Promotion of Bone Regeneration by CCN2 Incorporated into Gelatin Hydrogel

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ABSTRACT

CCN family protein 2/connective tissue growth factor (CCN2/CTGF) is a unique molecule that promotes the entire endochondral ossification process and regeneration of damaged articular cartilage. Also, CCN2 has been shown to enhance the adhesion and migration of bone marrow stromal cells as well as the growth and differentiation of osteoblasts; hence, its utility in bone regeneration has been suggested. Here, we evaluated the effect of CCN2 on the regeneration of an intractable bone defect in a rat model. First, we prepared two recombinant CCN2s of different origins, and the one showing the stronger effect on osteoblasts *in vitro* was selected for further evaluation, based on the result of an *in vitro* bioassay. Next, to obtain a sustained effect, the recombinant CCN2 was incorporated into gelatin hydrogel that enabled the gradual release of the factor. Evaluation *in vivo* indicated that CCN2 continued to be released at least for up to 14 days after its incorporation. Application of the gelatin hydrogel–CCN2 complex, together with a collagen scaffold to the bone defect prepared in a rat femur resulted in remarkable induction of osteoblastic mineralization markers within 2 weeks. Finally, distinct enhancement of bone regeneration was observed 3 weeks after the application of the complex. These results confirm the utility of CCN2 in the regeneration of intractable bone defects *in vivo* when the factor is incorporated into gelatin hydrogel.

INTRODUCTION

BONE LOSS IS ONE OF the most common complications in the field of orthopedics. For example, fractures in cancellous bone, such as in the proximal humerus, the distal radius, or the tibial plateau, often lead to impaction of bone; consequently, a defect results after reduction. Autologous bone grafting is widely accepted as the gold standard for the treatment of bone defects and nonunions. However, autologous bone grafting has some serious drawbacks, such as prolonged operation time and donor site morbidity in about 10–30% of the cases.[1] To avoid the problems associated

with autologous bone grafting, there has been a continuous interest in the use of synthetic bone grafts during the past few decades. The most frequently used synthetic bone grafts are calcium phosphate ceramics, such as hydroxyapatite, which is known for its excellent biocompatibility.[2–4] However, in addition to such a physical scaffold, it has been recognized that additional factors to stimulate intrinsic osteogenesis may be necessary for generating new bone with structural and functional integrity.

Bone regenerative therapy is one of the advanced therapeutic methods based on tissue engineering techniques, which has been developed recently in the field of orthopedics

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and dental medicine. In general, it is believed that efficient regeneration of bone defects can be achieved by the combination of three regenerative elements—a scaffold, cells, and growth factor(s). As scaffolds, a number of biomaterials, such as bioceramics, biopolymers, and synthetic polymers, have been innovated, improved, and actually applied clinically to date.[5,6] Utility of mesenchymal stem cells has been recently established as a potential component for use in bone repair.[7,8] Also, several growth factors, including fibroblast growth factors (FGFs)[9,10] and bone morphogenetic proteins (BMPs),[11,12] have been evaluated to be used in bone regeneration therapy. Although a few of them are already shown to be functional and applied to bone regeneration, further investigation to discover, or specify another ideal one is still expected today.

CCN family protein 2/connective tissue growth factor (CCN2/CTGF) is one such candidate to be utilized in mesenchymal tissue regeneration.[13–17] Unlike other growth factors, this factor belongs to a novel family of signal modulators, the CCN family,[18–22] which comprises six members in mammals. CCN is an acronym of the assemblage of three classical members, Cyr61, CTGF, and NOV, and all of the members are commonly characterized by their unique primary structure. Except for trimodular CCN5, four conserved modules are connected tandem therein. The modules are designated: insulin-like growth factor binding protein (IGFBP), von Willebrand factor type C repeat (VWC), thrombospondin type 1 repeat (TSP1), and C-terminal (CT) modules.[18,19,22] All of these modules are highly interactive with various biomolecules, and thus CCN proteins are capable of conducting the extracellular signaling network to exert multiple functions in a variety of tissues.

Among the six members, the effect of CCN2 in mesenchymal tissue regeneration has been best evaluated, and its utility is firmly indicated. Indeed, CCN2 promotes the proliferation and differentiation of fibroblasts, chondrocytes, vascular endothelial cells, and osteoblasts *in vitro*.[23–27] It should be also noted that CCN2 enhances the adhesion and migration of human bone marrow mesenchymal stem cells, which differentiate into osteoblasts to play a central role in bone regeneration.[28,29] Further, application of CCN2 to full-thickness articular cartilage defects and osteoarthritic lesions in rat models remarkably promoted articular cartilage repair *in vivo*.[16] In that study, a gelatin hydrogel was employed to enable gradual and sustained supply of the growth factor *in vivo*.[16] Importantly, *CCN2* gene expression was shown to be upregulated during fracture healing in a mouse model and during distraction osteogenesis in a rat model,[30] suggesting that CTGF/CCN2 may play a critical role in bone repair *in vivo*. In addition, the fact that CCN2 is abundantly involved in platelets and is released upon their activation indicates CCN2 to be a natural regenerator of damaged tissues.[31,32] Recent reports clearly indicate the utility of platelet-rich plasma (PRP) in accelerating bone and cartilage regeneration, and their findings further suggest the utility of CCN2 in bone regenerative therapy.[33] Here, we evaluated the effect of CCN2 in relatively intractable bone defects in a rat model *in vivo*, in combination with gelatin hydrogel, which had successfully assisted CCN2 in regenerating articular cartilage in our previous study.

