Efficacy of atelocollagen honeycomb scaffold in bone formation using KUSA/A1 cells

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Abstract: To induce new bone formation, mesenchymal stem cells were seeded onto atelocollagen honeycomb scaffold. We evaluated the efficacy of this scaffold combined with KUSA/A1 cells *in vivo*. KUSA/A1 cells alone and with atelocollagen 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-atelocollagen showed some radiopaque areas formation but the latter disclosed a larger amount. Histologically, 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-atelocollagen revealed abundant new bone formation as well as cellular proliferation. We also determined the immunolocalization of type I collagen, CD34, osteocalcin, osteopontin, and PCNA in this newly formed bone. Our results indicated that collagen scaffold plays an important role allowing vessel formation and cell anchorage, especially through the proliferation and differentiation process in a confined space. This study supports tissue engineering as an alternative for treating different target diseases, such as trauma or congenital defects, and enhances existing therapeutic applications. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 707–717, 2006

Key words: tissue engineering; KUSA/A1 cells; atelocollagen; honeycomb scaffold; bone formation; immunohistochemical staining

INTRODUCTION

Bone regeneration is a biological process to renew damaged bone tissue. When the bony defect is small, it usually heals by regeneration. However, if the bony defect is large, it is necessary to use tissue grafting to allow bone repair.[1] 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. Tissue engineering is defined as an interdisciplinary field where the engineering and life sciences principles are applied toward biological substitute generation.[2]

The general strategies in tissue engineering are (A) infusion of isolated cells or cell substitutes, (B) infusion or implantation of tissue-inducing substances such as polypeptide growth factors or morphogens, and (C) implantation of cells on or within matrices.[3] Autologous cells can be used to repair articular cartilage. A small piece of cartilage is removed from the healthy section of a patient's injured knee.[4] Chondrocytes are isolated, expanded in culture, and then implanted at the injured site.[4] In a variation of this approach, mesenchymal stem cells have been harvested from patient bone marrow, expanded in culture, and then induced to differentiate into cells that can help to repair damaged bone, cartilage, tendon, or ligament.[5] In bone engineering, bone marrow stromal cells combined with calcium alginate are capable to induce new bone formation in cranial bone defects.[6] However, some strategies demonstrated inflammatory reaction,[7] few amount of bone formation in old age,[8] and high risk of tumor formation.[9]

Recently, implantation of cells onto scaffold has taken a great importance in tissue engineering and is expected to provide new applications for the creation of new organs and tissues. These scaffolds must be biodegradable without inducing adverse reaction.

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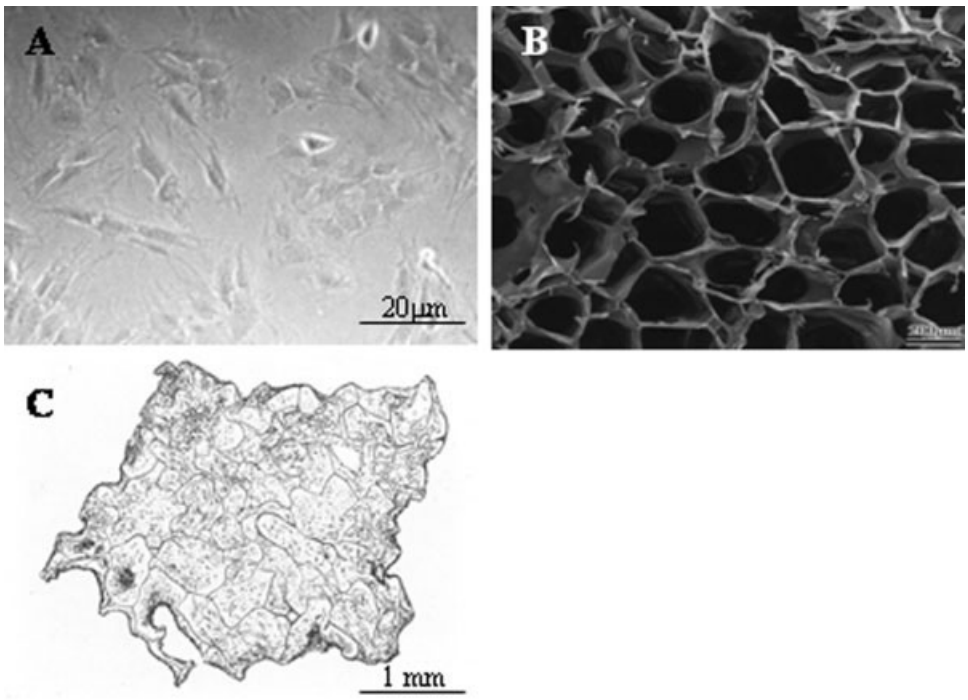

Figure 1. A: *In vitro* characteristics of KUSA/A1 cells at the semiconfluent stage. B: A scanning electron microscopic view of the honeycomb scaffold. C: Microscopic examination of KUSA/A1 cells on atelocollagen honeycomb scaffold on 10 days *in vitro*. KUSA/A1 cells connected each other forming a reticular network (H&E stain).

