

## Review

# Biological Analysis of a Candidate Stem Cell -KUSA/A1 cell - for Bone Tissue Engineering

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**Abstract:** The basic principle of bone induction for tissue engineering is to use stem cells, growth factors and organic matrix. KUSA/A1 cell is an example of bone marrow stromal stem cell, capable of differentiating into osteoblasts, chondrocytes and myotubes under inducing conditions. It has been reported that mature KUSA/A1 osteoblasts cultured in osteogenic condition, were able to induce a few bone formation in collagen hybridized PLGP sponge *in vivo*. This may be due to their low proliferation potential thereby not being able to obtain sufficient number of cells to promote large tissue repair.

Because of this, in order to use KUSA/A1 cells with high cell proliferation activity to induce large amount of new bone, we evaluated whether KUSA/A1 cells in non-induction condition will maintain their immature stage. The result demonstrated that KUSA/A1 cells cultured in  $\alpha$ -MEM maintained their immature stage *in vitro*. We further examined the osteoblastic differentiation under the influence of the host microenvironment in intraperitoneal diffusion chamber. The results indicated that immature KUSA/A1 cells *in vivo* cell culture differentiated into osteoblasts and produced mineralized bone-like tissue. Finally, we evaluated the effect of honeycomb scaffold to produce abundant bone formation using KUSA/A1 cells implanted in subcutaneous tissues of SCID mice.  $1 \times 10^6$  KUSA/A1 cells with honeycomb scaffold showed abundant new bone formation. While,  $5 \times 10^6$  KUSA/A1 cells alone showed only few small islands of new bone formation.

This study support that KUSA/A1 cell is a good candidate as stem cells for basic research in bone tissue engineering.

**Key words:** Biological analysis, Stem cell, Bone formation, Carrier chamber culture

## Introduction

Bone regeneration is a biological process to renew damaged bone tissue. Autografts, bone obtained from another site of the same subject, are currently the gold standard for bone repair and substitution, but the use of autografts has several serious disadvantages, such as additional expense and trauma to the patient, the possibility of donor-site morbidity, and importantly limited availability<sup>1,2)</sup>. Because of these problems, bone tissue engineering has been heralded as an alternative strategy for bone regeneration<sup>3-5)</sup>. However, some strategies used in this method have demonstrated inflammatory reaction<sup>6)</sup>, few amount of bone formation in old age<sup>7)</sup>, and high risk of tumor formation<sup>8)</sup>.

Recent progress in tissue engineering offers the prospect of sophisticated physiological *in vitro* models with the aim of developing materials that will help the body to heal itself. Temporary three-dimensional scaffolds play an important role in the manipulating the functions of osteoblasts<sup>9)</sup> and guiding new bone formation in their desired shapes<sup>10,11)</sup>. These scaffolds should be biocompatible, osteoconductive, biodegradable, highly porous with a large surface-to-volume ratio, mechanically strong, and malleable into the desired shapes<sup>12)</sup>. Collagen is the primary component of extracellular bone matrix and has been demonstrated to produce good osteoconductivity<sup>13)</sup>. Atelocollagen is produced by telopeptides removal from natural collagen molecules resulting to extremely low antigenicity<sup>14)</sup>. It is most widely used as a wrinkle eraser by injecting it into the skin<sup>15)</sup>. Atelocollagen is also used as a scaffold for keratinocytes and fibroblasts in artificial skin<sup>15)</sup>. It is even used to minimize allergic reactions in cosmetics<sup>16)</sup>. In the

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present study, we selected atelocollagen type I honeycomb scaffold because it has high cell affinity, and maintains its structural integrity, original size and shape during cell growth *in vitro*<sup>17)</sup>. This scaffold could enhance bone regeneration allowing high cellular proliferation and differentiation at the same time maintain the precise size and shape of the newly formed bone *in vivo*.

Recently, implantation of cells on scaffold has taken a great importance in tissue engineering and is expected to provide new applications for the creation of new organs and tissues. KUSA/A1 cells are marrow stromal cell line from primary bone marrow culture of female C3H/He mouse, which possess osteoblastic properties *in vivo*. Immortalized cells were obtained by frequent subculture for more than a year<sup>18)</sup>. Moreover, mature KUSA/A1 osteoblasts cultured in osteogenic medium were able to induce few bone formation in collagen hybridized PLGP sponge *in vivo*<sup>12)</sup>. This may be due to their low proliferation potential thereby not being able to obtain sufficient number of cells to promote large tissue repair. Because of this, in order to use KUSA/A1 cells with high cell proliferation activity to induce large amount of new bone, the biological analysis of KUSA/A1 cells for bone tissue engineering *in vitro* and *in vivo* condition was performed.

### Materials and Methods

#### Experiment I

In order to use KUSA/A1 cells with high cell proliferation activity to induce large amount of new bone, we evaluated whether KUSA/A1 cells in non-induction condition will maintain their immature stage.

#### Cell isolation (prepared by Dr. Umezama A.)

Primary culture of the marrow cells was performed according to Dexter's method<sup>19)</sup>. Female C3H-He mice (n=10) were anesthetized with ether, femurs were excised, and bone marrow cells were obtained for primary bone marrow cultures. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum (FBS) and penicillin (100 mg/ml)/streptomycin (250 ng/ml) at 37°C in humid air with 5% CO<sub>2</sub>. Immortalized cells were obtained by frequent subculture for more than a year. Cell lines from different dishes were subcloned by limiting dilution.

#### Cell culture

KUSA/A1 cells were courtesy of Dr. A. Umezawa from Keio University, Tokyo, Japan. The cells were cultured in minimum essential medium alpha medium ( $\alpha$ -MEM, GIBCO BRL, Inc., USA) supplemented with 10% FBS (SIGMA, USA) and 1% antibiotic-antimycotic agent (GIBCO, USA). Then, they were seeded in 10cm petridishes (Falcon, Inc., USA) and incubated at 37°C in humid air with 5% CO<sub>2</sub>.

In order to induce cell differentiation *in vitro*, the cells were exposed to ascorbic acid (AA, 50 mg/ml) and  $\beta$ -glycerophosphate

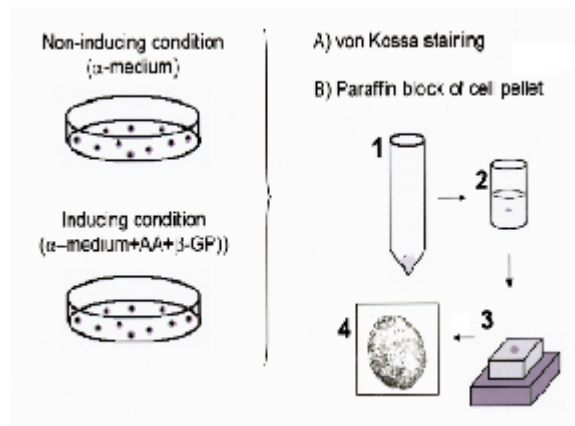


Fig.1. Scheme of *in vitro* experimental design. (A) The cells were stained with Von Kossa staining. (B) 1: The confluent cells were separated from culture dishes and centrifuged. 2: The cell pellets were removed to plastic vial. 3: Then, they were prepared for paraffin block. 4: Finally, they were sectioned at 4mm in thickness and stained.

( $\beta$ -GP 6 mM). The growth medium was changed every 3 days until the cells were nearly confluent. The cells were analyzed at 1, 3, 7, 10 and 14 days by Von Kossa staining and also paraffin embedded cell pellets were prepared for hematoxylin-eosin staining (H-E) and immunohistochemical studies (Fig.1).

#### Experiment II

In order to examine the osteoblastic differentiation under the influence of the host microenvironment, KUSA/A1 cells were seeded in diffusion chamber and implanted in the peritoneal cavity.

#### Diffusion Chamber implantation

Twelve SCID mice were subjected to intramuscular anesthesia with Nembutal (Dianabot, USA). The ventral surface of the skin was shaved, disinfected with 70% alcohol and iodine and cut by blunt dissection to form peritoneal pockets.  $5 \times 10^6$  of KUSA/A1 cells were placed in 6 diffusion chambers, and implanted in the peritoneum. The animals were sacrificed with an overdose of ether at 1, 2, 4 and 6 weeks after implantation. The chambers were removed, fixed with 4% paraformaldehyde (PFA), embedded in paraffin, sectioned at 4  $\mu$ m in thickness, and stained with routine

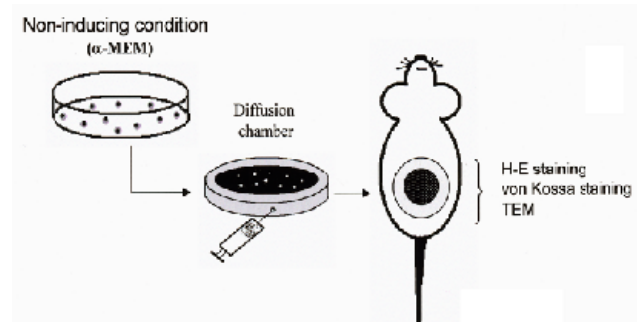


Fig.2. Scheme of *in vivo* cell culture method. The cells were seeded in diffusion chambers and implanted in the peritoneal cavity. The animals were sacrificed at 1, 2, 4 and 6 weeks after implantation. The implants were subjected to H-E staining, Von Kossa staining and TEM.

