Physical interaction of CCN2 with diverse growth factors involved in chondrocyte differentiation during endochondral ossification

Hany Mohamed Khattab¹ · Eriko Aoyama¹ · Satoshi Kubota^{1,2} · Masaharu Takigawa¹

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Abstract CCN family member 2 (CCN2) has been shown to promote the proliferation and differentiation of chondrocytes, osteoblasts, osteoclasts, and vascular endothelial cells. In addition, a number of growth factors and cytokines are known to work in harmony to promote the process of chondrogenesis and chondrocyte differentiation toward endochondral ossification. Earlier we showed that CCN2 physically interacts with some of them, suggesting that multiple effects of CCN2 on various differentiation stages of chondrocytes may be attributed to its interaction with these growth factors and cytokines. However, little is known about the functional interaction occurring between CCN2 and other growth factors and cytokines in promoting chondrocyte proliferation and differentiation. In this study we sought to shed light on the binding affinities between CCN2 and other essential growth factors and cytokines known to be regulators of chondrocyte differentiation. Using the surface plasmon resonance assay, we analyzed the dissociation constant between CCN2 and each of the following: TGF- β 1, TGF- β 3, IGF-I, IGF-II, PDGF-BB, GDF5, PTHrP, and VEGF. We found a strong association between CCN2 and VEGF, as well as a relatively high association with TGF- β 1, TGF- β 3, PDGF-BB, and GDF-5. However, the sensorgrams obtained for possible interaction between CCN2 and IGF-I, IGF-II or PTHrP showed no response. This study underlines the

correlation between CCN2 and certain other growth factors and cytokines and suggests the possible participation of such interaction in the process of chondrogenesis and chondrocyte differentiation toward endochondral ossification.

Keywords CCN protein 2 (CCN2) · Growth factors · Chondrocyte · Binding affinity

Abbreviations

PDGF	Platelet-derived growth factor
TGF- β	Transforming growth factor beta
IGFs	Insulin-like growth factors
PTHrP	Parathyroid hormone-related protein
VEGF	Vascular endothelial growth factor
GDF-5	Growth-differentiation factor 5
SPR	Surface plasmon resonance
BMP	Bone morphogenetic protein
FGF	Fibroblast growth factor
CTGF	Connective tissue growth factor
OPG	Osteoprotegerin

Introduction

The CCN family of proteins is a complex family of multifunctional cysteine-rich secretory proteins containing 6 members designated as CCN family member 1 (CCN1) to CCN family member 6 (CCN6). It has been determined that these proteins have a significant degree of structural resemblance to one another. Yet, they exhibit different biological functions. CCN2 protein was previously known as CTGF and hypertrophic chondrocyte-specific gene product 24 (Hcs24; Takigawa et al. 2003; Perbal and Takigawa 2005). CCN2 is composed of 4 conserved modules, i.e., insulin-like growth factor binding-protein-like, von Willebrand factor type C, thrombospondin

✉ Masaharu Takigawa
takigawa@md.okayama-u.ac.jp

¹ Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan

² Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan

type 1 repeat, and C-terminal modules, by which it modulates the effects of a variety of growth factors/cytokines and extracellular matrix component organization (Takigawa 2003; Takigawa et al. 2003; Perbal and Takigawa 2005; Aoyama et al. 2009, 2012, 2015).

