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Promotion of attachment of human bone marrow stromal cells by CCN2

Mitsuaki Ono [a,b], Satoshi Kubota [b], Takuo Fujisawa [a], Wataru Sonoyama [a], Harumi Kawaki [b], Kentaro Akiyama [a], Masamitsu Oshima [a], Takashi Nishida [b], Yasuhiro Yoshida [c], Kazuomi Suzuki [c], Masaharu Takigawa [b], Takuo Kuboki [a,*]

[a] *Department of Oral and Maxillofacial Rehabilitation, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8525, Japan*
[b] *Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
[c] *Department of Biomaterials, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*

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Abstract

Cell attachment is a crucial step in tissue regeneration. In this study, human bone marrow stromal cells (hBMSCs) were isolated, and the effects of CCN2 on their attachment were examined. CCN2 significantly enhanced the hBMSC attachment, and this enhanced cell attachment was mainly regulated by the C-terminal module of CCN2. This enhancement was negated by the anti-integrin $\alpha_v\beta_3$ antibody and p38 MAPK inhibitor, and phosphorylation of p38 MAPK was detected upon the enhanced cell attachment mediated by CCN2. We thus conclude that CCN2 enhances hBMSC attachment *via* integrin–p38 MAPK signal pathway. Enhanced hBMSC attachment on hydroxyapatite plates by CCN2 further indicated the utility of CCN2 in bone regeneration.

Keywords: Bone marrow stromal cell; CCN2/connective tissue growth factor; Cell attachment; p38 MAPK; Hydroxyapatite

Reconstruction of bone defects is one of the major clinical issues in orthopedics and dentistry. Most classical techniques are based on the transplantation of homologous bone tissue. However, transplantation of allograft bone causes immunological reaction and transfer of viruses. Tissue engineering, a relatively new and challenging research area, offers attractive potential solutions for repair of bone defects.

In tissue engineering for bone, appropriate artificial bioresorbable and biocompatible scaffold materials may be combined with cells from various sources and growth factors for the stimulation of new bone. These three basic components of tissue engineering can be used in different combinations [1–9]. Natural mineral phase of bone contains a hard calcium phosphate mineral salt phase called hydroxyapatite (HAp). Many clinicians have been using bioceramics such as HAp-based materials for bone implants. It is also well known that HAp scaffolds seeded with osteoblasts before subcutaneous implantation have increased bone formation [10–12].

CCN2/connective tissue growth factor (CTGF), which has been regarded as one of the growth factors, is a member of the CCN protein family. The CCN family has six distinct members (CTGF/Fisp12, Cyr61/Cef10, Nov, rCOP-1/WISP-1, Elm-1/WISP-2, and WISP-3) with a high degree of amino acid sequence homology and 38 conserved cystein residues. This family has four distinct protein modules: an insulin-like growth factor-binding protein-like module; a von Willebrand factor type C repeat; a thrombospondin type 1 repeat; and a C-terminal (CT)-module [13–20]. Although their exact functions are still unknown, these proteins are involved in a number of biological processes such as embryonic development, tissue repair, and tumor suppression [21–23]. Previously, it was reported that

* Corresponding author. Fax: +81 86 235 6684.
E-mail address: kuboki@md.okayama-u.ac.jp (T. Kuboki).

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recombinant CCN2 promoted the regeneration of cartilage and angiogenesis that plays a major role in bone regeneration *in vivo* [24,25]. However, effect of CCN2 on the proliferation and differentiation of hBMSC remains to be clarified. Since hBMSC is a key player of bone regeneration, this is a critical issue to be immediately explored.

In this study, under the hypothesis that CCN2, BMSCs, and HAp scaffold could be an ideal combination for bone regeneration therapy, we isolated human BMSCs (hBMSCs) and investigated the effects of CCN2 adsorption on initial human BMSC attachment *in vitro*. The mechanisms of action was also analyzed with respect to intracellular signal transduction and the modular architecture of the CCN2 molecule.