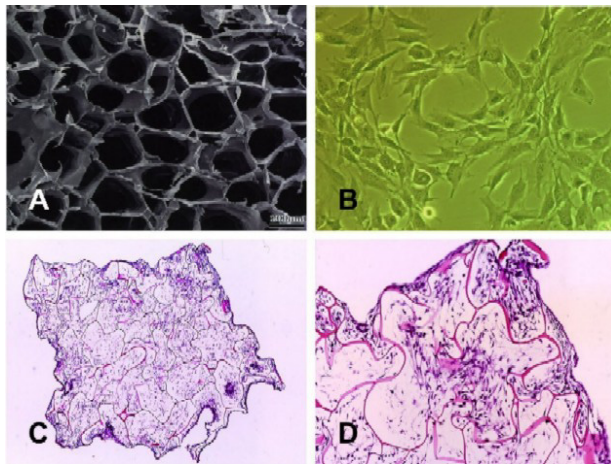


Fig.3. (A) A scanning electron microscopic view of the honeycomb scaffold. (B) *In vitro* characteristics of KUSA/A1 cells at the semiconfluent stage. (C) Low and (D) high magnification of microscopic examination of KUSA/A1 cells on atelocollagen honeycomb scaffold on 10 days in vitro. KUSA/A1 cells connected each other forming a reticular network. H-E.

H-E, Von Kossa staining and immunohistochemical studies (Fig.2).

#### Transmission electron microscopy (TEM)

The chambers were fixed with 2% glutaraldehyde and 2% PFA buffer with a pH of 7.4 for 2 hours at 4 °C, postfixed in 2% OsO<sub>4</sub> buffer with pH of 7.4 for 2 hours at 4°C, then dehydrated in ethanol series and embedded in EPON 812. Ultrathin sections were cut with a diamond knife using Reichert Ultracut Microtome (Germany). The sections were stained with 2% uranylacetate and nitrate and examined using H-800 TEM.

### Experiment III

In order to evaluate the effect of honeycomb scaffold to induce abundant bone formation, KUSA/A1 cell alone and combined with atelocollagen honeycomb scaffold were implanted in subcutaneous tissue of mice.

#### Atelocollagen honeycomb scaffold

The atelocollagen honeycomb scaffold (KOKEN, Japan), 3x3x2 mm in size, composed by multiple collagen membranes (1mm in thickness) with honeycomb-shaped were used. The scaffold is composed of parallel pores extended from surface to surface (Fig. 3A).

#### Three-dimensional culture

When the cells became nearly confluent (Fig. 3B), they were harvested with trypsin-EDTA and placed at a concentration of 2x10<sup>5</sup> 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<sub>2</sub>. The medium was changed every 3 days (Fig. 3C, D).

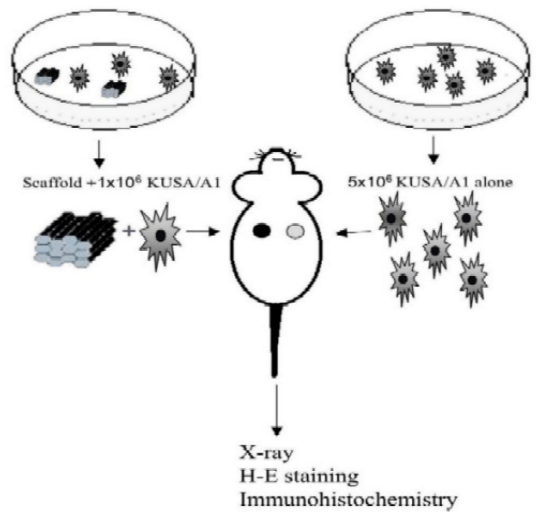


Fig.4. Scheme of *in vivo* experimental design. Atelo collagen honeycomb scaffold combined with KUSA/A1 cells were implanted in the subcutaneous pockets on the left side of KUSA/A1 cells alone, as control, were injected subcutaneously on the right side. The animals were sacrificed at 1, 2 and 4 weeks after implantation and the specimens subjected to Soft X-ray, H-E staining and immunohistochemical examination

#### Subcutaneous implantation and explantation

SCID mice were subjected to intramuscular anesthesia with Ketamine (Fuji Chemical Industry Co., Ltd. Japan) and Dormitor (Meiji Seika LTD. Japan). The skin of the back was shaved, disinfected with 70% alcohol and iodine and cut by blunt dissection to form subcutaneous pockets. On the left side, ten atelocollagen honeycomb scaffolds combined with 1x10<sup>6</sup> KUSA/A1 cells were implanted in the subcutaneous pockets and sutured. On the right side 5x10<sup>6</sup> of KUSA/A1 cells alone, as control, were injected in subcutaneous tissues. The animals were sacrificed with an overdose of ether at 1, 2 and 4 weeks after implantation. All specimens and surrounding tissues were removed, fixed by 4% paraformaldehyde and subjected to soft X-ray (SOFTTEX). Then, they were decalcified with 10% EDTA, embedded in paraffin, sectioned at 4 mm in thickness, and stained with H-E. The implants were also subjected to immunohistochemical studies (Fig. 4).

#### Immunohistochemical staining of CD34, Proliferating Cell Nuclear Antigen (PCNA), and Osteopontin (OSP)

The sections were immunostained with monoclonal antibodies against CD34 (Abcam.UK) using Vectastain ABC (Avidin-Biotin-peroxidase Complex, USA) Rat Kit method, OSP (IBL, Japan) and PCNA (Novocastra, UK) using Vectastain ABC Mouse Kit method (USA). The main steps were as follows: 1) inactivation of endogenous peroxidase with hydrogen peroxide in methanol for 30 minutes; 2) the activation of antigenicity was done with microwave treatment before blocking nonspecific protein binding with rabbit normal serum and horse normal serum respectively for 10 minutes at room temperature; 3) incubation with primary antibody at 4°C overnight. The optimal dilutions of each primary

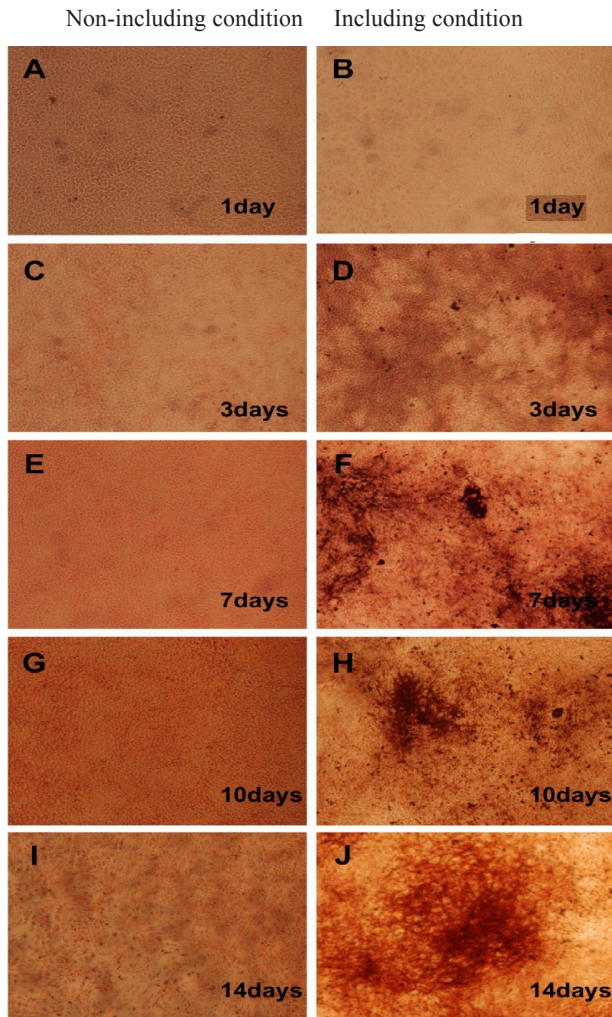


Fig.5. Von Kossa staining of KUSA/A1 cells seeded in non-inducing condition and inducing condition, 4x. (A, C, E, G, I) There was no evidence of calcium accumulation in KUSA cultured in non-inducing condition and (B) in inducing condition on day 1. (D, F, H, J) Calcium deposits was observed in KUSA/A1 cells exposed to inducing condition after 3 days and increased with the course time.

antibodies were as follows: (CD34) 1:100, (OP) 1:50 and (PCNA) 1:100; 4) incubation with anti-rat IgG (1:200) and anti-mouse IgG (1:200) respectively for 30 minutes; 5) incubation with ABC at a dilution of 1:50 for 30 minutes; 6) treatment with DAB color development and counterstaining with Mayer's hematoxylin.

