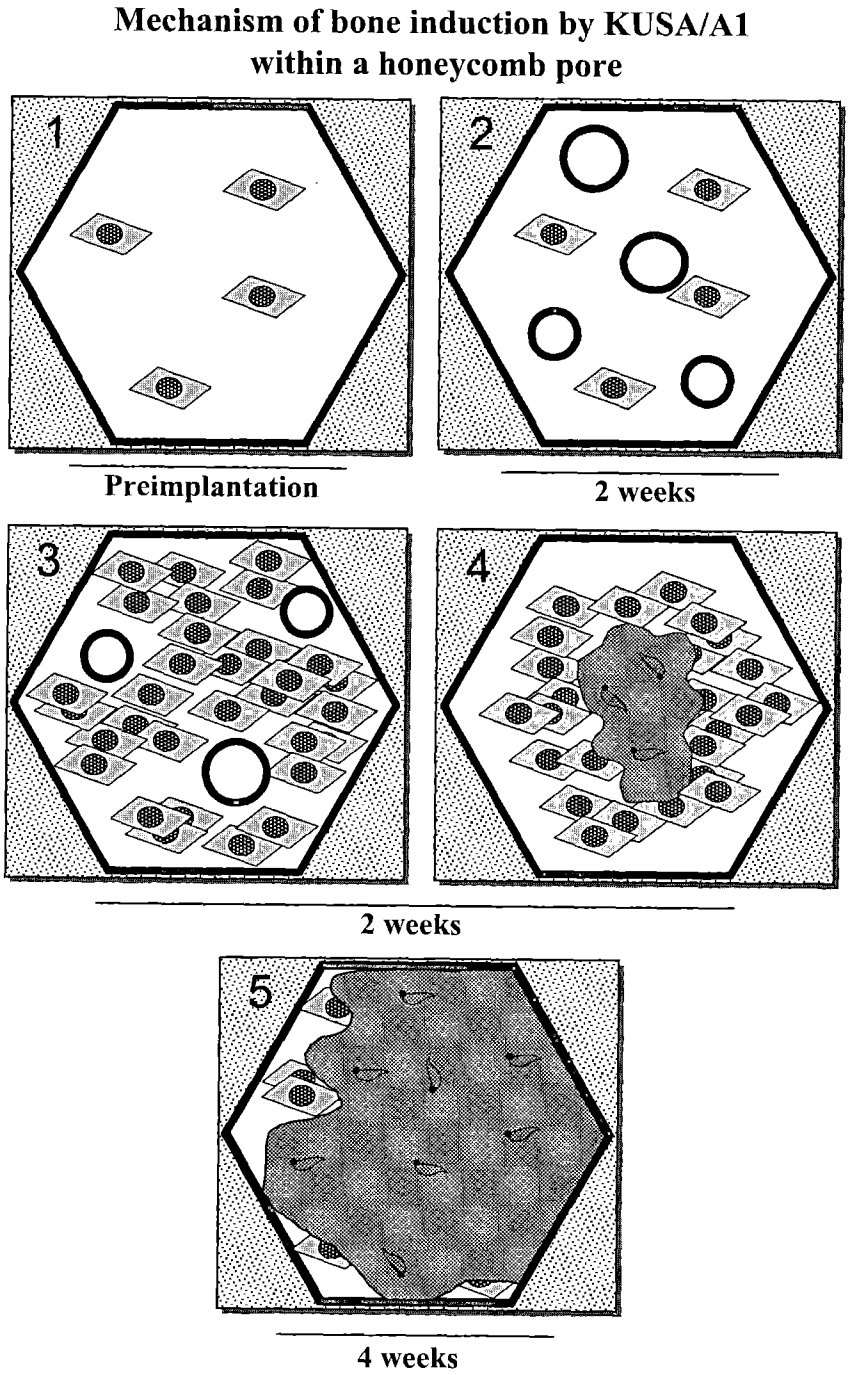

Figure 5. Scheme of process of bone induction by KUSA/A1 within a honeycomb pore. (1) Pre-implantation. (2) Vessel formation. (3) Cellular proliferation and differentiation. (4) Matrix formation. (5) Bone formation.

implanted as pre-confluent KUSA/A1 cells, they differentiated into osteoblast-like cells after implantation *in vivo*. At 4 weeks, the scaffold was filled with new bone formation and many proliferating cells. As it has been reported, the micromass culture indicated that cell–cell contacts were important for the differentiation process [15, 16]. The micromass inoculation corresponds to the state of condensation of mesenchymal cells during membranous bone formation, which amplifies the number of

pre-osteoblasts [17]. We believe that these cells are capable to form micromass-like structure within porous of the scaffold that would permit cell–cell contact inducing osteoblast differentiation expressed by Col I and OC markers (Figure 4B, Stage III).

In summary, the process of bone formation by KUSA/A1 cells within a pore of the scaffold is: (1) presence of few KUSA/A1 cells within the scaffold; (2) stimulation of angiogenesis; (3) cellular proliferation forming micromass followed by osteoblast differentiation with initial matrix formation; (4) induction of bone matrix from the center of the micromass, and also from the collagen membrane; (5) bone formation attaching the collagen membrane of the scaffold at 4 weeks (Figure 5).

In conclusion, less amount of stem cells are capable to induce the precise amount of new bone within a confined area compared to KUSA/A1 cells alone. The new bone within a pore of the scaffold starts from the center of the micromass and also from the collagen membrane of the scaffold. This study support that atelocollagen honeycomb scaffold plays an important role in cellular anchorage and in vessel invasion, giving the precise shape and size for the new bone formation.

Acknowledgements

This study was supported by Grant-in-Aid for JSPS and Scientific research from Japanese Ministry of Education, Culture, Sport, Science and Technology (A) No. 15209060 and (C) No. 17591910.

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