Materials and methods

Isolation of hBMSCs. hBMSCs were isolated according to the method used for colony forming unit–fibroblast (CFU–F) assay [26]. Briefly, fresh bone marrow was obtained from the iliac crest of healthy adult males (23 or 29 years-old) under sufficient informed consent and the permission of the Ethical Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences. Bone marrow aspirates were plated in Mesenchymal Stem Cell Basal Medium (MSCBM: CAMBREX, Walkersville, MD) containing 10% fetal bovine serum (FBS). Then, the cells were cultured at 37 °C under 5% CO_2 in air. Twenty-four hours after seeding, the medium was changed to remove the non-adherent cells. Then, the adherent clonogenic cells were cultured and their population was allowed to expand. The hBMSCs at the 4–9th passages were used for the experiments.

In vitro differentiation of hBMSC. Osteogenic differentiation was induced by culturing the cells in Dulbecco's modified Eagle's minimum essential medium (DMEM: SIGMA, St. Louis, MO) supplemented with 10% FBS, 50 μg/ml ascorbate (SIGMA), 10 mM β-glycerophosphate (SIGMA), and 0.1 μM dexamethasone (SIGMA) for 21 days. Then, calcium deposition was confirmed by staining with alizarin red (SIGMA). Adipogenic differentiation was induced by culturing the cells in Adipogenic Induction Medium (CAMBREX) for 28 days according to the manufacturer's instructions. Then, cytoplasmic lipid vacuoles were identified by staining the cells with oil red O (SIGMA). Chondrogenesis of pellet cultures was induced by incubating the cells in Chondrogenic Induction Medium (CAMBREX) for 28 days according to the manufacturer's directions, and confirmed by staining with alcian blue (SIGMA).

Immunofluorescent staining. Cells were plated at 3×10^3 cells/cm^2 onto glass chamber slides that had been coated with a collagen gel (Cellmatrix Type1-P: Nittazeratin, Osaka, Japan). For detection of STRO-1 or integrin $\alpha_v\beta_3$ expression, cells were stained with mouse anti-STRO-1 antibody (R&D Systems, Minneapolis, MN) or mouse anti-integrin $\alpha_v\beta_3$ antibody (LM609: CHEMICON, Temecula, CA). Subsequently, the cells were stained with Alexa Fluor® 488-labeled anti-mouse IgG (Molecular Probes, Coralville, IA). Nuclei were visualized with Hoechst 33342 (Molecular Probes).

Recombinant proteins and cell attachment assay. Recombinant full-length CCN2 was purified as previously described [27], and CT-module protein was obtained from PeproTeck (London, UK).

Attachment assays were performed on plastic culture plates or HAp (Pentax, Tokyo, Japan) coated with CCN2 or CT-module at the desired concentration in 0.1% BSA in PBS overnight at 4 °C. Next, the cells were seeded onto the plates at 3×10^3 cells/cm^2 and allowed to attach to the surface in serum-free MSCBM. After the incubation for 1 or 3 h, MSCBM containing 10% FBS was added to fill the wells, and the plates were sealed and placed bottom up for 15 min to remove the floating cells. The MTS assay was carried out to evaluate the number of attached cells (CellTiter 96 AQueous® One Solution: Promega, Madison, WI) according to the manufacturer's instructions. The attachment efficiency was determined from data obtained by measuring the optical absorbance at a wavelength of 490 nm with a microplate reader (Bio-Rad, Hercules, CA).

For the inhibition assay with the anti-integrin $\alpha_v\beta_3$ antibody or p38 MAPK inhibitor (SB 203580: CALBIOCHEM, San Diego, CA), hBMSCs were incubated with 10 μg/ml of anti-integrin $\alpha_v\beta_3$ antibody or p38 MAPK inhibitor (1, 10 μM) for 30 min, and then the same cell attachment assay was carried out.