*Immunohistochemical staining of Collagen type I (Coll I) and Osteocalcin (OSC)*

The sections were also immunostained with polyclonal antibodies against Coll I (LSL, Japan) and OSC (LSL, Japan) using PAP method (DAKO, Denmark). The main steps were as follows: 1) inactivation of endogenous peroxidase with hydrogen peroxide in methanol for 30 minutes; 2) treatment with microwave before blocking nonspecific protein binding with swine normal serum (DAKO) containing 1% bovine serum albumin for 10 minutes; 3) incubation with primary antibody at a dilution of 1:500

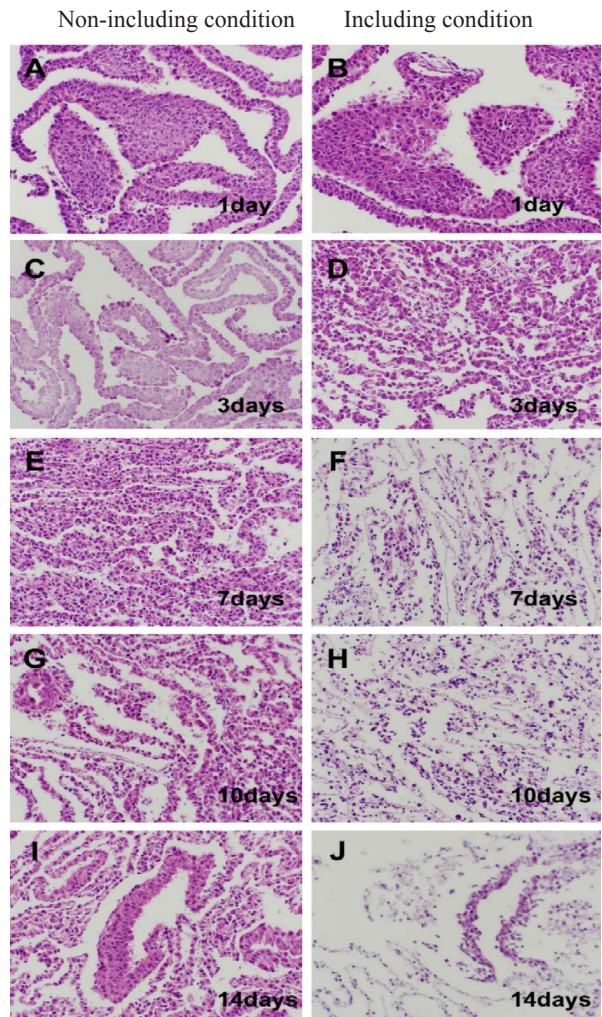


Fig.6. Histological examination of KUSA/A1 cells seeded in non-inducing condition and inducing condition, 40x. (A, C, E) Cords of spindle-shaped of KUSA/A1 cells was observed in non-inducing condition on 1, 3 and 7 days and (B) in inducing condition on day 1. Presence of marked cell degeneration was demonstrated (G, I) in non-inducing condition after 10 days and (F, H, J) in inducing condition after 7 days.

of Collagen type I and 1:100 of osteocalcin containing 1% bovine serum albumin at 4°C overnight; 4) incubation with anti-rabbit IgG (DAKO) at a dilution of 1:40 for 30 minutes; 5) incubation with PAP at a dilution of 1:40 for 30 minutes; 6) treatment with DAB and counterstaining with Mayer's hematoxylin. They were examined by optical microscopy.

*Quantification of bone induction, vessel formation and cellular proliferation*

In order to clearly emphasize the efficacy of this scaffold, the quantity of the whole new bone formed in both groups was measured using NIH image. The specimens stained by CD34 and PCNA were subjected to histometrical studies at 1, 2 and 4 weeks after implantation in order to know the vessel and cellular proliferation amount, respectively. The counting process was performed by using an eyepiece micrometer at three separate areas:

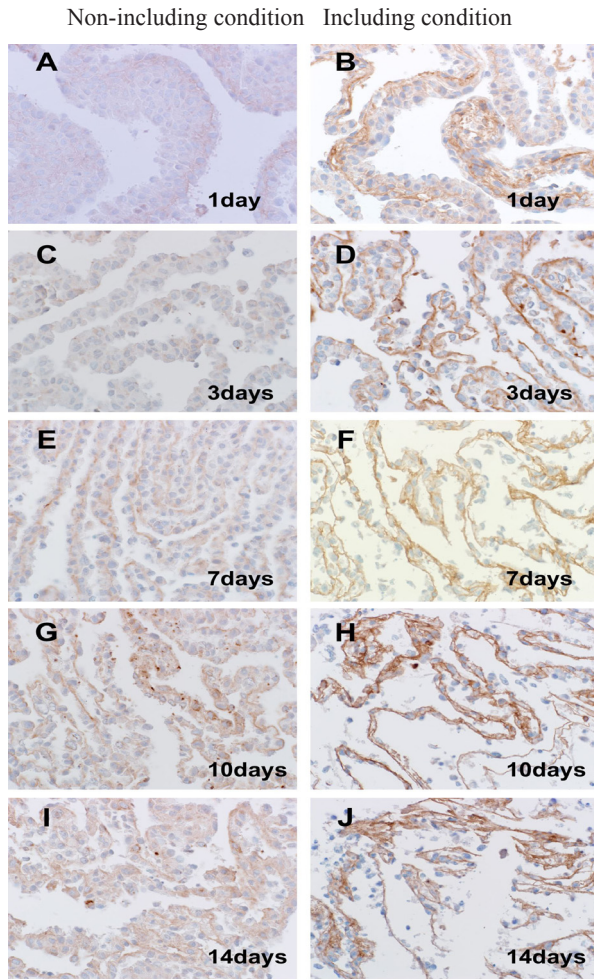


Fig.7. Collagen type I expression of KUSA/A1 cells seeded in non-inducing condition and inducing condition, 40x. (A,C,E,G,I) KUSA/A1 cells cultured in non-inducing condition showed weak expression for Collagen type I. (B,D,F,H,J) In contrast, strong expression was observed in KUSA/A1 cells exposed to inducing condition.

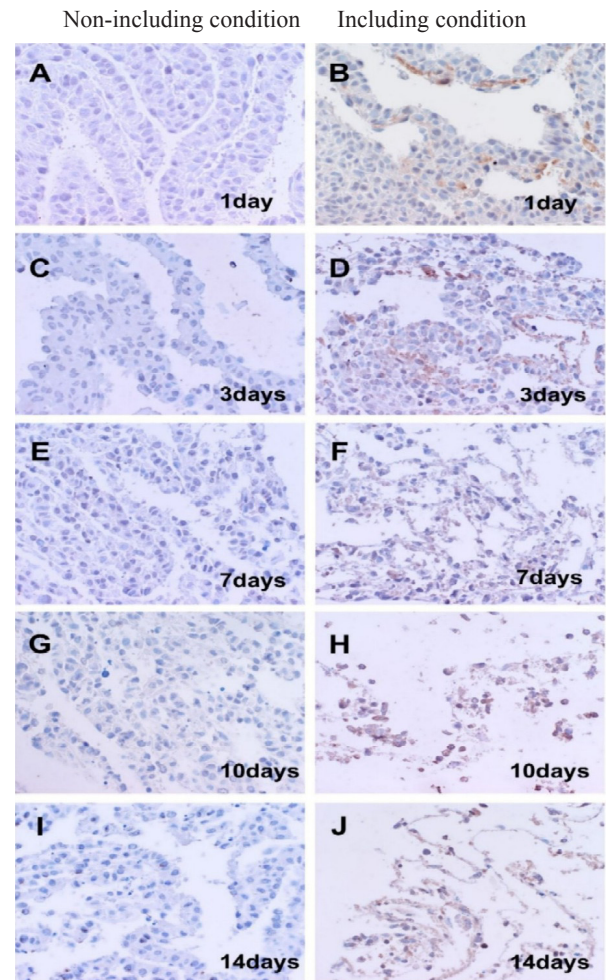


Fig.8. Osteocalcin expression of KUSA/A1 cells seeded in non-inducing condition and inducing condition, 40x. (A,C,E,G,I) KUSA/A1 cells cultured in non-inducing condition were completely negative for osteocalcin. (B,D,F,H,J) In contrast, in KUSA/A1 cells exposed to inducing condition revealed positive reaction for osteocalcin.

center, right and left side of the slide at 40x magnification, and repeated three times to minimize the operator error. For vessel formation, the number of vessels positively stained with CD34 was counted in each of KUSA/A1 alone and KUSA/A1-Atelocollagen at 1, 2 and 4 weeks as described above. For cellular proliferation, the percentage of the cells positively stained with PCNA was calculated in each of KUSA/A1 alone and KUSA/A1-Atelocollagen at 1, 2 and 4 weeks as described above. The differences in vascular formation and cell proliferation were subjected to statistical analysis.