MATERIALS AND METHODS

Preparation of recombinant CCN2

Two different types of recombinant CCN2 proteins were prepared. One was an affinity-purified human CCN2 from a stable HeLa cell clone that had been transformed by a CCN2 overexpression plasmid, as described previously.[26] The other was a commercially available (Biovender, Heidelberg, Germany) *Escherichia coli*–produced CCN2 protein. These two proteins were functionally characterized *in vitro*, to select the most efficient one to be used in subsequent *in vivo* experiments.

Preparation of gelatin hydrogel

Gelatin with a molecular weight of 99,000 and an iso-electrical point (pi) of 5.0 was prepared by alkalinization followed by thermal denaturation of bovine bone (kindly supplied by Nitta Gelatin, Osaka, Japan). Gelatin hydrogel microspheres were prepared by cross-linking acidic gelatin with glutaraldehyde, as described previously.[10] The resultant hydrogel was composed of 95% H_2O and 5% gelatin microspheres of 30–37 μm.

In vitro *CCN2 retention studies*

To verify the incorporation of the CCN2 into the gelatin hydrogel, 0.5 μg of HeLa CCN2 was mixed with 1 mg of the gelatin hydrogel in 10 μL of phosphate-buffered saline (PBS) and was incubated at 25°C for 1 h. Afterward, the hydrogel–CCN2 complex was suspended in 100 μL of PBS and immediately recollected by centrifugation. Then, CCN2 retained in, or immediately released from the hydrogel was analyzed by Western blotting analysis with an anti-human CCN2 monoclonal antibody, as described previously.[31] Similarly, the hydrogel–CCN2 complex was further incubated in PBS at 37°C for 3, 7, or 14 days, and the hydrogel was thereafter recollected by centrifugation. CCN2 retained in the hydrogel was extracted by a lysis buffer,[31] and was quantified by an established ELISA system.[31]

In vivo *CCN2 release studies*

For the evaluation of the gradual release of CCN2 *in vivo* (Fig. 2), ^{125}I-labeled CCN2 (160,000 cpm) was prepared and incorporated as described previously[34] into the gelatin hydrogel, and the gel (without a scaffold) was then implanted into the back subcutis of 6-week-old female ddY mice (Shimizu Laboratory Supply, Kyoto, Japan) at the midline 15 mm from the tail root, following an established protocol.[10] At selected time points, the skin on the back of

mice including the remaining gel was excised as a 3×5 cm strip, after which the radioactivity was measured with a gamma counter (Aloka, Tokyo, Japan) to assess the retention profile of CCN2 *in vivo*, as previously described.[35] The experimental group comprised three mice unless otherwise stated.

Preparation of CCN2–gelatin hydrogel–collagen complex for in vivo bone regeneration

CCN2 was absorbed into the gelatin hydrogel by mixing 1 mg of lyophilized gelatin hydrogel microspheres with 0 (control), 0.5, or 1 μg of CCN2 in 10 μL of a PBS, which was then incubated at 25°C for 1 h. The CCN2–gelatin hydrogel–collagen sponge composite was prepared by lyophilizing the mixture of CCN2 (0.5 or 1 μg), gelatin hydrogel (1 mg) and an acidic collagen solution (100 μL: I-PC containing type I athelocollagen from *Bovine* dermis; Koken, Tokyo, Japan).

Cell culture

Normal osteoblasts were obtained from the calvaria of mice and seeded into wells of a 48-well plate at a density of 20,000 per cm^2 in Dulbecco's modified Eagle's minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). After 2 days, the cells were replenished with D-MEM with 0.5% FBS for 24 h and then treated with 50 ng/mL of CCN2 or an equal volume of PBS (control) for another 24 h in the same medium for the comparative evaluation of the metabolic activity.

Determination of cell metabolic activity

The metabolic activity of the mouse osteoblasts was evaluated by the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay, as previously described.[36] Briefly, 50 μL of 0.5% MTT in distilled water was added to 500 μL of medium in each well. After incubation for 4 h at 37°C, the cells were lysed in 500 μL of 0.04 M hydrochloric acid 2-propanol. Then, the absorbance of the lysate was measured at a wavelength of 570 nm (excitation: 630 nm).

In vivo implantation studies

Bone regeneration was evaluated by using the established rat model described previously.[37] Briefly, 11-week-old Wistar rats were anesthetized with an intraperitoneal injection of Nembutal at a dose of 40 mg/kg body weight. After a parapatellar incision had been made, the articular part of the femur was exposed, and a bone defect was prepared in the left distal femur at the medial condyle by drilling a channel of 2.8 mm in diameter with a drill bit. Immediately after the preparation of the bone defect, the CCN2–gelatin hydrogel–collagen sponge composite was applied to each defect. The femur was repositioned, and the

incision was closed with nylon sutures. After the operation, the animals were fed *ad libitum* and allowed to move around freely in their cages. One, 2, or 3 weeks after the operation, the rats were deeply anesthetized with an intraperitoneal injection of ketaral (100 mg/kg body weight) and processed for sampling. Four animals were prepared, processed, and analyzed in each group in all of the experiments. The Animal Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences approved all of these experimental procedures.