Atelocollagen is produced by telopeptides removal from natural collagen molecules, resulting in extremely low antigenicity.[10] It is most widely used as a wrinkle eraser by injecting it into the skin.[11] Atelocollagen is also used as a scaffold for keratinocytes and fibroblasts in artificial skin.[11] It is even used to minimize allergic reactions in cosmetics.[12]

Atelocollagen type I honeycomb scaffold is a biocompatible and reabsorbable biomaterial. It has a high cell affinity, maintains its structural integrity, original size, and shape during cell growth *in vitro*.[13] This scaffold could enhance bone regeneration allowing cellular proliferation and differentiation at the same time, and maintaining the precise size and shape through bone formation *in vivo*.

KUSA/A1 cell line is a marrow stromal cell line from primary bone marrow culture of female C3H/He mouse. Immortalized cells were obtained by frequent subculture for more than a year.[14] These cells are capable of expressing three mesenchymal phenotypes; osteocytes, adipocytes, and myocytes by treatment with 5-azacytidine during cell culture.[15]

Moreover, the induction of new bone formation using atelocollagen honeycomb scaffold with KUSA/A1 cells, that could be a good candidate for bone regeneration therapy, has not been reported. Thus, to examine the efficiency of this scaffold in bone regeneration, we evaluated atellocollagen honeycomb scaffold combined with KUSA/A1 cells implanted in SCID mice subcutaneous tissues.

MATERIALS AND METHODS

Cell culture

KUSA/A1 cell line was kindly provided by Dr. Umezawa from Keio University, Tokyo, Japan. These cells, fibrocytic in appearance, are marrow stromal cells obtained from primary bone culture of female C3H/He mouse [Fig. 1(A)]. The cells were cultured in minimum essential medium alpha medium (α medium, Gibco BRL, USA) supplemented with 10% fetal bovine serum (Sigma, USA) and 1% antibiotic–antimycotic (Gibco). Then, they were seeded in 10-cm petri dishes (Falcon, 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 of multiple collagen membranes (1 μm in thickness) with honeycomb-shape were used. [Fig. 1(B)]. The scaffold presents parallel pores extended from surface to surface.

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 [Fig. 1(C)].

Animals

Eight 4-week-old male severe combined immunodeficient (SCID) mice were used in this study in accordance with the Guidelines for Animal Experiments at Graduate School of Medicine and Dentistry Okayama University, 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, Japan) and Dormitol (Meiji Seika, 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, 10 atelocollagen honeycomb scaffolds combined with 1×10^6 KUSA/A1 cells were implanted in the subcutaneous pockets and sutured. On the right side, 5×10^6 of KUSA/A1 cells alone, as control, were injected in subcutaneous tissues. The animals were killed with an overdose of ether at 2 and 4 weeks after implantation. All specimens and surrounding tissues were removed, 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). The implants were also subjected to immunohistochemical studies.

Immunohistochemical staining of CD-34, proliferating cell nuclear antigen, and osteopontin

The sections were immunostained with monoclonal antibodies against CD-34 (abcam'UK) using Vectastain ABC (Adivin-Biotin-peroxidase Complex, USA) Rat Kit method, osteopontin (OSP) (IBL, Japan) and proliferating cell nuclear antigen (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 min; (2) the activation of antigenecity was done with microwave treatment before blocking nonspecific protein binding with rabbit normal serum and horse normal serum, respectively, for 10 min at room temperature; (3) incubation with the primary antibody at 4°C overnight. The optimal dilutions of each primary were performed as follows: (CD-34) 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 min; (5) incubation with ABC at a dilution of 1:50 for 30 min; and (6) treatment with DAB color development and counterstaining with Mayer's hematoxylin.

Immunohistochemical staining of type I collagen (Coll I) and osteocalcin

The sections were also immunostained with polyclonal antibodies against Coll I (LSL, Japan) and osteocalcin (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 min; (2) treatment with microwave before blocking nonspecific protein binding with swine normal serum (Dako) containing 1% bovine serum albumin for 10 min; (3) incubation with the primary antibody at a dilution of 1:500 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 min; (5) incubation with PAP at a dilution of 1:40 for 30 min; and (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

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 2 and 4 weeks after implantation so as to know the vessel and cellular proliferation amount, respectively. The counting process was performed by using an eyepiece micrometer at three separate areas: center, right, and left side of the slide at 40× of magnification, and the process was repeated three times to decrease 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 2 and 4 weeks as described earlier. 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 2 and 4 weeks as described earlier. The differences in vascular formation and cell proliferation were subjected to statistical analysis.