#### Statistical Analysis

The statistical study was performed with SPSS for Windows Statistical program by using Student's t-test.

### Results

#### I. In vitro study

#### Calcium accumulation

KUSA/A1 cells cultured in non-inducing condition ( $\alpha$ -MEM alone) did not show accumulation of calcium deposits during the time course. In contrast, KUSA/A1 cells exposed to the inducing condition ( $\alpha$ -MEM+AA- $\beta$ GP) exhibited small areas with calcium deposition on day 3, whereas marked accumulation of calcium deposits were observed after 7 days and increased with the time course. (Fig.5)

#### Histological examination of cell pellet

KUSA/A1 cells cultured in non-inducing condition ( $\alpha$ -MEM alone) showed cords of spindle-shaped cells on day 1 and 3. Moreover, marked cellular proliferation was observed on day 7 and degenerated cells were seen after 10 days. While, the cells cultured in inducing condition ( $\alpha$ -MEM+AA- $\beta$ GP) demonstrated marked cell degeneration on day 7, 10 and 14. (Fig.6)

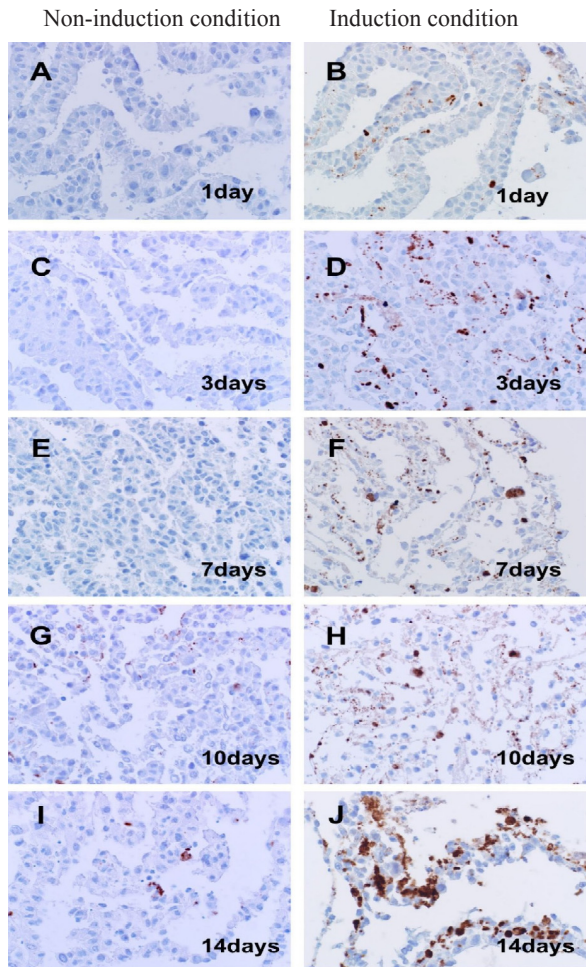


Fig.9. Osteopontin expression of KUSA/A1 cells seeded in non-inducing condition and inducing condition, 40x. (A, C, E) No evidence of osteopontin expression on days 1, 3 and 7, but (G,I) it was detected only after 10 days cultured in non-inducing condition. (B, D, F, H, J) While, strong expression was observed in KUSA/A1 cells exposed to inducing condition

*Immunohistochemical examination of cell pellet*

KUSA/A1 cells placed in non-inducing condition ( $\alpha$ -MEM alone) demonstrated weak expression for Coll I after 1 day of culture (Fig.7 A, C, E, G, I), and were completely negative for OPN and OCN in all groups (Fig.8, 9 A,C, E,G, I). While, the cells cultured in inducing condition ( $\alpha$ -MEM+AA- $\beta$ GP) revealed strong expression for Coll I, OPN and OCN after 3 days and increased with the time course (Fig.7, 8, 9 B, D, F, H, J).

**II. In vivo Cell culture condition**

*Histological analysis*

KUSA/A1 cells at 1 week demonstrated evidence of multilayer of spindle-shaped cells (Fig.10 A). KUSA/A1 cells at 2 weeks revealed early immature bone formation attached to the membrane of the chamber. Osteoid formation, osteocytes, and active osteoblasts were observed. Matrix protein secretion was also observed within the pores of the membrane (Fig.10 C). KUSA/A1 cells at 4 weeks showed more amount of bone with many

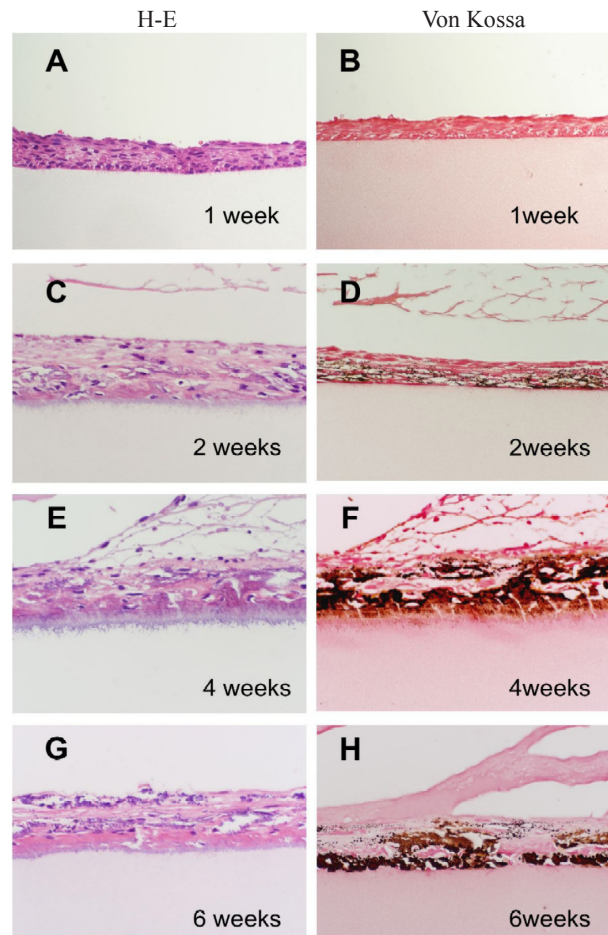


Fig.10. Histological examination of KUSA/A1 cells seeded in diffusion chamber, H-E, 40x. (A) KUSA/A1 cells at 1 week demonstrated multilayer of spindle-shaped cells. (C) At 2 weeks, early immature bone formation occurred within the chamber. (E) At 4 weeks, large amount of new bone with osteocytes within the lacunae and secreted proteins inside the porous of the chamber membrane were observed. (G) The presence of degenerative cells and bone tissues at 6 weeks, Calcification examination of KUSA/A1 cells seeded in diffusion chamber, Von Kossa staining, X40. (B) Calcium accumulation was not detected in KUSA/A1 cells at 1 week. (D, F, H) In KUSA/A1 cells at 2 weeks calcium deposit in the bone matrix were observed, and calcification was also seen in degenerated cells apart from the membrane of the scaffold at 4 and 6 weeks.

osteocytes within the lacuna formation, osteoid and mineralized matrix. Matrix proteins were also detected within the pores of the membrane (Fig.10 E). KUSA/A1 cells at 6 weeks revealed degenerative cells and bone (Fig.10 G).

*Calcification analysis (Von Kossa staining)*

Calcium accumulation was not seen in KUSA/A1 cells at 1 week (Fig.10 B). At 2 weeks, initial calcium deposition appeared were observed like “small spots”, distinctly separate from the non-calcified areas (Fig.10 D). The cells apart from the membrane of the chamber, and the tissue attached to the membrane of the chamber showed calcium accumulation at 4 and 6 weeks (Fig.10 F, H).