RNA extraction and real-time reverse transcription–mediated polymerase chain reaction analysis

One and 2 weeks after the operation, tissues including the operated area in the center were dissected, isolated, and subjected to total RNA extraction. RNA extraction was performed by the acid guanidium phenol-chloroform method using a commercially available reagent (ISOGEN; Nippon Gene, Tokyo, Japan). Total RNA was then reverse-transcribed to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase with oligo d(T) as a primer, which was supplied as a kit package (RNA PCR kit; Takara, Tokyo, Japan), following exactly the manufacturer's protocol. Quantitative real-time PCR was carried out by using a LightCycler system (v1.5; Roche, Basel, Switzerland) with LC DNA Master SYBR Green I (Roche). Primers used for the amplification of each cDNA were as follows: osteocalcin sense, 5′-agctcaaccccaattgtgac-3′, and antisense, 5′-agct caaccccaattgtgac-3′; alkaline phosphatase sense, 5′-gagcag gaacagaagtttgc-3′, and antisense, 5′-gttgcagggtctggagagta-3′; glyceraldehyde 3-phosphate dehydrogenase sense, 5′-catc aagaaggtggtgaagcag-3′, and antisense, 5′-cgtcaaaggtggagg agtgg-3′. PCR reactions consisted of 5 min of constant heating at 95°C, followed by 50 cycles of amplification comprising 10 s of denaturation at 95°C, 10 s of annealing at 60°C, and 10 s of extension at 72°C, followed by a melting curve analysis to confirm the formation of the proper PCR product, as described previously.[38]

Histological examination and quantification of regenerated bone

After the operation, bone regeneration was monitored along a time course by X-ray radiography. Animals were processed for histological analysis at 3 weeks after the operation, when bone regeneration became evident in the CCN2-treated groups by X-ray examination. At the day of sampling, the rats were anesthetized and perfused with 4% paraformaldehyde, and the femur was then dissected and further fixed overnight at 4°C in the same solution. After having been decalcified with 10% ethylene-diamine tetraacetate (EDTA) for 2 weeks, the samples were embedded in paraffin according to an established procedure.[30] Paraffin sections were prepared from these samples, deparaffinized

with xylene, rehydrated stepwise, and washed with PBS. The sections (6 μm in thickness) were stained with hematoxylin and eosin by a standard protocol, and examined under a light microscope.

Quantification of the regenerated bone was performed by examining the images of the stained sections. Total area of regenerated bone in the region of the defect prepared was computed by using version 1.62 of NIH image public domain software. With the data obtained, the bone regeneration index was calculated as the percentage of the area occupied by the regenerated bone against the whole area evaluated.

Statistical analysis

The results obtained from quantitative experiments were reported as the mean values ± standard deviation (SD). Statistical comparisons between the groups were performed by using the nonparametric one-way analysis of variance (ANOVA) and Tukey test at 5% significance ($p < 0.05$) with StatView software for Windows, version 5.0 (SAS Institute, Cary, NC).

RESULTS

Comparative analysis of the effect of human recombinant CCN2s produced by mammalian cells and E. coli on the metabolic activity of osteoblasts in vitro

It was previously reported that CCN2 promotes both proliferation and differentiation of osteoblastic cells *in vitro*. Therefore, to obtain best results by using the better material *in vivo*, we initially evaluated the effects of the two recombinant CCN2s (rCCN2s) currently available on the metabolic activity of mouse primary osteoblasts *in vitro*. One was a commercially obtained recombinant human CCN2 produced by *E. coli*, and the other was human CCN2 produced by a HeLa cell clone that had been transduced with a mammalian expression vector of human CCN2 and purified through two steps of affinity chromatography, as previously described.[16] Osteoblasts were cultured with either protein, and their metabolic activity was monitored by conducting the MTT assay. Consequently, both CCN2s were capable of promoting the survival and metabolism of osteoblasts (Fig. 1); however, the HeLa-derived one was more potent. Therefore, we selected the HeLa-derived rCCN2 for the subsequent evaluation with gelatin hydrogel *in vivo*.