Statistical analysis

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

RESULTS

Radiological examination

At 2 weeks, KUSA/A1-atelocollagen implants showed many weak spotted areas of diffuse and wide

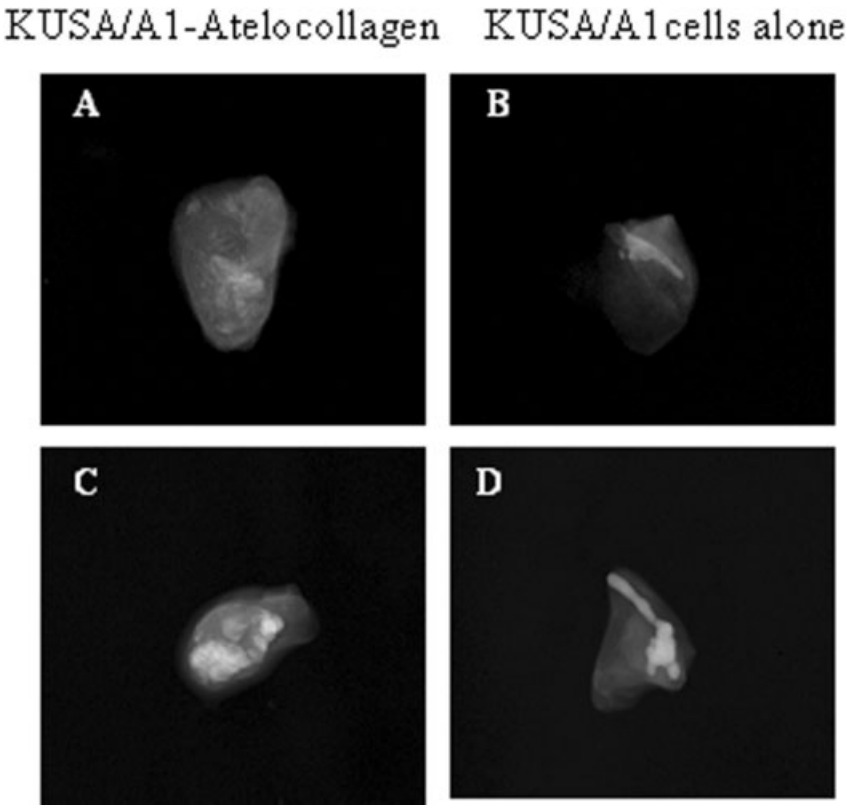

Figure 2. Soft X-ray of KUSA/A1-atelocollagen and KUSA/A1 alone after implantation. KUSA/A1–atelocollagen at 2 weeks (A) and 4 weeks (C) showed larger radiopacity compared with KUSA/A1 alone at 2 weeks (B) and 4 weeks (D).

radiopacity [Fig. 2(A)]. KUSA/A1 alone implants revealed a length, weak, and small radiopaque area [Fig. 2(B)].

At 4 weeks, in KUSA/A1-atelocollagen, the scaffolds were filled with many ovoid or rounded radiopaque areas, showing different degrees of calcification. They had an unclear or diffuse border [Fig. 2(C)]. KUSA/A1 alone showed an irregular, prolonged radiopaque area. The radiopacity were homogeneous and dense with well-defined border [Fig. 2(D)].

Histological examination

KUSA/A1-Atelocollagen at 2 weeks

The specimens showed three different types of areas: (1) cellular proliferation and vessel formation, (2) few cells with many vessels formation, and (3) woven bone formation. The newly formed bone was present on scaffold collagen membranes, but not attached [Fig. 3(B)]. It was developed in the spaces of collagen membranes, especially at its periphery [Fig. 3(A)]. Muscle and fat tissues were also detected.

KUSA/A1 Alone at 2 weeks

The samples showed few small islands of new bone formation, some surrounded by KUSA/A1 cell proliferation, but others covered by few lines of them [Fig. 3(C)]. Inside the new bone, osteocytes with lacunae as well as many vessels were observed [Fig. 3(D)]. Fat and connective tissue were also detected.

KUSA/A1-Atelocollagen at 4 weeks

The slides showed the scaffolds fully filled by new bone with high cellularity. The new hard tissue was observed at the scaffold periphery, and cellular proliferation was found in its center [Fig. 3(E)]. The new bone formed was composed of irregular trabeculae bordered by active osteoblasts, and osteocytes with lacunae were seen in the bone [Fig. 3(F)].