*Immunohistochemical analysis*

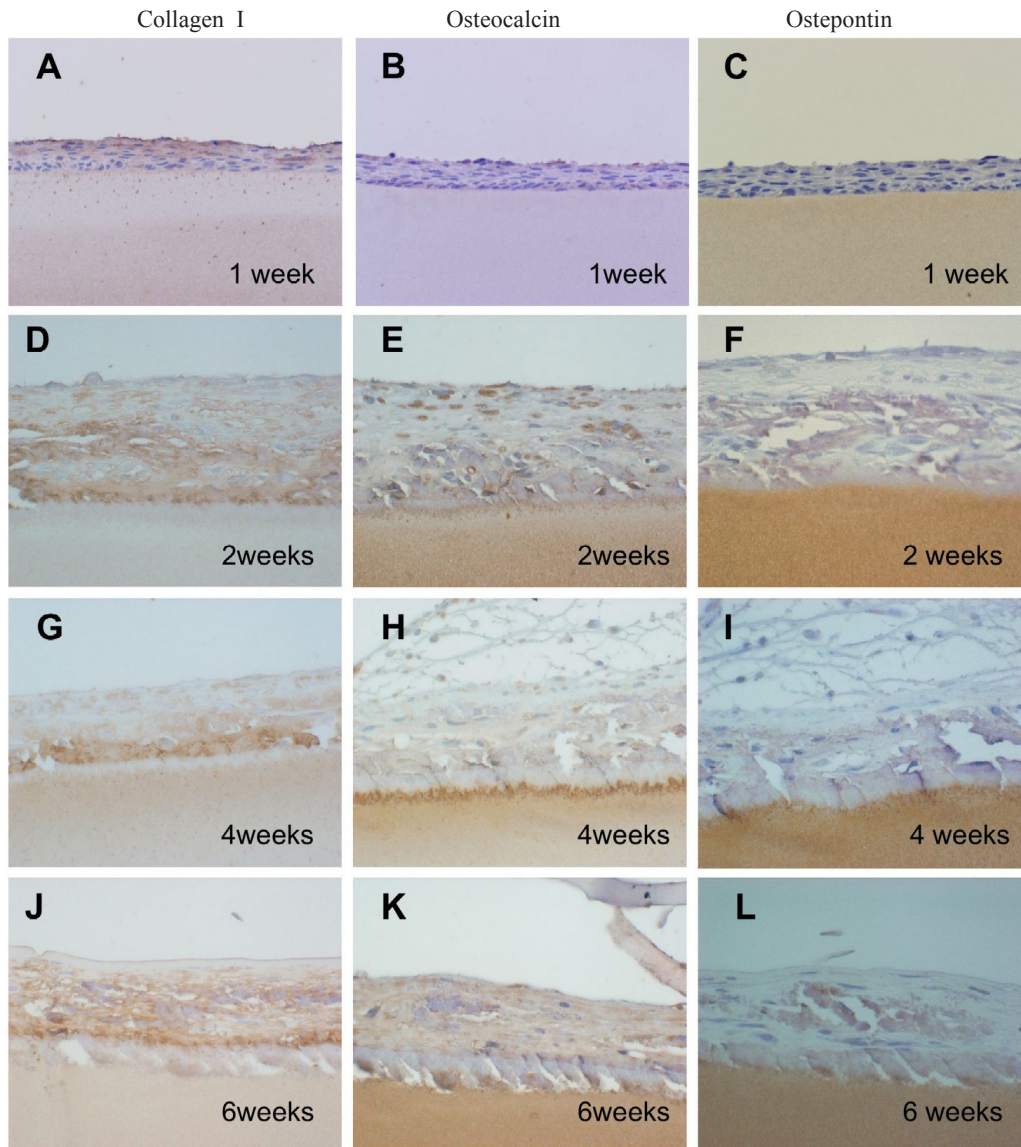


Fig.11. Immunohistochemical examination of KUSA/A1 cells seeded in diffusion chamber,40x. Osteoblastic differentiation was observed at 1 week, which was positive for (A) Collagen I, (B) Osteocalcin and (C) Osteopontin. The new bone exhibited positive reaction for (D, G, J) Collagen I, (E, H, K) Osteocalcin and (F, I, L) Osteopontin at 2, 4 and 6 weeks.

KUSA/A1 cells at 1 week revealed weak expression for Coll I, OPN and OCN (Fig.11A, B, C). After 2, 4 and 6 weeks, these cells induced new bone showing positive reaction for Coll I, OPN and OSN observed in bone matrix, osteoblasts and osteocyte-like cells (Fig.11 D-L).

#### Transmission electron microscopy

TEM was performed, in order to examine the initial calcification foci in the hard tissue induced by KUSA/A1 cells within the diffusion chamber.

Cytoplasmic processes within the membrane of the chamber and multilayer of undifferentiated KUSA/A1 cells were observed at 1 week (Fig.12 A, B). The initial calcification foci were seen in the collagen fiber and also in the matrix vesicles within the

membrane of the diffusion chamber at 2 weeks (Fig.12 C, D). Dystrophic calcification was also detected in degenerated cells apart from the membrane of the chamber at 4weeks (Fig.12 E, F).

### 3. In vivo study

#### Radiological examination

At 1 week, both groups revealed weak radiopacity areas (Fig.13A, B). At 2 weeks, KUSA/A1-Atelocollagen implants showed many weakly spotted areas of diffuse and wide radiopacities (Fig.13 C). In KUSA/A1 alone implants revealed a weak and small radiopaque area (Fig.13 D). At 4 weeks, in KUSA/A1-Atelocollagen, the scaffolds were filled with many ovoid or rounded radiopaque areas showing different degrees of calcification with unclear or diffuse border (Fig.13 E). KUSA/

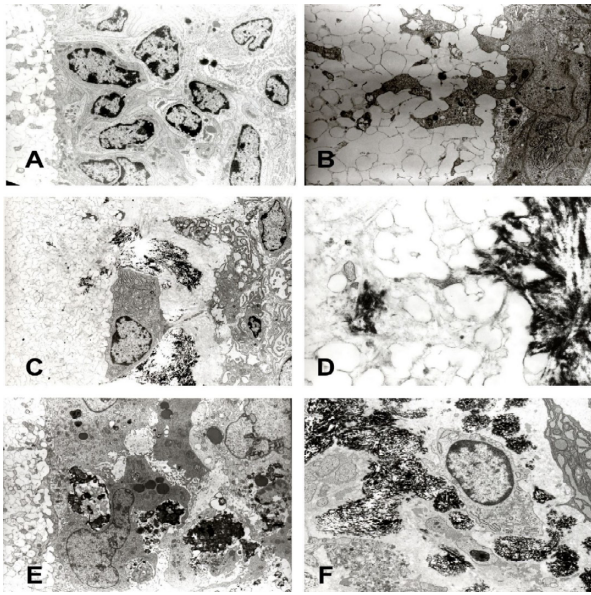


Fig.12. TEM image of KUSA/A1 cells seeded in diffusion chamber. (A) Multilayer of undifferentiated KUSA/A1 cells and (B) cytoplasmic processes within the membrane of the chamber were seen at 1 week. No evidence of calcification was observed. (C) The initial calcification foci were presented in the collagen fiber and also in the matrix vesicles within the membrane of the diffusion chamber at 2 weeks. (D) Enlargement of calcification foci were exhibited. (E, F) Dystrophic calcification was also detected in degenerated cells apart from the membrane of the chamber at 4weeks.

A1 alone showed irregular radiopaque areas. The radiopacities were homogeneous and dense with well-defined border (Fig.13 F).

**Histological Examination**

*KUSA/A1-Atelocollagen at 1 week*

The implants demonstrated scanty immature bone formation at the periphery of the scaffold (Fig.14 A). Areas with several amount of cell debris without vessel formation were noted. However, evidence of other areas with vessel formation and healthy cells were also observed.

*KUSA/A1 alone at 1 week*

The transplants exhibited small islands of immature bone formation surrounded by proliferating KUSA/A1 cells. (Fig.14 B)

*KUSA/A1-Atelocollagen at 2 weeks*

The specimens showed three different areas: 1) cellular proliferation and vessel formation, 2) few cells with many vessels formation, and 3) more woven bone formation (Fig.14 C). The newly formed bone was present on scaffold collagen membranes, but not attached. The immature bone formation was seen especially at the periphery of the scaffold. Muscle and fat tissues were also detected.