Gradual and sustained release of CCN2 by gelatin hydrogel in vivo

The gelatin hydrogel has been shown to be effective in realizing a prolonged supply of certain growth factors when implanted in tissues *in vivo*. In our previous work, we showed that CCN2 can be efficiently incorporated into the
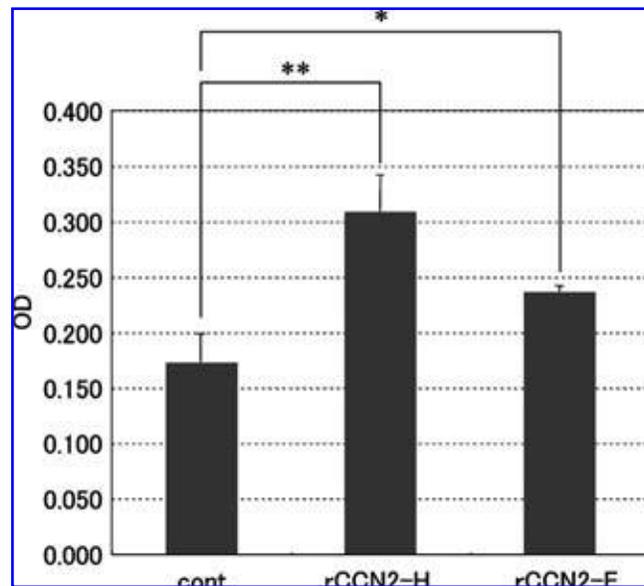
FIG. 1. Effect of the two rCCN2 proteins on the proliferation of mouse osteoblasts in primary culture. The cells were treated with 50 ng/mL of a HeLa-derived human rCCN2 (rCCN2-H) or an *E. coli*–derived human rCCN2 (rCCN2-E) for 5 h, and their survival and metabolism were then evaluated by conducting the MTT assay. Results are presented as the mean values of four sets of evaluations with standard deviations (SDs). Statistically significant differences (*$p < 0.05$ and **$p < 0.01$) between two experimental groups are indicated by the brackets.

hydrogel by absorption without allowing immediate diffusion out of the hydrogel.[16] First of all, we experimentally confirmed the efficient incorporation and gradual release of the CCN2 in combination with the gelatin hydrogel *in vitro*. As previously described, CCN2 was successfully incorporated into the gelatin hydrogel and was retained in the hydrogel even after 14 days of incubation in PBS at 37°C (Fig. 2A). To confirm that CCN2 absorbed to the gelatin hydrogel was also released gradually during degradation of the hydrogel *in vivo*, we prepared radiolabeled HeLa-derived recombinant CCN2 and absorbed it to the gelatin hydrogel, after which the complex was implanted subcutaneously into the dorsal skin of mice, according to an established prodedure.[10] Quantitative evaluation of the radioactive CCN2 remaining at the implant site up to 2 weeks after the impregnation indicated that at least more than 30% of the CCN2 was still present in the hydrogel at 3 days after the implantation (Fig. 2). Further, even after 2 weeks, implanted CCN2 was still detectable in the local area around the implanted gel suggesting that CCN2 was continuously supplied from the gelatin hydrogel complex as long as 14 days by a single local administration. Therefore, although the speed of CCN2 release was observed to be slightly faster than the disintegration speed of the hydrogel *in vivo*,[10] the combination of CCN2 and gelatin hydrogel was proven to be effective in enabling a sustained supply of this factor
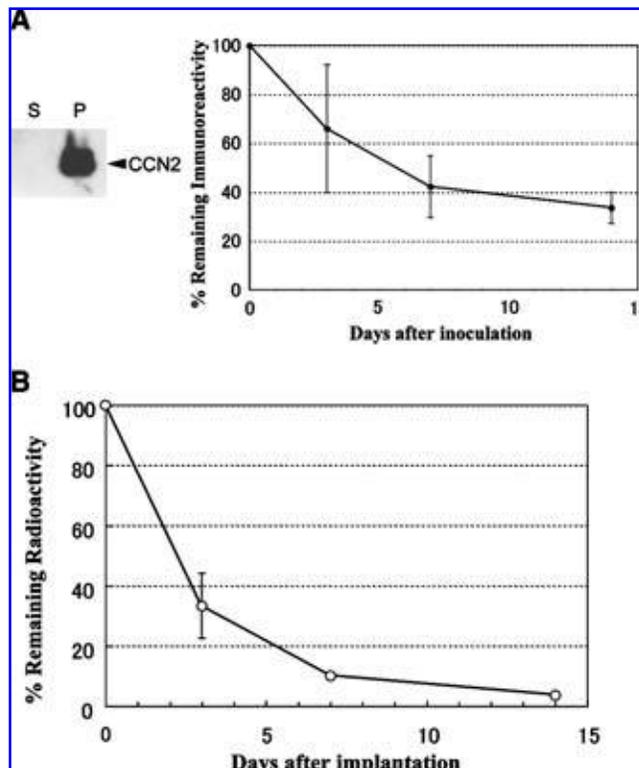
FIG. 2. (**A**) Retention of the rCCN2 in the gelatin hydrogel *in vitro*. Efficient incorporation of the HeLa-derived rCCN2 in the gelatin hydrogel was confirmed by Western blotting of the pellet (P) and supernatant (S) fractions of the gelatin hydrogel in PBS immediately after suspension (left panel). The gelatin hydrogel–rCCN2 complex was further incubated in PBS at 37°C for the indicated time periods, and the CCN2 retained in the hydrogel was quantified by ELISA. Amount of the CCN2 retained is represented as a percentage versus the original incorporated amount at time 0. Data represent the results of three independent experiments. Mean values are shown with error bars of SDs. (**B**) Retention of the rCCN2 in the gelatin hydrogel in the back subcutis of mice. After the implantation of the radiolabeled rCCN2 adsorbed onto the gelatin hydrogel, the tissue surrounding the implanted gel was collected, and the time course of loss of radioactivity was determined. Radioactivities in the collected samples were counted, and the relative amounts of remaining rCCN2 were computed as percentages of the collected radioactivity versus the initial total radioactivity in the implant. Data represent the results of the experiments with three animals for each group. Mean values are shown with error bars indicating SDs.

around the local region of implantation for tissue regeneration *in vivo*.