KUSA/A1 Alone at 4 weeks

The specimens showed islands of new bone surrounded by a thin layer of cells [Fig. 3(G)]. Bone was

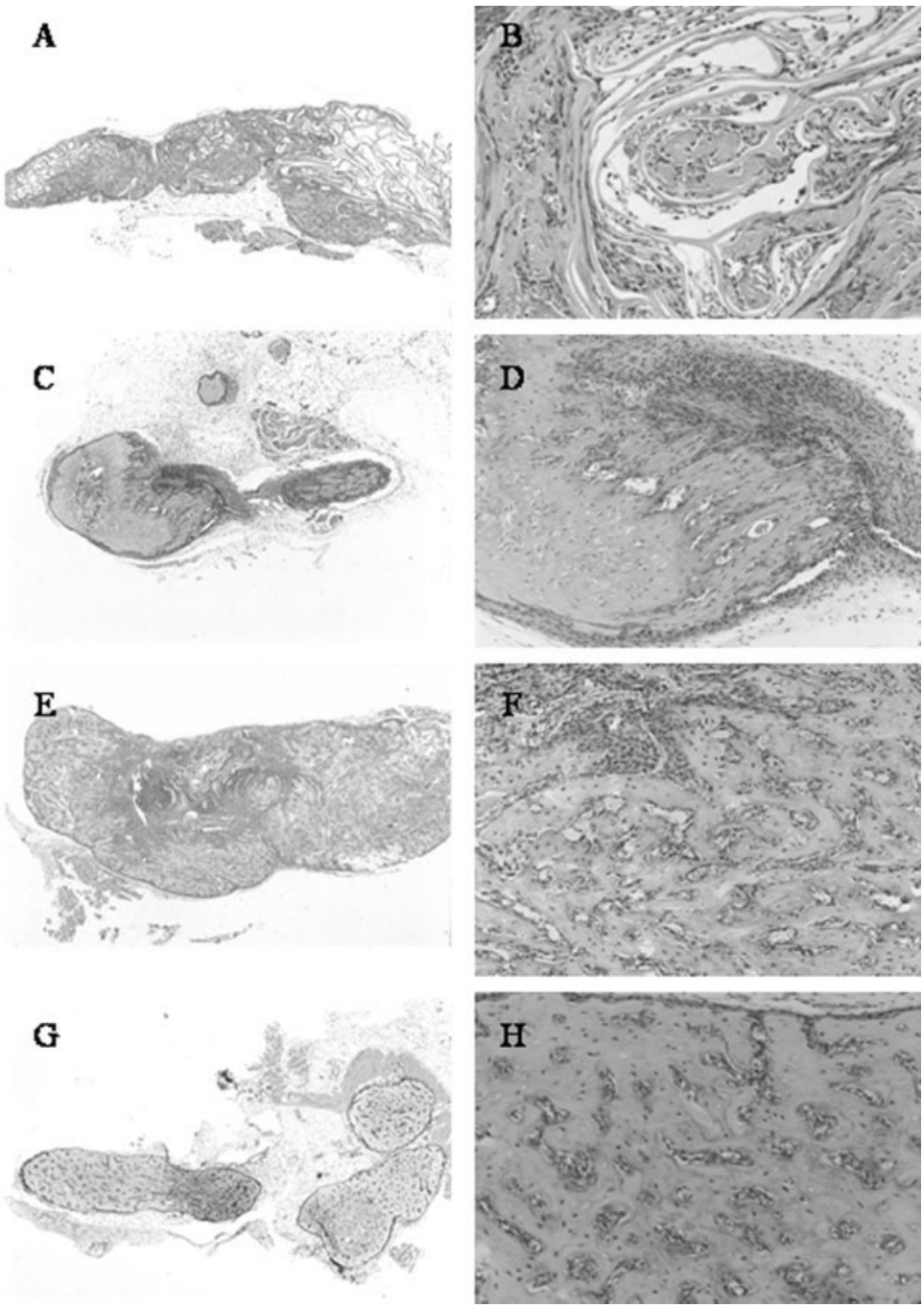

Figure 3. Histological examination of KUSA/A1-atelocollagen at 2 weeks. A: Areas of new bone and cellular proliferation within the scaffold were observed (H&E, ×5). B: Note the presence of immature bone not attached to the scaffold and vessels formation (H&E, ×33). Histological examination of KUSA/A1alone at 2 weeks. C: Islands of new bone and cellular proliferation were clearly seen (H&E, ×5). D: Note the presence of mineralized bone, vessel formation, osteoid, and cellular proliferation (H&E, ×33). Histological examination of KUSA/A1-atelocollagen at 4 weeks. E: All scaffolds were filled of new bone and cellular proliferation (H&E, ×5). F: Presence of woven bone with reabsorption of collagen membranes, high cellularity, and vessel formation were also seen. Histological examination of KUSA/A1 alone at 4 weeks. G: Small islands of new bone surrounded by few cells were seen (H&E, ×5). H: The new bone was composed of immature bone with formation of vessels (H&E, ×33).

composed of lamellae irregular rings and populated by a variable number of osteocytes within the lacunae as well as vessel formation [Fig. 3(H)]. These lamellae were arranged in central canals that contain blood vessels, and osteoblasts. Loose connective tissue, fat, and muscle were also shown. Note that the newly formed bone is more immature in KUSA/A1-atelocollagen than in KUSA/A1 alone.