*KUSA/A1 alone at 2 weeks*

The samples showed few small islands of new bone formation,

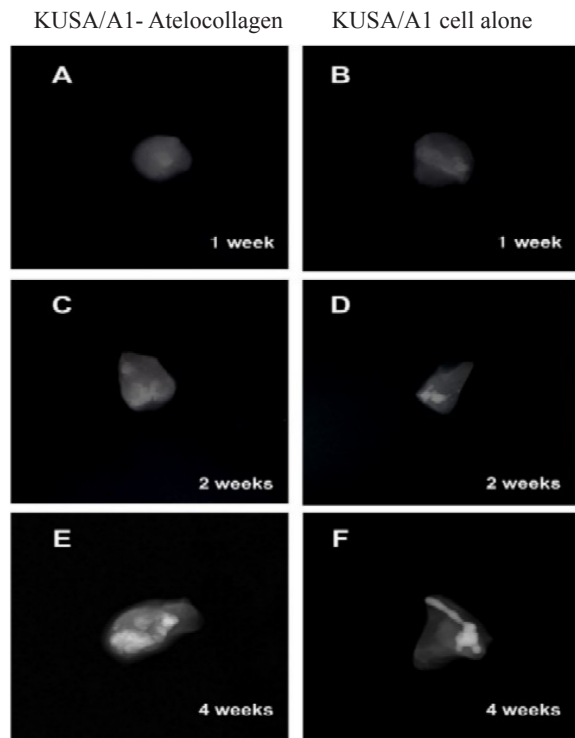


Fig.13. Soft X-ray of KUSA/A1-Atelocollagen and KUSA/A1 alone after implantation. KUSA/A1-Atelocollagen at (A) 1 week, (C) 2 weeks and (E) 4 weeks showed larger radiopacity compared to KUSA/A1 alone at (B) 1 week, (D) 2 weeks and (F) 4 weeks.

some surrounded by KUSA/A1 cell proliferation (Fig.14 D). Inside the new bone, osteocytes as well as many vessels were observed. Fat and connective tissue were also detected.

*KUSA/A1-Atelocollagen at 4 weeks*

The slides showed scaffolds filled with new bone and high cellularity (Fig.14E). The newly formed hard tissue was observed at the scaffold periphery and cellular proliferation was found in the center. The newly formed bone was composed of irregular trabeculae bordered by active osteoblasts, and osteocytes were seen within the bone matrix.

*KUSA/A1 alone at 4 weeks*

The specimens showed islands of new bone surrounded by a thin layer of cells (Fig.14F). Bone was composed by lamellae irregular rings and populated by a variable number of osteocytes within the lacunae as well as vessel formation. Loose connective tissue, fat and muscle were also seen. Note that the newly formed bone is more immature in KUSA/A1-Atelocollagen than in KUSA/A1 alone.

*Immunohistochemical examination of KUSA/A1-Atelocollagen at 1 week*

Presence of areas with cell debris without vessel formation (Fig.15A atage 1), areas with few cells with many developing vessels strongly positive for CD34 and areas with high cellularity intensely positive for PCNA were observed.



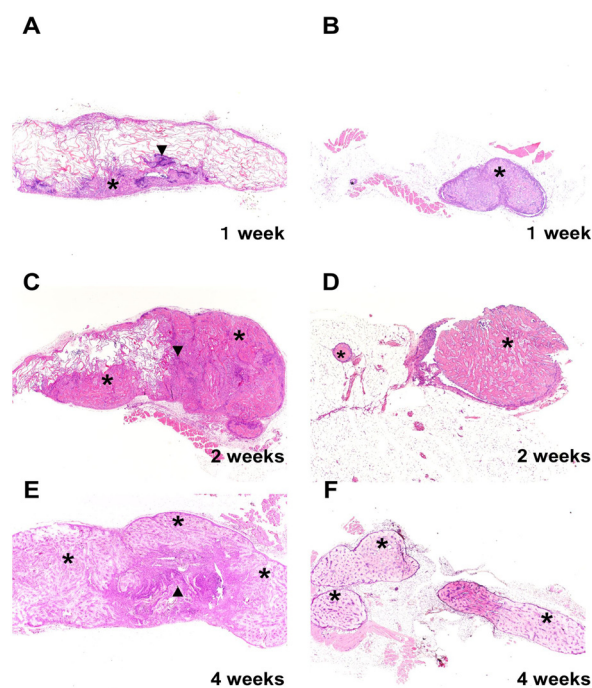


Fig.14. Histological examination of KUSA/A1-Atelocollagen, H-E, 4x. (A) Areas of new bone and cellular proliferation at the periphery of the scaffold were observed at 1 week. (C) Areas of newly bone formation and high cellularity within the scaffold were revealed at 2 weeks. (E) All scaffolds were filled of new bone and cellular proliferation at 4 weeks. Histological examination of KUSA/A1 alone, 4x. (B, D, C) Nests of new bone were clearly seen at 1, 2 and 4 weeks. Areas of new bone (\*), areas of cellular proliferation (arrow head).

#### *KUSA/A1 alone at 1 week*

Small islands of immature bone formation surrounded by proliferating cells positive for PCNA and also evidence of many vessel formations intensely positive for CD34 were seen.

#### *KUSA/A1-Atelocollagen at 2 weeks*

Excessive developing vessels within the pore of the scaffold showing positive immunoreaction for CD34 were observed (Fig.15A stage 2). In areas with high cellular proliferation, many nuclei were strongly positive for PCNA (Fig.15A stage 3). However, Coll I and OSP, were negative. OSC was generally negative though few cells showed positive reaction. Central nucleations of mineralization in areas with high cellularity, which are intensively positive for OPN were observed (Fig.15B stage 3). In areas with bone formation, differentiated osteoblasts and young osteocytes were positive for Coll I and OSC, while the bone matrix was weakly positive for Coll I. The mineralized front was positive for OSP. Moreover, at woven bone and low cellular proliferation areas, many endothelial cells were clearly positive for CD34.

#### *KUSA/A1 alone at 2 weeks*

Few small vessels were positive for CD34. Some KUSA/A1 cells surrounding the hard tissue were positive for PCNA. However, these cells were negative for OSP and weakly stained

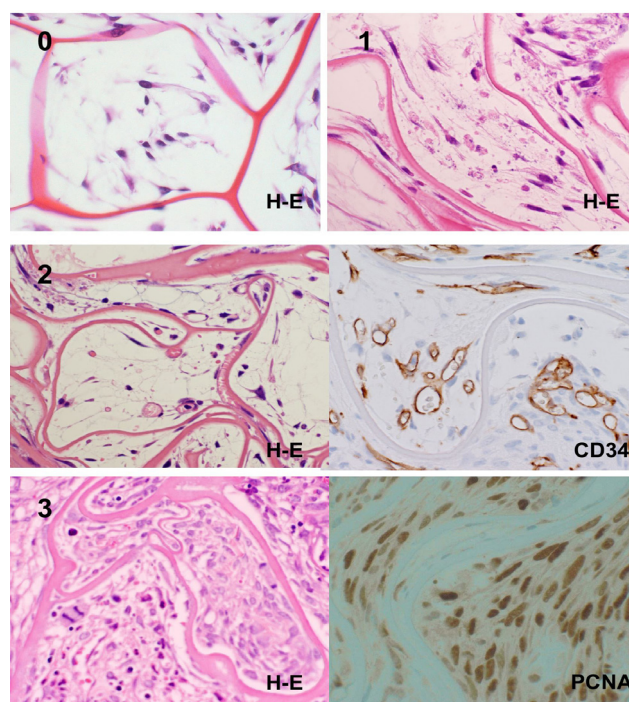


Fig.15A(stage 0-3). Mechanism of bone induction by KUSA/A1 cells within a pore of the scaffold, 40x. (0) Preimplantation, H-E; (1) Cell debris formation, H-E; (2) Excessive developing vessels within the pore of the scaffold (H-E). These zones were strongly positive for CD34. (3) Presence of areas with high cellularity of KUSA/A1 cells, H-E. These proliferating KUSA/A1 cells were strongly positive reaction for PCNA within their nuclei.

for Coll I and OSC. On the other hand, osteoblasts and young osteocytes in areas of new bone formation were positive to Coll I and OSC. Bone matrix was weakly positive for Coll I. The mineralized fronts were strongly positive for OSP.

#### *KUSA/A1-Atelocollagen at 4 weeks*

In areas with bone formation, differentiated osteoblasts and young osteocytes were positive for Coll I and OSC. Bone matrix was weakly positive for Coll I (Fig. 15B stage 4,5). The mineralized fronts were strongly positive for OSP (Fig.15B stage 4,5). Many endothelial cells in developing vessels were clearly positive with CD34 immunostaining in the whole scaffold. In areas with high cellularity, some cells were positive for PCNA.

#### *KUSA/A1 alone at 4 weeks*

Few vessels positive for CD34 were observed. By PCNA immunostaining, some KUSA/A1 layers were positive and few nuclei from woven bone were also positive. Osteoblasts in woven bone as well as young osteocytes were positive for Coll I and OSC. Bone matrix was weakly positive for Coll I. The mineralized fronts were strongly positive for OSP.