Western blotting analysis. Tyrosine-phosphorylated ERK, p38 MAPK, and JNK were detected by Western blotting. Briefly, total cellular protein was prepared by lysing cells in a lysis buffer [20 mM Tris–HCl (pH 8.0), containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 5% glycerol, 40 mM ammonium molybdate, and 1 mM methanol] at various time points. Twenty-microgram protein samples were separated by SDS–PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 1% BSA/TBS, each membrane was incubated for 24 h at 4 °C with one of the following antibodies: anti-ERK (Cell Signaling Technology, Boston, MA), p38 MAPK (CALBIOCHEM), JNK (Cell Signaling Technology), active ERK (Promega), active p38 MAPK (Promega), and active JNK (Promega). The membranes were then incubated with HRP-labeled anti-rabbit IgG (DAKO, Trappes, France). The signals were detected by using an enhanced chemiluminescence method (ECL Western Blotting Detection System: Amersham Biosciences, Piscataway, NJ).

Statistical analysis. The results obtained from quantitative experiments were reported as mean values ± standard deviation (SD). Significant differences were examined by one-way factorial ANOVA followed by Scheffe's multiple comparison test.

Results

Involvement of mesenchymal stem cells (MSCs) in BMSCs confirmed by STRO-1 expression and their multipotency

STRO-1 antigen is known to potentially define the MSC subset [28,29]. We confirmed that most cells did display this stem cell marker (see Supplementary Fig. 1A).

Next, we evaluated whether these hBMSCs retained their multipotency, which is one of the major characteristics of stem cells. Following osteogenic induction, the cells showed remarkable calcium deposits as confirmed by alizarin red staining (see Supplementary Fig. 1B). Along with calcium deposition, the expression of osteocalcin mRNA was increased as confirmed by the reverse transcription-mediated polymerase chain reaction (RT-PCR) method (data not shown). By 28 days in adipogenic induction medium, the hBMSCs had differentiated into adipocytes containing clusters of oil red O-stained lipid droplets (see Supplementary Fig. 1C). Also, distinct gene expression of fat-associated markers, e.g., peroxisome proliferator-activated receptor γ and lipoprotein lipase, was detected by RT-PCR (data not shown). Chondrogenic differentiation was evaluated in pellet cultures of hBMSCs that had been cultured for 28 days in a chondrogenic induction medium. Alcian blue staining clearly indicated the chondrogenic differentiation of the hBMSCs (see Supplementary Fig. 1D). Up-regulation of the expression of the type-II collagen gene, a cartilage-specific one, was also confirmed by real-time PCR (data not shown). The results above collectively indicate that our hBMSC preparation consisted of MSCs that had the capacity for multipotent differentiation toward mesenchymal tissue.

Promotion of the hBMSC attachment by full-length CCN2 and single CT-module via integrin $\alpha_v\beta_3$

To evaluate the effects of CCN2 on hBMSC, we carried out a cell attachment assay. As shown in Fig. 1A, the efficiency of cell attachment to plastic culture plates was enhanced by CCN2, being maximally 1.9 times ($p < 0.001$) higher at 100 ng/ml compared with the control efficiency. Next, an inhibition assay was carried out with anti-integrin $\alpha_v\beta_3$ antibody to confirm whether the effect of CCN2 on the cell attachment might be mediated by integrin $\alpha_v\beta_3$, one of the receptors of CCN2. Prior to conducting the inhibition assay, we confirmed that integrin $\alpha_v\beta_3$ was actually displayed on the hBMSCs (Fig. 1B and C). As expected, the cell attachment increased by CCN2 adsorption (50 ng/ml) was efficiently neutralized by the anti-integrin $\alpha_v\beta_3$ antibody ($p = 0.0115$, Fig. 1D). These results indicate that CCN2 effectively promoted the attachment of hBMSCs *via* its integrin $\alpha_v\beta_3$ receptors.