Immunohistochemical examination

KUSA/A1-Atelocollagen at 2 weeks

In areas with high cellular proliferation, many vessels were strongly positive for CD34, and PCNA was intensely positive in several nuclei [Fig. 4(A,C)]. However, Coll I and OSP were negative [Fig. 4(E,G)]. OSC was generally negative although few cells showed positive reaction. 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 [Fig. 4(B)]. Some KUSA/A1 cells surrounding layers were positive for PCNA [Fig. 4(D)]. However, these cells were negative for OSP and weakly stained for Coll I and OSC. On the other hand, osteoblasts and young osteocytes at new bone formation areas were positive for Coll I [Fig. 4(F)] and OSC. Bone matrix was weakly positive for Coll I [Fig. 4(F)]. The mineralized fronts were strongly positive for OSP [Fig. 4(H)].

KUSA/A1-Atelocollagen at 4 weeks

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

KUSA/A1 Alone at 4 weeks

Few vessels positive for CD34 were observed [Fig. 5(B)]. By PCNA immunostaining, some KUSA/A1 layers were positive and few nuclei from woven bone were also positive [Fig. 5(D)]. Osteoblasts in the woven bone as well as young osteocytes were positive for Coll I and OSC [Fig. 5(F)]. Bone matrix was weakly positive for Coll I. The mineralized fronts were strongly positive for OSP [Fig. 5(H)].

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 25 mm^2 and 81 mm^2 in KUSA/A1-atelocollagen at 2 and 4 weeks, respectively, while they were 7 mm^2 and 45 mm^2 in KUSA/A1 alone at 2 and 4 weeks, respectively. A significant increase of bone formation in KUSA/A1-atelocollagen compared with KUSA/A1 alone at 2 and 4 weeks was observed [Fig. 6(A)]. The average of vessel numbers were 54 and 63 in KUSA/A1-atelocollagen at 2 and 4 weeks, respectively, while they were 15 and 14 in KUSA/A1 alone at 2 and 4 weeks, respectively. The vessel formation was approximately threefold at 2 weeks and fourfold at 4 weeks in KUSA/A1-atelocollagen group as compared with KUSA/A1 alone [Fig. 6(B)]. The percentages of cells positive for PCNA immunostainings were 76 and 45 in KUSA/A1-atelocollagen at 2 and 4 weeks, respectively, while they were 32 and 33 in KUSA/A1 alone at 2 and 4 weeks, respectively. The difference in cell proliferation was approximately twofold in KUSA/A1-atelocollagen at 2 weeks as compared with KUSA/A1 alone, although the change at 4 weeks was not marked[Fig. 6(C)]. Thus, KUSA/A1 cells combined with this scaffold type showed a significant increase in vessel formation both at 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 at 2 and 4 weeks were almost the same.

DISCUSSION

The basic principle of tissue engineering is to use seeded 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 mammalians (including rodents and human); and their ability to produce a bone-like mineralized tissue has been demonstrated both *in vivo*, that is, in diffusion chambers loaded with bone marrow cells[15,16] and *in vitro*, where under suitable culture conditions bone-like tissue is synthesized by various marrow stromal cell populations.[5,17] 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 to differentiate into osteoblast-like cells and induce new bone *in vivo*.