Effects of the CCN2-absorbed gelatin hydrogel on osteoblastic differentiation of the cells in the regenerating bone defect *in vivo*

To evaluate the effect of the rCCN2–gelatin hydrogel complex on bone regeneration, we prepared an intractable

bone defect model by following the methodology described in a previous study.[37] In that study, the effect of TGF-β on bone regeneration was examined, but no significant effect was found. According to the same methodology, we prepared bone defects in rat femur, as illustrated in Figure 3A, and implanted the gelatin hydrogel containing PBS only (control) or 0.5 μg of the rCCN2, which was built in a collagen sponge scaffold. After 1 and 2 weeks, the tissue in the original defect was sampled for RNA analysis. Quantitative real-time RT-PCR evaluation revealed that gene expression of an osteoblastic differentiation marker gene, osteocalcin (*ocn*), was remarkably elevated by the application of the rCCN2–gelatin hydrogel complex (Fig. 3B). Another such gene, the mineralization-associated alkaline phosphatase gene (*alp*), appeared to be induced by rCCN2 as well, although the increase in its expression was not statistically significant (Fig. 3C). Therefore, the rCCN2–gelatin hydrogel complex was effective in promoting osteogenesis by acting on either osteoblasts or bone marrow mesenchymal stem cells or both, as both are targets of CCN2, through the gradual release of rCCN2 as suggested by the data shown in Figure 2.

Enhanced regeneration of the bone defect by the CCN2-absorbed gelatin hydrogel *in vivo*

Utilizing the same bone defect model as employed above, next we evaluated the effect of the CCN2-absorbed gelatin hydrogel on bone regeneration. After preparation of the bone defect, gelatin hydrogel that had been incubated with PBS only (control), 0.5 μg of rCCN2, or 1 μg of rCCN2 and built in a collagen sponge was impregnated therein, and the time course of bone regeneration was initially monitored by X-ray radiomicrography. Since regenerated bone was clearly observed at 3 weeks after the operation, animals were processed and the sections of the corresponding areas were analyzed at this time point. Histological examination of the sections clearly showed that both 0.5 and 1 μg of rCCN2 incorporated into the gelatin hydrogel clearly promoted bone regeneration, which resulted in almost complete repair of the cortical bone removed by the operation (Fig. 4). Of note, woven bone formation indicating active bone regeneration was clearly observed only with rCCN2 along the cortical bone–bone marrow boundary (Fig. 4E). In contrast, control group showed only partial repair of the bone defect with sparse bone trabeculae at the same time point (Fig. 4A). In these cases, fibrous structure that is supposed to be the remnant of the collagen scaffold can be observed at the area without efficient bone formation (Fig. 4B). Further, quantitative analysis of the histological sections revealed significant acceleration of bone regeneration by rCCN2 (Fig. 5). The effects obtained with 0.5 and 1 μg of rCCN2 were comparable without statistical significance, suggesting that 0.5 μg was an optimal dose to yield a maximal effect in promoting the regeneration of bone in a defect of this size and location. According to previous
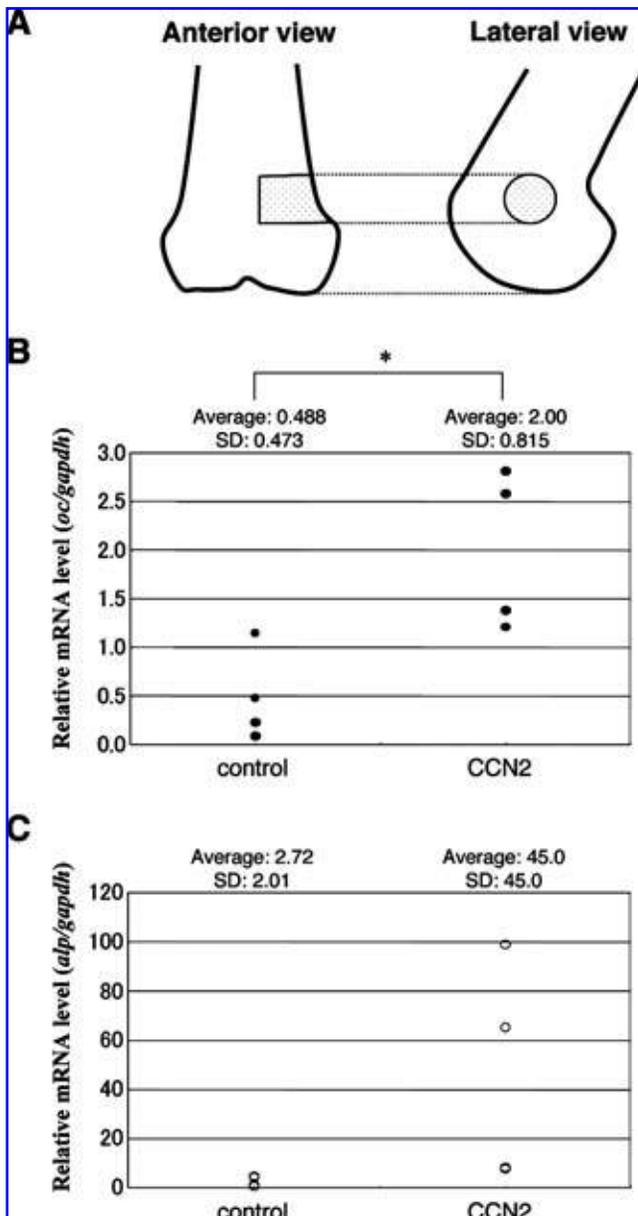
FIG. 3. (**A**) Schematic representation of the bone defect prepared in the rat model. The cortical bone (2.8 mm in diameter) of the operated area was completely removed by drilling as illustrated, and the gelatin hydrogel complex was implanted therein. (**B, C**) Effect of the gelatin hydrogel–absorbed CCN2 built in a collagen sponge on the expression of osteocalcin and alkaline phosphatase genes. One or 2 weeks after the application of the hydrogel complex, RNA was extracted from the regenerating tissues, including the operated areas, and subjected to quantitative real-time PCR evaluation. Four animals were analyzed per group. Data are presented as relative mRNA levels against those of the *gapdh* gene and are plotted independently, with the average and SD values indicated for each group. A statistically significant difference (*$p < 0.05$) between the groups is indicated by the bracket.