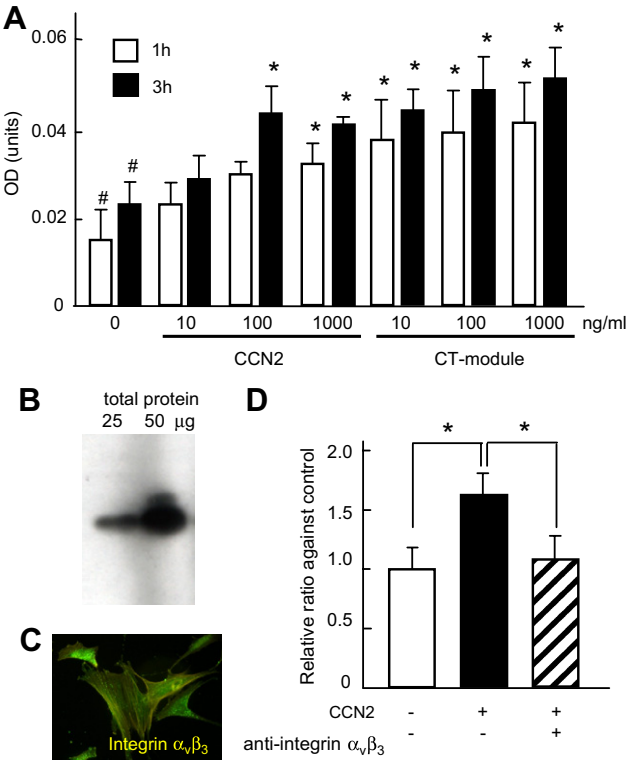

Fig. 1. Effect of CCN2 and CT-module on hBMSC attachment and involvement of integrin $\alpha_v\beta_3$. (A) hBMSCs were seeded and allowed to attach onto CCN2 or CT-module coated plastic culture plates for 1 or 3 h. The MTS assay was carried out to estimate relative number of attached cells. Bars represent means ± SD ($n = 8$) (*$p < 0.05$ vs. #). (B) Expression of integrin $\alpha_v\beta_3$ on hBMSCs. Western blotting of hBMSC lysate of indicated protein quantities was performed with a specific antibody against integrin $\alpha_v\beta_3$. (C) Expression of integrin $\alpha_v\beta_3$ on hBMSCs as demonstrated by immunofluorescence technique. (D) Inhibition assay using anti-integrin $\alpha_v\beta_3$ antibody. hBMSC suspensions were pre-incubated with anti-integrin $\alpha_v\beta_3$ (10 μg/ml), and then plated into plastic culture plates that had been pretreated with CCN2 (50 ng/ml). After further incubation for 3 h, the MTS assay was carried out. Bars represent the means ± SD ($n = 8$) (*$p < 0.05$ as indicated by the brackets).

According to a previous report, one of the ligands of integrin $\alpha_v\beta_3$ is located in the CT-module of CCN2 [30]. Therefore, similar attachment analysis of hBMSC onto plastic culture plates was next comparatively performed with single CT-module (10–1000 ng/ml). Surprisingly, the recombinant single CT-module protein at 10, 100, or 1000 ng/ml also enhanced the cell attachment significantly ($p < 0.0001$) up to 2.87-fold, which was more striking than the effect obtained with the full-length CCN2 (Fig. 1A). Thus, the CT-module was shown to be necessary and sufficient for the induction of hBMSC attachment mediated by integrin $\alpha_v\beta_3$.

Intracellular signaling cascade mediating the CCN2 signal to promote the hBMSC attachment

Next, to clarify the intracellular signaling pathway involved in the promotion of hBMSC attachment mediated by CCN2, we evaluated the activation of major MAPKs by Western blotting. As shown in Fig. 2A, CCN2 significantly induced the phosphorylation of p38 MAPK from 5 min after cell seeding, with maximal activation at 30 min. In contrast, no significant activation/phosphorylation of ERK or JNK was observed. Next, an inhibition assay was carried out by using a p38 MAPK inhibitor. As expected, the increased cell attachment caused by CCN2 was efficiently and significantly ($p = 0.0065$) neutralized by 10 μM p38 MAPK inhibitor (Fig. 2C). Morover, as shown in Fig. 2B, CT-module significantly induced the phosphorylation of p38 MAPK as well as full-length CCN2. These results clearly indicate that CCN2 effectively promoted the attachment of hBMSC *via* p38 MAPK.