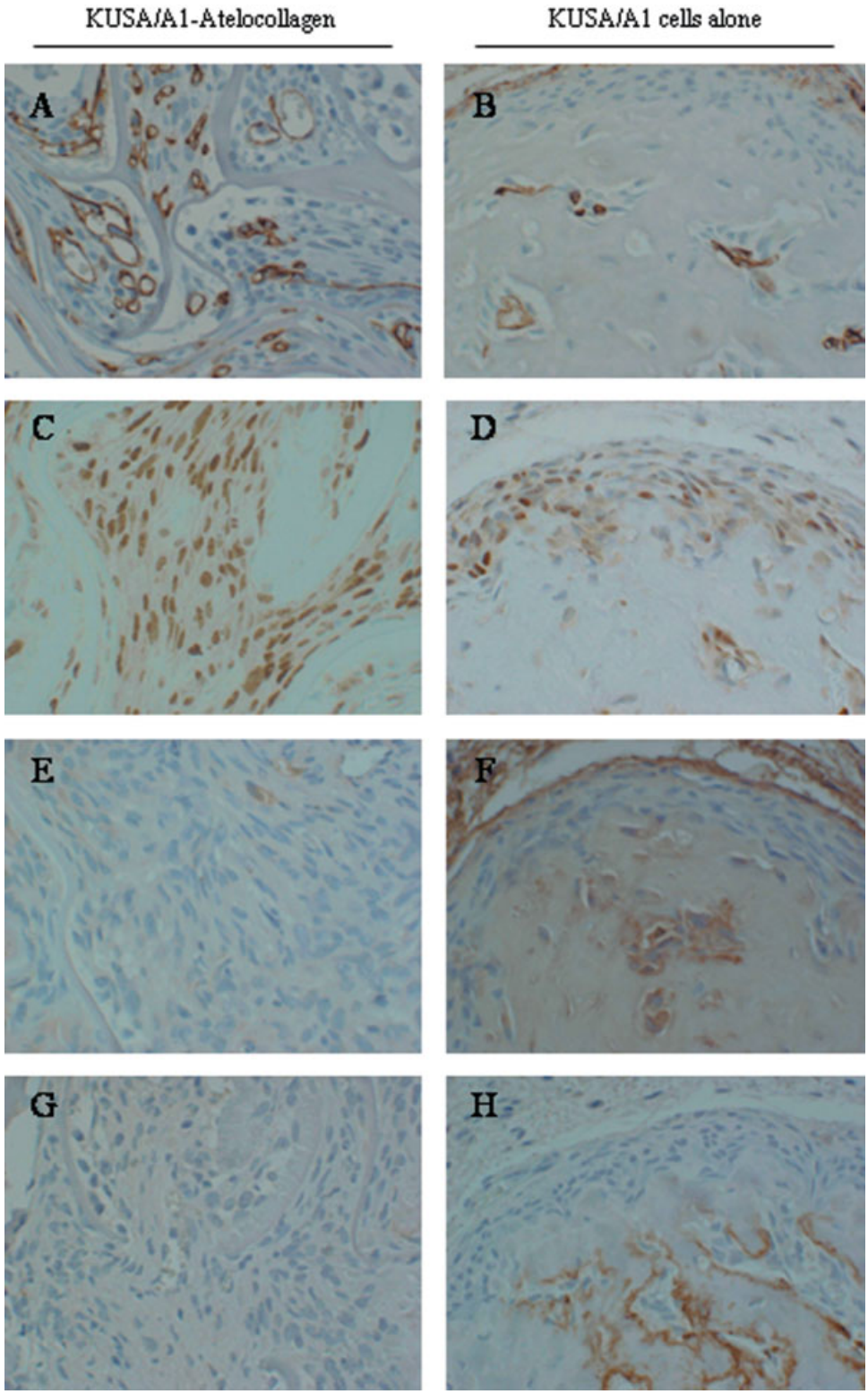

Figure 4. Immunohistochemical examination of KUSA/A1-atelocollagen and KUSA/A1 cells alone at 2 weeks. A and B: Immunostaining for CD34 localized in developing vessels. Note the excessive vessel formation in KUSA/A1-atelocollagen compared with KUSA/A1 cells alone (×400). C and D: Immunostaining for PCNA within the nucleus of proliferating KUSA/A1 cells. Note that PCNA is strongly positive in areas with high cellularity of KUSA/A1-atelocollagen (×400). E and F: Immunostaining for Coll I and (G, H) OSP are completely negative in KUSA/A1-atelocollagen. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