#### *Quantification of bone induction, vessel formation and cellular proliferation in KUSA/A1 alone and KUSA/A1-Atelocollagen*

The areas of the new bone formation are 36, 170 and 268

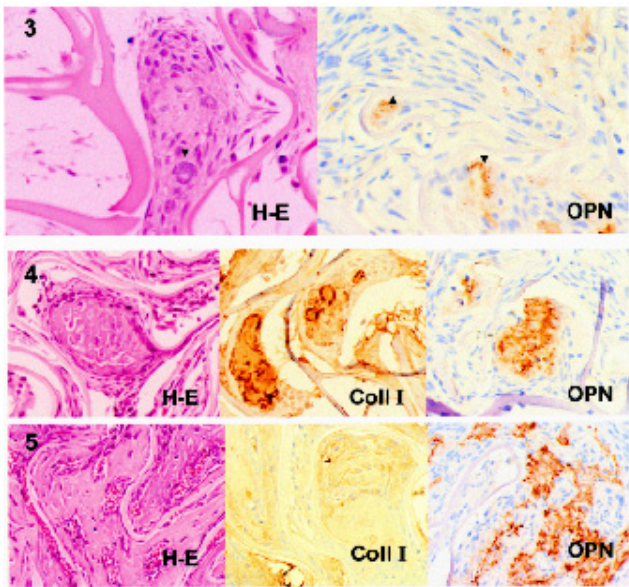


Fig.15B (stage 3-5). Mechanism of bone induction by KUSA/A1 cells within a pore of the scaffold, 40x. (3) Central nucleation of mineralization in areas with high cellularity (H-E), which are intensely positive for OPN; (4) Evidence of bone matrix production (H-E), which showed immunoreaction for osteogenic markers such as Coll I and OPN. (5) Presence of new bone formation (H-E), which demonstrated immunolocalization for Coll I and OPN. Nucleation of mineralization ( arrow ).

mm<sup>2</sup> in KUSA/A1-Atelocollagen at 1, 2 and 4 weeks, respectively. While that they were 10, 43 and 48 mm<sup>2</sup> in KUSA/A1 alone at 1, 2 and 4 weeks respectively. A significant increase of bone formation in KUSA/A1-Atelocollagen compared to KUSA/A1 alone at 2 and 4 weeks was observed (Fig.16A). The average of vessel numbers were 42, 54 and 63 in KUSA/A1-Atelocollagen at 1, 2 and 4 weeks, respectively, while they were 28, 15 and 14 in KUSA/A1 alone at 1, 2 and 4 weeks, respectively. The vessel formation was approximately three fold at 2 weeks and four fold at 4 weeks in KUSA/A1-Atelocollagen group as compared with KUSA/A1 alone (Fig.16B). The percentages of cells positive for PCNA immunostainings were 38, 76 and 45 in KUSA/A1-Atelocollagen at 1, 2 and 4 weeks, respectively, while they were 35, 32 and 33 in KUSA/A1 alone at 1, 2 and 4 weeks, respectively. The difference in cell proliferation was approximately two folds in KUSA/A1-Atelocollagen at 2 weeks compared to KUSA/A1 alone, although the change at 4 weeks was not dramatic (Fig.16C). Thus, KUSA/A1 cells combined with this scaffold type showed a significant increase in vessel formation at 1, 2 weeks (\*P = 0.0010) and 4 weeks (\*\*P < 0.0001) as well as cellular proliferation both at 2 weeks (\*\*P < 0.0001) and 4 weeks (\*P = 0.0079). Note that vessel formation and cellular proliferation in KUSA/A1 cells alone

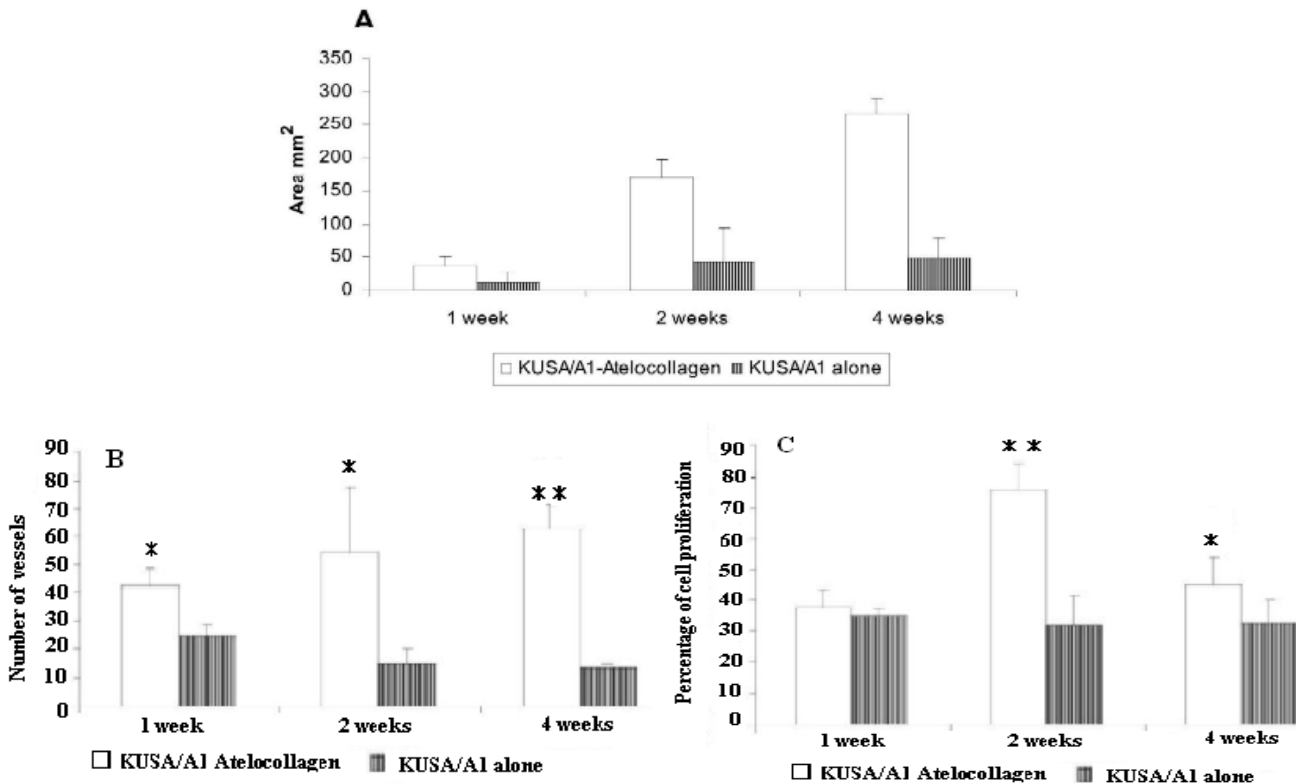


Fig.16. A: Effects of Atelocollagen Honeycomb scaffold on the quantity of bone induction, Student's *t*-test. Note excessive new bone formed by differentiated KUSA/A1 cells within the scaffold at 2 (\*P = 0.022) and 4 weeks (\*\*P < 0.001) compared with KUSA/A1 alone. B: Effects of Atelocollagen Honeycomb scaffold on the number of vessels marked with CD34, Student's *t*-test. In KUSA/A1-Atelocollagen, the number of vessels was significantly higher and different compared with KUSA/A1 alone at 1 and 2 (\*P=0.0010) and 4 weeks (\*P < 0.0001). C: Effects of Atelocollagen honeycomb scaffold on cellular proliferation marked with PCNA Student's *t*-test. In KUSA/A1-Atelocollagen, cells positively stained for PCNA was significantly higher and different compared with KUSA/A1 alone at 2 (\*\*P < 0.0001) and 4 weeks (\*\*P = 0.0079).

at 2 and 4 weeks were almost the same.

### Discussion

KUSA/A1 cells have been isolated from adult marrow and are capable to differentiate into osteoblast, chondrocytes and myotubes under inducing condition<sup>18</sup>). Thus, KUSA/A1 after cultured in osteogenic condition (composed by ascorbic acid and b-glycerophosphate), expressed high ALP activity, calcium deposition, osteocalcin release with low PTH responsibility<sup>20</sup>). It has been reported that ascorbic acid is required for the synthesis of collagen, the regulation of alkaline phosphatase activities and protein synthesis in cultures of osteoblasts-like cells<sup>21</sup>). The organic phosphate,  $\beta$ -glycerophosphate, has been used *in vitro* to provide a potential source of phosphate ions<sup>21</sup>). In order to investigate whether KUSA/A1 cells cultured in a-medium maintain their immature stage *in vitro*, the cells were exposed to  $\alpha$ -MEM alone and  $\alpha$ -MEM+AA+ $\beta$ -GP as positive control. KUSA/A1 cells exposed to the inducing condition demonstrated extracellular matrix positive for Von Kossa staining with marked accumulation of calcium deposits (Fig.5 B, D, F, H, J) and positive expression for Coll I, OCN and OPN in all period of times (Fig.7, 8, 9 B, D, F, H, J). In contrast, KUSA/A1 cells cultured in non-inducing condition ( $\alpha$ -MEM alone) did not show accumulation of calcium deposits (Fig.5 A, C, E, G, I), Coll I was weakly expressed (Fig.7A, C, E, G, I) and was completely negative for OCN and OSP during the time course (Fig.8, 9 A, C, E, G, I). This result indicates that KUSA/A1 cells are capable to maintain their immature stage in non-inducing condition, which shows osteogenic potential activity in inducing condition.

