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Abundant Retention and Release of Connective Tissue Growth Factor (CTGF/CCN2) by Platelets

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Wound healing and tissue regeneration are usually initiated by coagulation followed by fibrous tissue formation. In the present study, we discovered an abundance of connective tissue growth factor (CTGF/CCN2) in human platelets, which was released along with the coagulation process. The CTGF/CCN2 content in platelets was 10-fold higher than that in arterial tissue. Furthermore, the CTGF/CCN2 content in a single platelet was computed to be more than 20-fold higher than that of any other growth factor reported. Considering that CTGF/CCN2 promotes angiogenesis, cartilage regeneration, fibrosis and platelet adhesion, it may be now regarded as one of the major functional components of platelets.

Key words: CCN2, CTGF, platelet, tissue regeneration, wound healing.

Platelets play central roles in hemostasis, clot formation, and the wound healing process. After adhesion to injured areas, platelets release their endogenous growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), and interleukins (*1*, *2*). Since these factors have been thought to participate in the subsequent granulation tissue formation which leads to wound healing and tissue repair, the local application of platelet-rich plasma (PRP) to the surgical area after an operation has been explored and has yielded significant effects (*3*).

Connective tissue growth factor (CTGF/CCN2) (*4*) is a multifunctional modulator molecule that is now classified as the second member of the CCN family (*5–8*). CTGF/CCN2 is composed of four conserved modules, by which it modulates the effects of a variety of cytokines and exerts pleurotropic functions. It is of note that it not only mediates the adhesion of platelets and monocytes (*9*, *10*), and is involved in wound healing and fibrosis, but also promotes the growth and differentiation of vascular endothelial cells (*11*), osteoblasts (*12*), and chondrocytes (*13*) *in vitro* as well as angiogenesis *in vivo* (*11*). Furthermore, a recent report described distinct effects of locally applied CTGF/CCN2 on cartilage regeneration that would not be expected under natural conditions (*14*). As such, CTGF/CCN2 may be regarded as a central driver of wound healing and regeneration of connective tissues (*15*). Previously, we had established (*16*) and have improved enzyme-linked immunosorbent assay (ELISA) systems for the quantitative analysis of CTGF/CCN2. Utilizing one of the improved systems, we found the presence of a remarkably high level of this factor in platelets, and compared the amount with those in other tissues and those of other growth factors in platelets quantitatively.

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In evaluating the utility of the ELISA system for the determination of serum CTGF/CCN2 levels, we noticed an unignorable effect of the procedure used to draw the blood on the outcome of the ELISA. As such, we examined the effect of the time period between blood sampling and plasma or serum separation on the level of the factor determined. Total blood was drawn from healthy donors and left at room temperature for the desired time periods to obtain serum. Other blood samples were drawn into commercially available vacuum sampling tubes (Venoject II: Terumo, Tokyo, Japan) containing a clinical dose of either sodium citrate or di-sodium ethylenediamine tetraacetic acid (EDTA-2Na) as an anticoagulant, following the manufacturer's instructions. After the incubation, each blood sample was centrifuged to remove the cellular fraction, and then the plasma/serum was directly used for CTGF/CCN2 quantification. The level of CTGF/CCN2 in each sample was determined by means of a sandwich ELISA system using two different anti-human CTGF monoclonal antibodies (mAb 8–64 and 8–86). The ELISA system utilized was an improved one developed from a previously-reported system (*16*). During incubation at 37°C for 2 h, CTGF/CCN2 in the samples was captured on ELISA strips pre-coated with 8–64 mAb. After washing, horseradish peroxidase-conjugated 8–86 mAb was added and incubation was carried out for 1 h at 37°C. Signals were developed with tetramethylbenzidine (TMB). Surprisingly, CTGF/CCN2 detected in the serum continuously increased along with the time course up to 160 min (Fig. 1A). Indeed, the concentration became 2-fold higher than the dose at which CTGF/CCN2 exerted its maximal stimulatory effects on the growth and differentiation of osteoblastic cells and chondrocytes (*12*, *13*). Moreover, the observed release of CTGF/CCN2 was completely blocked by the addition of sodium citrate or EDTA to the blood (Fig. 1). These results clearly indicate that CTGF/CCN2 was released or produced during or at a certain step(s) of the blood coagulation cascade.