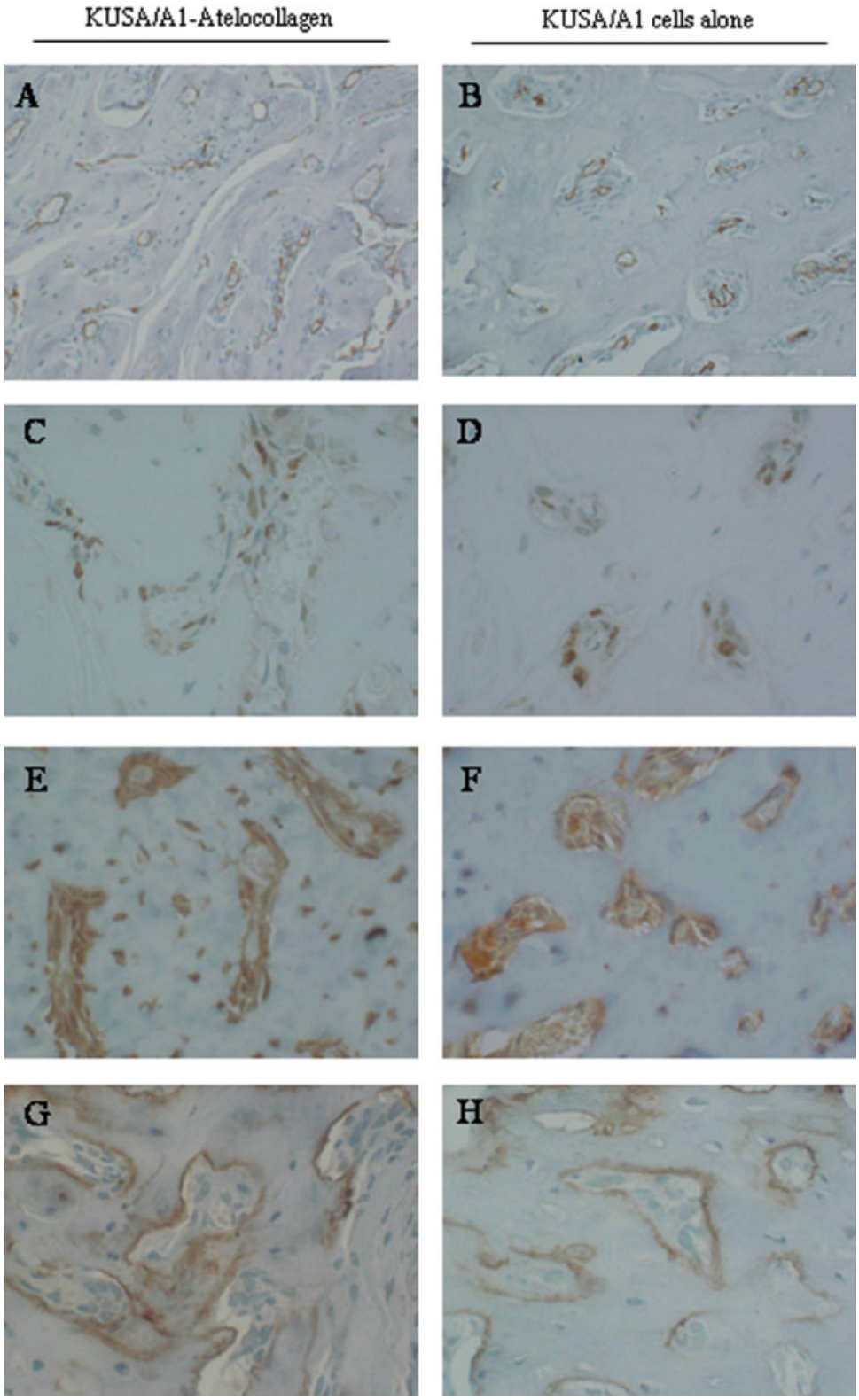

Figure 5. Immunohistochemical examination of KUSA/A1-atelocollagen and KUSA/A1 cells alone at 4 weeks. A and B: Immunostaining for CD34 localized in developing vessels. Note the excessive vessel formation in KUSA/A1-atelocollagen compared with KUSA/A1 cells alone (×400). C and D: Immunostaining for PCNA observed within the nucleus of proliferating KUSA/A1 cells (×400). E and F: Immunostaining for OSC localized in osteoblast-like cells and osteocyte-like cells of woven bone. G and H: Immunostaining for OSP noted in the calcification front of woven bone (×400). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