reports, the dose dependence of CCN2 in both chondrocytic and osteoblastic cells *in vitro* was found to be comparable (Table 1: 50 ng/mL was sufficient to yield maximal effects). However, an *in vivo* study has shown that higher dose is required for cartilage regeneration than the bone regeneration performed in this study (Table 1). This *in vitro–in vivo* discrepancy in CCN2 dose dependence also emphasizes the utility of this drug delivery system, particularly in the regeneration of this type of bone defects. Together with the cell biological effects illustrated in Figure 3, these results strongly indicate the efficacy of rCCN2, in combination with gelatin hydrogel, in promoting the regeneration of bone in intractable bone defects.

DISCUSSION

We initiated this study by reevaluating the biological activity of our original rCCN2 derived from a HeLa transformant in comparison with that of a commercially available one to obtain the best results *in vivo*. After confirming the better effect of our rCCN2 on osteoblast survival and metabolism, we next examined if the combination of the gelatin hydrogel and rCCN2 was suitable for the sustained administration of rCCN2 by local application *in vivo*. Since gradual release of rCCN2 was confirmed, we finally applied the CCN2–gelatin hydrogel complex to an intractable bone defect model prepared in a rat femur. As a result of the application of 0.5–1 μg of rCCN2 incorporated into the gelatin hydrogel with a collagen sponge scaffold, regeneration of the bone defect was markedly accelerated, which was preceded by the induction of several genes that were critical for osteoblast differentiation, mineralization, and bone remodeling. These results strongly indicate the utility of gelatin hydrogel–absorbed CCN2 as a therapeutic for the treatment of intractable bone defects.