As has been noted previously, bone is formed by 2 distinct processes, i.e., intramembranous ossification and endochondral ossification. The endochondral process has been described as comprising 5 phases. Mesenchymal cells are first committed to become chondrocytes by the action of paracrine factors. During the second phase, these committed cells condense and differentiate into chondrocytes. The third stage is characterized by the rapid proliferation of the chondrocytes, during which time their volume increases by 5–10 fold, thus resulting in hypertrophic chondrocytes. Afterwards, in the fourth phase the matrices become mineralized by calcium phosphate, and osteoclastic infiltration begins. Finally, vascular infiltration occurs in the fifth phase, leading to chondrocyte apoptosis and osteoblastic differentiation (Gilbert 2000; Oseni et al. 2011; Takigawa 2013). During the last decade, many researchers have identified a variety of growth factors that influence differentiation events. For instance, platelet-derived growth factor (PDGF) together with transforming growth factor beta (TGF- β) and insulin-like growth factors (IGFs) have been shown to regulate mesenchymal cell condensation as well as chondrogenitor proliferation and differentiation. In addition, parathyroid hormone-related protein (PTHrP) and vascular endothelial growth factor (VEGF) have long been recognized to regulate chondrocyte maturation and hypertrophy (Strohback et al. 2011). In comparative studies, our group and others have documented the crucial effects of CCN2 on all types of cells implicated in the route of endochondral ossification (Takigawa 2003; Takigawa et al. 2003; Kubota and Takigawa 2007a, b, 2011; Kawata et al. 2012; Takigawa 2013). Understanding how this protein interacts with various growth factors will inevitably help in providing a better understanding of the functional relationships between those proteins as well as yielding insight into protein-based associations involved in biological processes.

Previously we suggested that CCN2 acts as a “signal conductor” by modifying the actions of these growth factors, by binding not only to the growth factors, but also to their receptors as well as to extracellular matrices via its 4 modules (Kubota and Takigawa 2007b; Takigawa 2013). Recently, we published the binding affinities between CCN2 and certain growth factors and cytokines such as BMP2 (Maeda et al. 2009), FGF-2 (Nishida et al. 2011), FGF-1 (Abd El Kader et al. 2014), and OPG (Aoyama et al. 2015) as determined by use of the surface plasmon resonance assay.

To clarify further the molecular mechanism of the multifunctional action of CCN2 as a signal conductor in cartilage, we presently studied the kinetics of the interaction between CCN2 protein and other proteins such as TGF- β 1, TGF- β 3,

IGF-I, IGF-II, PDGF-BB, GDF-5, PTHrP, and VEGF, all of which have been shown to play important roles in the biological process of endochondral ossification.

Materials and methods

Materials

Recombinant protein CCN2 was purchased from BioVendor Laboratory Medicine (Brno, Czech Republic). TGF- β 1, TGF- β 3 (Cell Signaling, Danvers, MA, USA), IGF-I, IGF-II, GDF-5 (All R&D Systems, Minneapolis, MN, USA), PDGF-BB, VEGF, and PTHrP (All Wako Pure Chemical Industries, Osaka, Japan) came from the sources indicated. Sensor chips C1, CM5, and HBS-EP (GE Healthcare UK Ltd. Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England) were used for SPR analysis.

Methods

Surface plasmon resonance (SPR) spectroscopy has been used to compute the dissociation constant (Kd) among CCN2 protein and growth factors human recombinant proteins by using Biacore X (GE Healthcare). Briefly, diluted CCN2 protein was immobilized onto a sensor chip according to the amine coupling protocol (Aoyama et al. 2012, 2015; Abd El Kader et al. 2014). Human recombinant proteins were diluted with HBS-EP buffer (GE Healthcare) to different concentrations (Table 1), and were flushed over the CCN2-bearing surface. In like manner a CCN2-free surface was used as a control. The sensorgrams were corrected by subtracting the signal of the reference cell, as described previously (Abd El Kader et al. 2014; Aoyama et al. 2015). For affinity measurements, binding and dissociation were monitored. Data were fitted by using BIA evaluation software version 4.1 (GE Healthcare UK Ltd.) with the single-cycle kinetics support package (GE Healthcare UK Ltd.). Binding data were globally fit to the single-cycle kinetics 1:1 Langmuir binding model and single-cycle kinetics 1:1 Langmuir binding model with drifting. Chi square values of all data were under 10 % of Rmax.

Results

Although many studies have identified several growth and differentiation factors that are involved in cartilage and bone differentiation and development (Strohback et al. 2011; Khattab et al. 2014), much remains to be understood concerning the potential interaction between these growth factors and CCN2. Such understanding may further define the specific role of these growth factors together with CCN2 in mediating chondrocyte proliferation and differentiation.