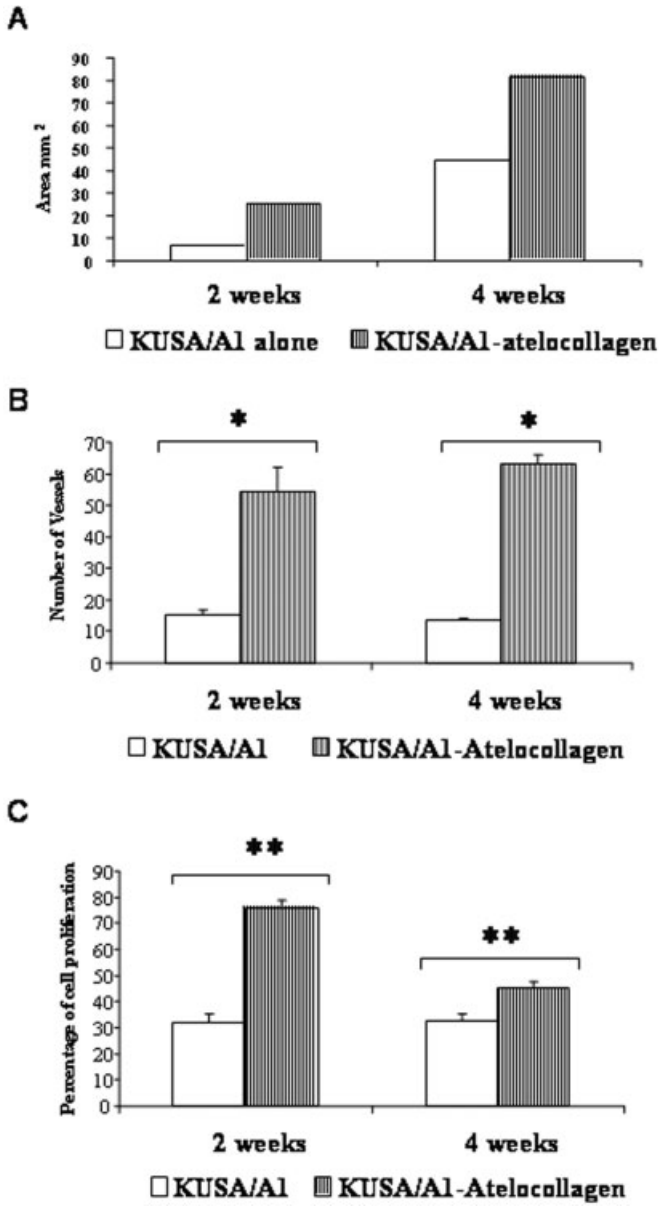

Figure 6. A: Effects of atelocollagen honeycomb scaffold on the quantity of bone induction. Note the excessive new bone formed by differentiated KUSA/A1 cells within the scaffold. B: Effects of atelocollagen honeycomb scaffold on the number of vessels marked with CD-34. In KUSA/A1-atelocollagen, the number of vessels was significantly higher and different in KUSA/A1 cells alone at 2 ($*p = 0.0010$) and 4 weeks ($*p < 0.0001$). C: Effects of atelocollagen honeycomb scaffold on cellular proliferation marked with PCNA. In KUSA/A1-atelocollagen, cells positively stained for PCNA were significantly higher and different in KUSA/A1 cells alone at 2 ($**p < 0.0001$) and 4 weeks ($**p = 0.0079$).

Another key issue for *in vivo* bone engineering is to use the 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 kind of material types such as polymers, partially demineralized bone, and calcium containing substances like calcium alginate.[6,18–20] We selected atelocollagen honeycomb scaffold as a carrier, because it offers a good environment for the cells and good mechanical stability, and also maintains its original size and shape during cell growth *in vitro*.[13] Importantly, KUSA/A1 cells with atelocollagen scaffold were able to proliferate and differentiate into osteoblasts, and then the degradation of the scaffold followed. 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 in both period of time (Fig. 2). 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 need more time to be mature compared with KUSA/A1 cells alone.

Also our study showed that the new bone formed is completely separated from the collagen membrane of the scaffold at 2 weeks [Fig. 3(B)]. 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.[21] At 4 weeks, the whole scaffold was filled with proliferated cells and woven bone. 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 know that vascularization is an important prerequisite for osteogenesis.[4,22] Atelocollagen honeycomb scaffold contains parallel tubes easily invaded by vessels *in vivo* [Fig. 1(B)]. Vascularization in the implant regions progressed with the proliferation of KUSA/A1 cells. The number of the vessels strongly positive for CD34 increased in KUSA/A1-atelocollagen at 2 and 4 weeks [Figs. 4(A) and 5(A)]. 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 toward 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 invasion 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.[23] KUSA/A1-atelocollagen at 2 weeks showed excessive cellular proliferation being strongly positive for

PCNA, 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 invaded 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 preconfluent 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 invasion 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.[24,25] The micromass inoculation corresponds to the state of condensation of mesenchymal cells during membranous bone formation, which amplifies the number of preosteoblasts.[26] 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, the new bone formation started from the periphery of the scaffold. First, there were vessel formation and cellular proliferation, followed by osteoblast differentiation and finally bone formation. KUSA/A1-atelocollagen scaffold was limited to form new bone only into the scaffold, whereas KUSA-A1 cells alone induced formation of many small islands of new bone in diffuse area.

Previously, we also demonstrated that the process of osteogenesis depends upon the physico-chemical nature and shape of the carrier that affects the microenvironment for cell differentiation.[27] The atelocollagen honeycomb scaffold plays an important role in providing vessel formation, cellular anchorage for proliferation and differentiation in a confined area, and gives the precise size and shape for the new bone. In conclusion, our study showed that the honeycomb scaffold is an efficient carrier to induce bone formation, and this scaffold combined with autologous stem cells could be considered as a future therapeutic strategy.

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