The difference in the biological activity observed *in vitro* between the two recombinant proteins would supposedly be due to the organism that produced these proteins. In mammalian cells, proteins can be posttranslationally modified in the endoplasmic reticulum and Golgi apparatus; this modification obviously results in molecules structurally different from those produced by prokaryotes. Indeed, in the case of mammalian cell–derived CCN2, N-linked glycosylation occurs at two asparagine residues, whereas this cannot occur in the *E. coli*–derived one.[39] Together with other possible side chain modifications, such glycosylation may well result in a tertiary structure different from that of *E. coli*–derived CCN2. However, in this study, we evaluated the CCN2 activity only in terms of osteoblast survival and metabolism. It should be noted that CCN2 is a multifunctional protein with multiple targets. Therefore, even if *E. coli*–derived CCN2 is not active enough in this particular situation, it can be as active as a mammalian cell–derived CCN2 in other functional aspects. Further analysis with
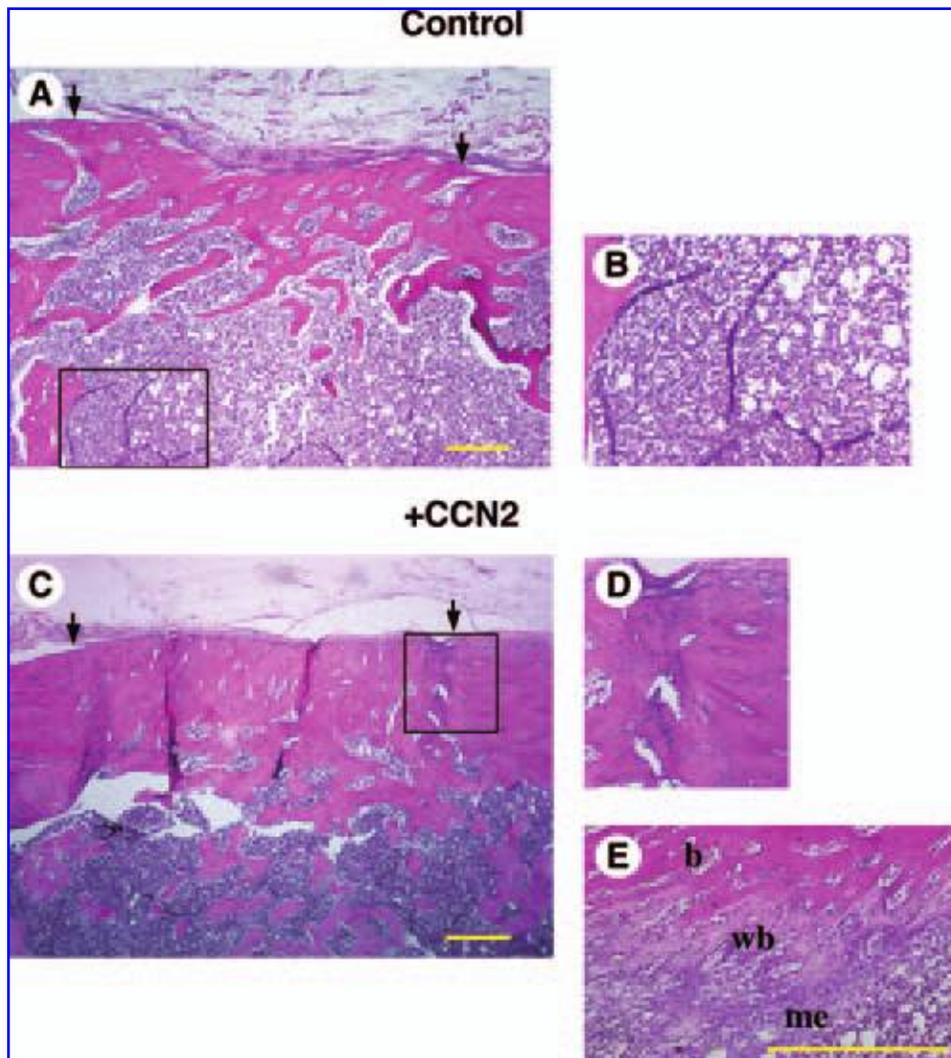
FIG. 4. Typical histological findings observed in the bone defect area treated with (+CCN2) or not (Control) in combination with gelatin hydrogel and collagen scaffold 3 weeks after the operation. Microscopic views of hematoxylin–eosin staining of sections representing the groups without (**A, B**) and with (**C–E**) CCN2 are displayed. Approximate position of the edges of the operated area is indicated by arrows. A higher magnification of the area specified in (**A**) and (**C**) in a rectangle is shown as (**B**) and (**D**), respectively. Atypical structure supposed to be the remnant of collagen scaffold is visible in the control group (**A, B**). Woven bone formation (wb) was clearly observed at the bone (b)–mesenchymal stromal tissue (me) boundary in the CCN2-treated groups (**E**). Scale bars: 400 μm. Regeneration of cortical bone is clearly observed only in the group treated with the CCN2–gelatin hydrogel–collagen sponge composite (**C–E**). Color images available online at www.liebertpub.com/ten.

different bioassay systems will provide an answer for this question, which is in progress.

As revealed in Figure 4, the cortical bone that regenerated with the assistance of CCN2 appeared to be quite natural, without a sign of overgrowth or atypical histological findings that are not usually observed in normal tissues. The regenerated cortical bone was connected seamlessly to the original bone cortex, as the border between the two can be noticed only by careful observation. Of note, it was previously shown that an intractable defect in articular cartilage could be also repaired in such an ideal manner by the same CCN2–gelatin hydrogel complex.[16] In either case,

it is critically important for the regenerative therapy to strictly induce cartilage/bone formation up to the necessary and sufficient level, avoiding any overcorrection that may cause dysfunction. Nevertheless, other bone-inducing growth factors are sometimes difficult to control to obtain a desired outcome, which is partly because of their direct and strong stimulatory effect toward bone regeneration. In contrast, CCN2 realized the bone repair exactly at the desired level without strict control of the dose. This novel action of CCN2 is probably based on the functional property of CCN2 as a conductor/modulator of the local extracellular signaling network. Instead of executing a specific
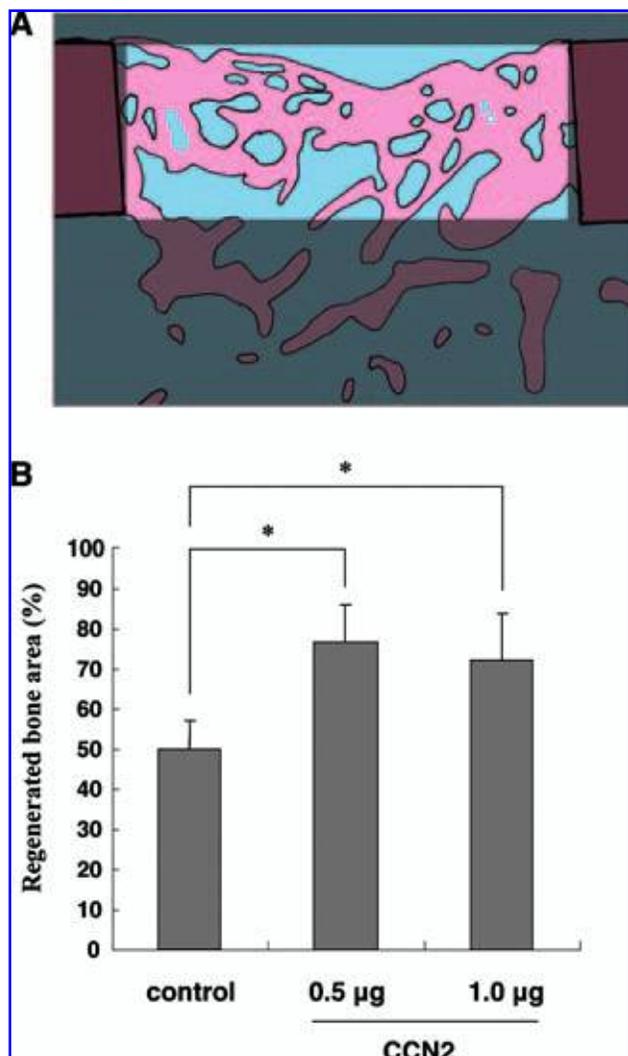
FIG. 5. Quantitative analysis of the regenerated bone in the bone defect area at 3 weeks after the operation. (**A**) Schematic representation of the histological area subjected to quantitative analysis. A maximum rectangle area within the original defect was selected, and both cortical and trabecular bone regions (shown in pink) were integrated to represent the total quantity of regenerated bone. (**B**) Results of the quantitative analysis. Four animals of each group were treated with a gelatin hydrogel/collagen sponge with or without CCN2, and were analyzed. Asterisks denote significant differences (*$p < 0.05$) between the two groups, as indicated by the brackets. Color images available online at www.liebertpub.com/ten.