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Suspecting platelets were the source of the CTGF/CCN2 during coagulation, we performed Western blotting analysis of platelet lysates with CTGF/CCN2–specific antibodies. Two module-specific antibodies were utilized for the strict identification of CTGF/CCN2. A von Willebrand factor type C-repeat (VWC)–specific rabbit antiserum, anti-CTGFw, and a C-terminal cysteine-knot (CT)–specific humanized monoclonal antibody, JMAb25, were used as primary antibodies, as described previously (*17*, *18*). Briefly, platelets (1×10^7) were collected by centrifugation and then lysed in 10 µl of 1× sodium dodecyl sulfate (SDS) sample buffer containing 2.5% 2-mercaptoethanol. The lysate was electrophoresed in a 15–25% SDS-polyacrylamide gel. The separated proteins were transferred to an Immobilon transfer membrane, and then incubated with a primary antibody and further incubated with a horseradish peroxide–conjugated secondary antibody against human or rabbit IgG, as described previously (*18*). Positive signals were visualized by an enhanced chemiluminescence method, utilizing an experimental kit (Renaissence, DuPont/NEN, Wilmington, DE, USA). As expected, CTGF/CCN2 molecules were detected in the platelet extract (Fig. 2). Indeed, the antibody and antiserum recognizing independent epitopes in different modules precisely indicated the same 36–38 kDa protein signals in normal platelet lysates. It is also of note that 10–13 kDa and 20–25 kDa fragments were detected with the antiserum against VWC and the antibody against CT, respectively, since certain biological functions of such fragments were previously suggested (*19*, *20*).

During the publication of this work, Cicha *et al.* reported the release of CTGF/CCN2 upon platelet activation, which was determined only by Western blotting with a single anti-CTGF antibody (*21*). We confirmed this finding more strictly by using two different antibodies. Furthermore, in order to gain more insights, we com-

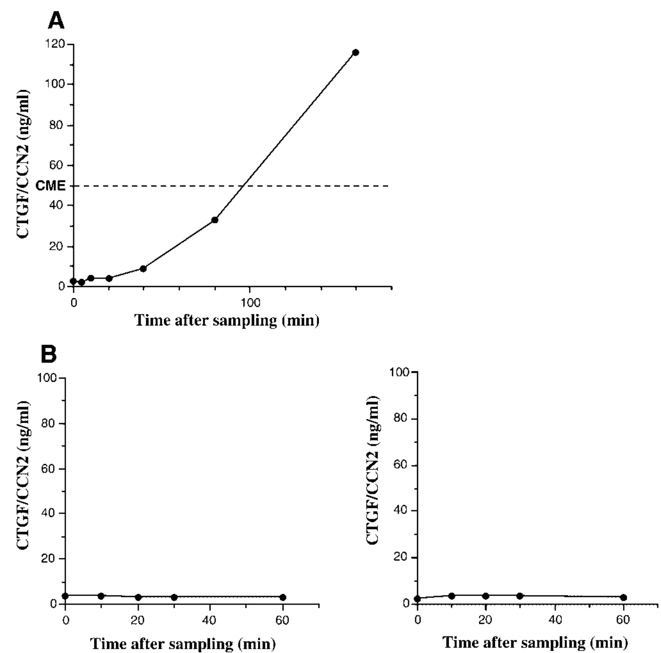

Fig. 1. **CTGF/CCN2 released along with blood coagulation.** A: CTGF/CCN2 concentrations detected in serum isolated from total blood after incubation at room temperature for the indicated time periods. CTGF/CCN2 concentrations were determined with an ELISA system. The concentration having maximal stimulatory effects on the growth and differentiation of chondrocytes and osteoblastic cells is indicated (CME). B: Effects of anticoagulants on the observed release of CTGF/CCN2 in plasma. The clot formation cascade was inhibited with either EDTA (left panel) or sodium citrate (right panel), and the CTGF/CCN2 content in the plasma was determined. These results are representative of those obtained for several independent samples.