CCN2-promoted hBMSC Attachment to HAp

HAp is one of the most promising scaffolds for the regeneration of large defects in bone tissue. Therefore, a cell attachment assay under the same protocol used for plastic plates was conducted to confirm the effects of CCN2 on the hBMSC attachment to HAp plates. Importantly, CCN2 adsorption (100 ng/ml) to HAp plates also promoted the attachment of hBMSCs up to 1.8-fold ($p = 0.0017$, Fig. 3), suggesting the utility of CCN2 for bone regenerative therapy in combination with HAp.

Discussion

In the present study, we evaluated the effects of CCN2 on hBMSCs and found that CCN2 promoted the hBMSC adhesion of the cells *via* an integrin $\alpha_v\beta_3$–p38 MAPK pathway *in vitro*. The utility of CCN2 in mesenchymal tissue regeneration has been suggested by previous findings [24,25,31–33]. Reports in the literature clearly indicate that CCN2 promotes the proliferation and differentiation of chondrocytes [31,32] and stimulates the proliferation, migration, and tube formation of vascular endothelial cells *in vitro* [33] and angiogenesis *in vivo* [24]. Nevertheless,

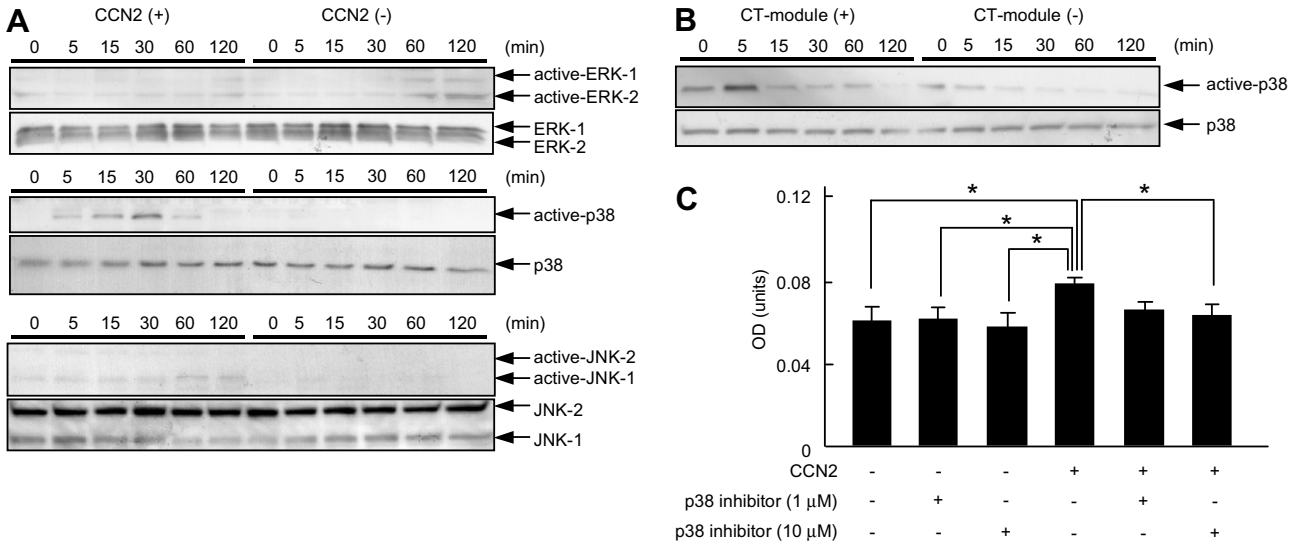

Fig. 2. Intracellular signaling cascade in hBMSC activated by CCN2 and effect of p38 MAPK inhibitor on the attachment. (A,B) Phosphorylation of p38 MAPK in hBMSC by CCN2 (A) and CT-module (B). hBMSCs were cultured with or without CCN2 (A) or CT-module (B), and cellular proteins were collected at the indicated time points. MAPKs signals were detected by Western blotting. (C) Attachment assay in the presence or absence of a p38 MAPK inhibitor. Cell suspensions were pre-incubated the p38 MAPK inhibitor (1 or 10 μM), and then the cells were plated into plastic culture plates that had been pretreated with CCN2. After incubation for 3 h, the MTS assay was carried out. Bars represent means ± SD ($n = 8$) ($^*p < 0.05$ as indicated by bracket).

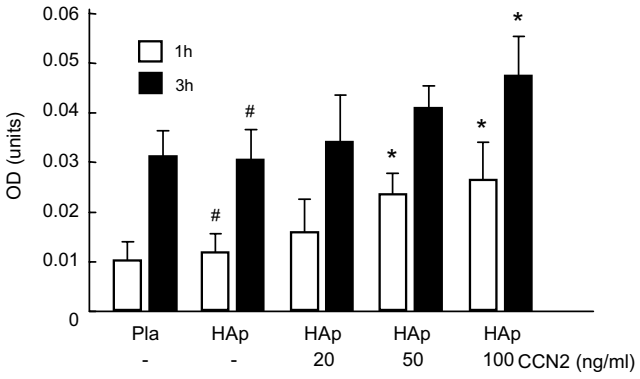

Fig. 3. Effect of CCN2 on hBMSC onto HAp plates. hBMSCs were seeded and allowed to attach onto CCN2-coated HAp plates for 1 or 3 h. The MTS assay was carried out to estimate the relative number of attached cells. Bars represent means ± SD ($n = 8$) ($^*p < 0.05$ vs. #).