mission through a specific receptor, CCN2 controls the overall status of extracellular signalings by interacting with a number of growth factors and extracellular matrix (ECM) components involved in osteogenesis, such as TGF-β, BMP-4, proteoglycans, and integrins.[17,19,22] Thus, the biological effects yielded by CCN2 is a net output from those growth factors and other molecules manipulated by the four modules of CCN2, rather than a direct and distinct effect of CCN2 *per se*. As a consequence, bone regeneration is promoted in a harmonized manner by CCN2 without atypical tissue formation or overgrowth. The property of CCN2 as a natural tissue-remodeling molecule is also indicated by the fact that this factor is abundantly released from platelets upon activation.[31,32]

From a cell biological point of view, the effect of CCN2 to promote harmonized regeneration of bone tissue can be also interpreted as an outcome of simultaneous enhancement of the growth and differentiation of most of the cell types involved. The major producer of bone matrix is the osteoblast; however, osteoblasts originate in mesenchymal stem cells in bone marrow. Needless to say, proper structure and remodeling potential of regenerated bone require the invasion by a newly formed microvasculature. In the case of bone fracture or large bone defects, chondrocytes temporarily appear, and the endochondral ossification process recurs to repair the defect.[30] According to previous reports, all of the cells mentioned above are targets of CCN2. Indeed, CCN2 promotes adhesion and migration of human bone marrow stem cells; the proliferation, differentiation, and mineralization of osteoblasts and growth plate chondrocytes; and the migration and proliferation of vascular endothelial cells *in vitro*.[25–29] On the other hand, we recently found that CCN2 promoted the formation of osteoclasts that are engaged in bone resorption (unpublished data). In fact, pathogenic role of CCN2 in osteolytic metastasis of breast cancer cells has been indicated.[40] However, bone resorption is rather an essential step in the remodeling of bone tissue during regeneration. In this way, since all of the key cellular players in bone formation/remodeling are together encouraged by CCN2 to regenerate bone defects, the regeneration process follows the physiological way of osteogenesis, which we consider is the cell biological background of the observed effects of CCN2.

The gelatin hydrogel–absorbed CCN2 could successfully promote the regeneration of bone in a relatively intractable

TABLE 1. DOSE DEPENDENCE OF THE CCN2 EFFECTS ON CARTILAGE AND BONE REGENERATION *IN VITRO* AND *IN VIVO*

Target cell/tissue	CCN2 dose in vitro (ng/mL)			CCN2 dose in vivo (µg/lesion)		
	25	50	100	0.5	1	1.5
Osteoblastic cell/bone	+	++	++	++	++	ND
Chondrocytic cell/articular cartilage	+	++	++	±	+	++

The data with osteoblastic cells and chondrocytic cells *in vitro* are from Nishida *et al.*[27] and Nakanishi *et al.*,[25] respectively. The *in vivo* data of bone regeneration are described in the present work, whereas those of cartilage regeneration are from Nishida *et al.*[16] ND: not determined.

defect of normal bone tissue to the exactly desired level. However, it may not be effective enough to regenerate more intractable cases with basal low regenerative potential of the tissue, which can occur under systemic and local pathological conditions. To obtain satisfactory results with such difficult cases, additional material may need to be combined with the present gelatin hydrogel–CCN2 complex. In our hands, the combination of CCN2 and a porous hydroxyapatite scaffold was earlier indicated to be effective for the repair of larger bone defects *in vivo*.[29] Also, it should be noted that CCN2 is a conductor/modulator of extracellular signaling networks to harmonize the effects of various factors involved in the microenvironment. If so, any other growth factor may be combined with CCN2 upon its application for tissue regeneration to control the mode of the action of the growth factor at a physiologically adequate level. Therefore, the utility of CCN2 as a second factor to be used in combination with a certain growth factor is strongly suggested, which would be expected to regenerate bone defects with pathological complexities, for example, as those in diabetes mellitus and postmenopausal osteoporosis.

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