Table 1 Binding constants (Kd) and the value CI^2 test measured by SPR between CCN2 and various growth factors involved in endochondral ossification

Growth factors	Dissociation Constant (Kd)	CI^2	Analyte(concentration)
TGF- β 1	64.7 nM	6.29	100 nM, 33 nM, 11 nM, 3 nM, 1 nM
TGF- β 3	260 nM	3.6	500 nM, 250 nM, 125 nM, 62.5 nM, 31.2nM
PDGF-BB	230 nM	2.88	100 nM, 50 nM, 25 nM, 12.5 nM, 6.25nM
GDF-5	26.3 nM	1.28	100 nM, 33 nM, 11 nM, 3 nM, 1 nM
IGF-I	No response	–	2500 nM, 500 nM, 100 nM, 20 nM, 4 nM
IGF-II	No response	–	500 nM, 125 nM, 30.1 nM, 8 nM, 2 nM
PTHrP	No response	–	100 nM, 50 nM, 25 nM, 12.5 nM, 6.25nM
VEGF	4.8 nM	2.52	100 nM, 33 nM, 11 nM, 3 nM, 1 nM

The influence of TGF- β super family and PDGF results in various biological responses during mesenchymal stem cell recruitment and differentiation toward chondrocytes (Ng et al. 2008; James et al. 2009; Andia and Maffulli 2013;

Augustyniak et al. 2014; Cassiede et al. 1996). Thereupon, we were primarily interested in determining the potential interaction between TGF- β 1, - β 3, and CCN2 proteins. As shown in Fig. 1, sensorgrams of the assay were obtained by

Fig. 1 Binding of human recombinant CCN2 protein to TGF- β 1, TGF- β 3, and PDGF-BB proteins analyzed by using SPR. CCN2 was immobilized on a CM5 (TGF- β 1) and C1 (TGF- β 3 and PDGF-BB) chips via amine coupling. Indicated concentrations of proteins were perfused over the biosensor surface, and injection started at time point 120 s with 60s time intervals between different dilutions to record sensorgrams. **a** SPR sensorgram was recorded for the interaction

between CCN2 and TGF- β 1 (100 nM, 33 nM, 11 nM, 3 nM, 1 nM). **b** SPR sensorgram was recorded for the interaction between CCN2 and TGF- β 3 (500 nM, 250 nM, 125 nM, 62.5 nM, 31.2 nM). **c** SPR sensorgram was recorded for the interaction between CCN2 and PDGF-BB (100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM). Data are representative of results from at least 2 different assays

using CCN2 immobilized on C1 and CM5 chips. For TGF- β 1, we found binding events at 100 nM, 33 nM, 11 nM, 3 nM, and 1 nM; whereas for TGF- β 3, these events were detected at 500 nM, 250 nM, 125 nM, 62.5 nM, and 31.2 nM. Then, the dissociation constant (K_d) for each TGF was calculated under the same conditions. In agreement with TGF- β 1 SRP findings by Abreu et al. (2002), our results showed a dissociation constant of 64.7 nM for TGF- β 1 and one of 260 nM for TGF- β 3. (Fig. 1a, b, Table 1). Correspondingly, the K_d value for the interaction between CCN2 and PDGF-BB was 230 nM (Fig. 1C). Biacore analysis thus confirmed that the binding affinity of CCN2 for PDGF-BB was slightly stronger than that for TGF- β 3. However, the interaction with TGF- β 1 was much stronger than that with those proteins (Table 1).