Diffusion chamber contains impermeable membranes for cells, which offers a natural condition permitting only free passage of molecules present in the humoral phase of the host<sup>11</sup>). Moreover, we reported that diffusion chamber is a good alternative to analyze gene and protein expression of implanted cells *in vivo*, which could be considered as “cell culture *in vivo* method” to analyze cell phenotype in basic research<sup>22</sup>). In order to evaluate the cell differentiation under the influence of the microenvironment, KUSA/A1 cells were implanted in intraperitoneal diffusion chamber. We selected intraperitoneal cavity, because in experimental animals this provides a powerful tool for studying stem cells *in vivo*<sup>23</sup>). Surprisingly, KUSA/A1 cells at 2, 4 and 6 weeks revealed bone formation only inside of chamber with matrix protein secretion within the pores of the membrane (Fig.10 C, E, G). We believe that the new bone was formed only by KUSA/A1 cells within the chamber, because the size of the pore in this membrane is too small (0.22 mm diameter) to permit cell migration. These new hard tissues exhibited presence of calcium deposition stained by Von Kossa (Fig.10 D, F, G) and the initial calcification foci observed by TEM, were seen in the collagen fiber of the osteoid, in the matrix vesicles within the membrane of the diffusion chamber (Fig.12 C, D) and also degenerated cells apart from the membrane of the chamber at 4weeks (Fig.12 E, F).

Moreover, the new bone showed immunopositive reaction for osteogenic markers (Fig.11). These results suggest that KUSA/A1 cells seeded within a host microenvironment are capable to differentiate into osteoblasts and produce mineralized bone matrix.

The basic principle of tissue engineering is to use seeded mature cells or stem cells combined with biocompatible and biodegradable scaffold to generate a certain type of tissue; either *in vitro* or *in vivo*. Most studies related to bone tissue engineering are focused on searching the ideal osteogenic seeded cells and an optimal scaffold. Osteogenic cells are present in bone marrow stroma from mammals (including rodents and human) and their ability to produce bone-like mineralized tissue has been demonstrated both *in vivo*, i.e., in diffusion chambers loaded with bone marrow cells<sup>18,24</sup>) and *in vitro*, where under suitable culture conditions bone-like tissue is synthesized by various marrow stromal cells populations<sup>21,25</sup>). In this study, we used KUSA/A1 cells, a marrow stromal stem cell line with a potential to induce new bone-like tissue, which is positively stained for Coll I, OSP and OSC markers. Our results indicated that KUSA/A1 cells were capable of differentiating into osteoblast-like cells and induce new bone *in vivo*.

Another key issue for *in vivo* bone engineering is to use three-dimensional cell distribution in a scaffold. The scaffold material requires biocompatibility to both, seeded cells and interfaced surrounding tissues. Currently, bone engineering is based on the use of different kinds of material such as polymers, partially demineralized bone, and calcium containing substances like calcium alginate<sup>26-29</sup>). We selected atelocollagen honeycomb scaffold as a carrier, because it offers a good environment for the cells and good mechanical stability, and maintains its original size and shape during cell growth *in vitro*<sup>17</sup>). Importantly, KUSA/A1 cells on Atelocollagen scaffold were able to proliferate and differentiate into osteoblasts, followed by the degradation of the scaffold. The cells generated their own extracellular matrix, suggesting that atelocollagen honeycomb scaffold was biocompatible for KUSA/A1 cells and interfaced surrounding tissues *in vivo*.

Radiographically, KUSA/A1-Atelocollagen showed larger, but less degree of radiopacity compared with KUSA/A1 cells alone (Fig.13). Histologically, KUSA/A1-Atelocollagen formed more immature bone than KUSA/A1 cells alone, suggesting that this scaffold enhanced the amount of the new bone, but needed more time to be mature bone compared to KUSA/A1 cells alone.

Moreover our study showed that the new bone formed is completely separated from the collagen membrane of the scaffold at 2 weeks. In contrast, it has been reported that, BMP-collagen carrier was important to form new bone, because most osteoid was close to the carrier fibers<sup>30</sup>). At 4 weeks, the whole scaffold was filled with proliferated cells and woven bone (Fig.14.E). Collagen membranes were absent and replaced by hard tissues. Interestingly, the new bone was only formed into the scaffold,

suggesting that this scaffold plays an important role in carrying the cells giving the precise size, shape and comfortable environment.

It is well-known that vascularization is an important prerequisite for osteogenesis<sup>31,32</sup>. Atelocollagen honeycomb scaffold contains parallel tubes easily invaded by vessels *in vivo*. Vascularization in the implant regions progressed with the proliferation of KUSA/A1 cells. The number of vessels, strongly positive for CD34 increased in KUSA/A1-Atelocollagen at 2 and 4 weeks (Fig.16 B). The presence of many hypoxic KUSA/A1 cells in a large scaffold required more blood supply stimulating angiogenesis. The endothelial cells from the neighboring vessels were stimulated to grow. The junctions between endothelial cells were altered, cell projections passed through the space created, and the newly formed sprout grew towards the source of the stimulus. On the other hand, KUSA/A1 cells alone consisted of small hypoxic areas with the formation of few vessels. These results suggest that this scaffold is an efficient conductor for vessel formation and enhances the vessel formation.

As it has been previously reported, PCNA is immunopositive in cellular proliferation of osteo/chondrogenic cells during the process of bone formation<sup>33</sup>. KUSA/A1-Atelocollagen at 2 weeks showed excessive cellular proliferation being strongly positive for PCNA (Fig.16C), and negative for Coll I, OSC, and OSP markers. In contrast, KUSA-A1 cells alone revealed only few nuclei stained with PCNA, but Coll I, OSC and OSP were positive. On the other hand, Coll I, OSC and OSP were strongly positive in both groups at 4 weeks. These results showed that this scaffold enhanced cellular proliferation being easily penetrated with vessels in the whole carrier offering an oxygen supply for the cells, which permitted KUSA/A1 cells to proliferate within collagen spaces of the scaffold during the first 2 weeks.

Interestingly in this study, although the cells were cultured without osteogenic medium and 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. These results demonstrated that after oxygen supply by vessel formation within the scaffold, the cells were able to proliferate, and preosteoblasts condense and differentiate into osteoblasts with bone matrix deposition similar to intramembranous bone formation. As it has been reported, the micromass culture indicated that cell-cell contact and cell-cell communication were important for the differentiation process<sup>34,35</sup>. The micromass inoculation corresponds to the state of condensation of mesenchymal cells during membranous bone formation, which amplifies the number of pre-osteoblasts<sup>36</sup>. We believe that these cells are capable to form micromass-like structure within collagen membrane spaces of the scaffold that would permit cell-cell contact inducing osteoblast differentiation expressed by Coll I, OSC and OSP markers.

In summary, few KUSA/A1 cells are capable of forming large

amount of new bone within honeycomb scaffold compared to KUSA/A1 cells alone (Fig.16 A). The new bone started from the periphery of the scaffold. In KUSA/A1-atelocollagen groups, the new bone was formed only into the scaffold, whereas KUSA-A1 cells alone induced formation of many small islands of new bone in diffuse area. We speculate the sequence of bone formation by KUSA/A1 cells combined with honeycomb scaffold as follows (Fig.15A, B).

Presence of few cells after implantation.

- 1.The cells became hypoxic and form cell debris due to lack of blood supply.
- 2.Hypoxic condition brought about angiogenesis resulting in vessel formation.
- 3.Then, cellular proliferation around a central nucleation of mineralization formed by KUSA/A1 debris forming micromass-like structures.
- 4.Followed by osteoblastic differentiation with induction of bone matrix from the center of the micromass.
- 5.Large amount of bone formation filling the whole scaffold.

### Conclusions

The biological analysis of KUSA/A1 cells for bone tissue engineering demonstrated that: KUSA/A1 cells cultured in non-inducing condition maintained their immature stage, which would be an appropriate method for bone tissue engineering. These cells previously cultured in non-inducing condition, under host microenvironment, demonstrated osteogenic differentiation in intraperitoneal diffusion chamber. Honeycomb scaffold plays an important role for vessel formation, cell proliferation and cell differentiation forming abundant new bone produced by few KUSA/A1 cells

This study concluded that KUSA/A1 cell is a good candidate as stem cells for basic research in bone tissue engineering.

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