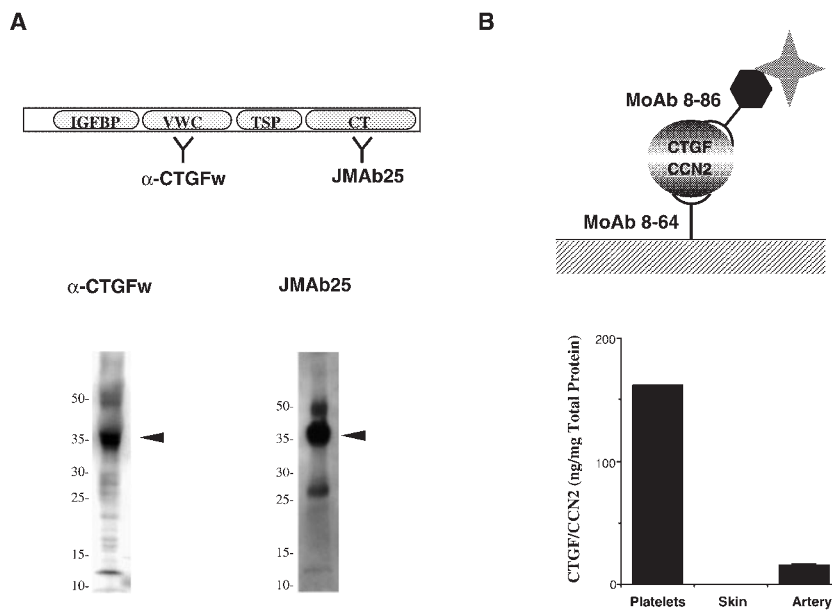

Fig. 2. **CTGF/CCN2 retained in platelets.** A: Upper panel: Modular structure of CTGF/CCN2 and the location of the epitopes of the antibodies used for Western blotting. IGFBP, insulin-like growth factor binding protein module; VWC, von Willebrand factor type C repeat module; TSP, thrombospondin type I repeat module; CT, C-terminal cysteine-knot module. The modules recognized by the two antibodies, α-CTGFw and JMAb25, are specified. Lower panel: Western blotting analysis of a platelet lysate with the anti-CTGF/CCN2 serum and monoclonal antibody. Major signals representing full-length CTGF/CCN2 are indicated by the arrowhead in each panel. B: Upper panel: Strategy for quantitative detection of CTGF/CCN2 with the ELISA system. The components of the sandwich-ELISA system are schematically illustrated. Note that the two monoclonal antibodies used in this system were different from those shown in A. The secondary antibody was conjugated with horseradish peroxidase (solid hexagon) to enable color development for quantification. Lower panel: Comparison of the CTGF/CCN2 contents of platelets and other CTGF/CCN2-associated tissues. Total protein extracts of normal human skin and artery were obtained commercially (Cosmo Bio Co., Ltd., Tokyo, Japan), as were human platelets from a healthy donor (AllCells, LLC, Berkeley, CA). CTGF/CCN2 was quantified with the ELISA system, and the data were standardized against each total protein level.

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pared the CTGF/CCN2 content in platelets with those in other tissues by quantitative analysis with our sandwich ELISA system. In skin tissue, CTGF/CCN2 is produced upon wounding, whereas blood vessel cells are known to produce CTGF/CCN2 under physiological conditions (*22*). As expected, no detectable CTGF/CCN2 was present in the skin protein extract, whereas a significant level of it was detected in the arterial protein extract, which served as a positive control level. Surprisingly, the platelet-retained CTGF/CCN2 level was far higher than the positive control. Based on the quantitative data, we calculated the content of CTGF/CCN2 in a single platelet. As a result, a single platelet was found to contain 2.5 fg of CTGF/CCN2. In comparison with the data presented in a previous report, the CTGF/CCN2 content appeared to be more than 20-times higher than that of any other growth factor examined, such as TGF-β (0.12 fg), IGF-I (0.06 fg), and PDGF-AB (0.08 fg) (*23*). The observed abundance of this factor in platelets strongly suggests its specifically-assigned critical functions therein.

As to the specific functions of platelet-derived CTGF/CCN2, earlier studies showed that CTGF/CCN2 gene expression was induced upon wounding in fibroblasts (*15*). This finding is of critical importance, since CTGF/CCN2 is believed to promote the regeneration of connective tissues including skin, cartilage and bone. However, even if the gene expression is stimulated by injury, production and accumulation of this matrix-associated growth factor requires a significant period of time. Indeed, upon tooth extraction in rats, CTGF/CCN2 production was evidently not observed in granulation tissues until 4 d after the operation (*24*). In light of the above findings together with those in this study, putative roles of CTGF/CCN2 in early stages of tissue regeneration are suggested. Immediately after injury, the initial aggregation of platelets results in CTGF/CCN2 release to provoke further platelet aggregation that realizes a large-scale rapid supply of exogenous CTGF/CCN2. Thereafter, accumulation of platelet-derived CTGF/CCN2 triggers the formation of granulation tissue. After that, endogenous CTGF/CCN2 is increasingly produced to take the place of the exogenous factor until its roles in tissue regeneration have been accomplished (*15*).

The effect of the addition of fresh platelets on tissue regeneration has been examined and positive effects have been confirmed clinically (*3*). According to the present study, such effects of platelets may be ascribed to the CTGF/CCN2 released from them. The regenerative potential of CTGF/CCN2 has already been confirmed in articular cartilage (*14*). Currently, the utility of CTGF/CCN2 in bone regeneration, and in osseointegration between bone and artificial biomaterials is being examined as well.

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