although BMSCs are thought to play the central roles in those regeneration procedures, little is known about the effects of CCN2 on BMSCs, particularly during the early events of mesenchymal tissue regeneration, such as attachment. In this study, we obtained clear answers to clarify these issues *in vitro*. Indeed, CCN2 promoted attachment of BMSCs, thus providing a rationale for using CCN2 in BMSC-based regenerative therapeutics.

As cell attachment is an initial crucial step for anchorage-dependent cell growth leading to tissue regeneration, it is thus quite important to clarify the mechanism of CCN2-mediated cell attachment of hBMSC. Here, we found that p38 MAPK play a crucial role in transmitting the signals needed for the cellular events leading to hBMSC attachment (Fig. 2A). On the other hand, CCN2 induces chondrocyte differentiation through a p38 MAPK and proliferation through a MEK/ERK pathway [34], whereas this factor promotes fibroblast attachment also through a MEK/ERK cascade [35]. A contribution of JNK pathway to the promotion of the proliferation and differentiation of chondrocytes was also suggested [35,36]. This functional complexity is supposedly due to the tetramodular structure of CCN2. Importantly, we also clarified that the single CT-module yielded rather stronger effects on hBMSC attachment than the full-length CCN2 (Fig. 1A). In terms of clinical application, such a modular fragment would be more feasible. According to a previous report, the major immunogenic site in the CCN2 molecule was specified to reside in the VWC module [37]. Therefore, the single CT-module would be expected to have less risk to cause adverse immune reactions. Other effects of the CT-module on hBMSCs are currently being evaluated.

In addition, our final results with HAp further indicated the utility of CCN2 in regenerative therapy, in combination with HAp scaffolds and BMSCs. Therefore, a CCN2–HAp–BMSC hybrid is now expected as a promising therapeutic material for the reconstruction of large and/or intractable bone defects in the field of orthopedics and functional rehabilitation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.052.

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