As several earlier studies assessed the role of IGF (Takigawa et al. 1997) and GDF-5 (Coleman et al. 2013; Luyten 1997) during chondroprogenitor proliferation and differentiation, we next examined the possible interaction between CCN2 and the above-mentioned proteins. For GDF-5

SPR analysis, CCN2 was immobilized onto a CM5 chip; and binding analysis was carried out with various dilutions (100 nM, 33 nM, 11 nM, 3 nM, 1 nM). For IGF-I and IGF-II an initial dilution of 2500 nM and 500 nM followed by five-fold and four-fold serial dilutions, respectively, was used, with the growth factors injected over the C1 chip surface. The sensorgrams in Fig. 2 show that a dose-dependent interaction between CCN2 and GDF-5 was detected (Fig. 2c, Table 1). On the other hand, IGF-I and IGF-II showed no response (Fig. 2a, b, Table 1).

Acting in concert with previously recognized signaling growth factors, PTHrP and VEGF have been found to be regulators of chondrocyte differentiation and maturation (Vinatier et al. 2009; Nishida et al. 2009). For the PTHrP experiment, the CCN2 ligand was immobilized on the surface of a sensor chip, followed by passage of the analyte in commercial HBS-EP buffers containing 0.005 % surfactant P. However, the response on the background cell associated with this experiment was too high to analyze the sensorgram, so we

Fig. 2 Binding of human recombinant CCN2 proteins to GDF-5, IGF-I, and IGF-II proteins analyzed by using SPR. CCN2 was immobilized on CM5 (GDF5) and C1 (IGF-I and IGF-II) chips via amine coupling. Indicated concentrations of proteins were perfused over the biosensor surface, and injection started at time point 120 s with 60 s time intervals between different dilutions to record the sensorgrams. **a** SPR

sensorgram was recorded for the interaction between CCN2 and GDF-5 (100 nM, 33 nM, 11 nM, 3 nM, 1 nM). **b** SPR sensorgram was recorded for the interaction between CCN2 and IGF-I (2500 nM, 500 nM, 100 nM, 20 nM, 4 nM). **c** SPR sensorgrams recorded for the interaction between CCN2 and IGF-II (500 nM, 125 nM, 33 nM, 8 nM, 2 nM). Data are representative of results from at least 2 different assays

attempted to assay with HBS-EP buffer containing 0.05 % surfactant P. Even in this case the sensorgrams obtained did not show high enough response for analysis. This phenomenon in the binding responses was also observed with IGF-I and II. Thus we were unable to present values for dissociation constants for the interaction between CCN2 and these proteins (PTHrP, IGF-I and II). In contrast, after CCN2 had become stabilized on a C1 chip surface, VEGF was injected (1 nM for up to 100 nM), the sensorgrams showed a gradual increase in the resonance units between CCN2 and VEGF (Fig. 3b, Table 1). This finding confirmed the results of the competitive affinity-binding assay previously reported by Inoki et al. (2002) to evaluate specific interaction between CCN2 and VEGF. Collectively, our results indicate interaction between CCN2 and certain growth factors that are known to be involved in chondrocyte differentiation.

Discussion

This study was performed to obtain new information for extending our current understanding of the behavior of CCN2 with other important growth factors noted as regulators of chondrocyte proliferation and differentiation. For this purpose, we used the SPR system to analyze and compare the affinities between several growth factors and CCN2. This system has some advantages over other methods such as the pull-down assay or solid-phase binding assay, which require specific antibodies against each protein. When antibodies are used, each antibody has a different titer, which makes the comparison of results difficult. In addition, these latter assays only reveal whether or not 2 or more proteins bind to each other, but they cannot provide values for binding affinity. On

the other hand, the SPR system can indicate kinetics parameters of these proteins; although there is a limitation in that it cannot show the presence of some third factor that might influence the binding between the 2 factors being examined. Therefore, SPR analysis provides at least useful information about the binding affinities between CCN2 and possible and known binding partners.

By SPR analysis we demonstrated that CCN2 significantly bound to GDF-5 and VEGF, moderately to TGF- β 1, and to a lesser extent to TGF- β 3 and PDGF-BB. Since these experiments were performed under the same conditions using the same analytical equipment, the results provide new comparable information for future research into the interaction between CCN2 and other binding partners.

CCN2, an endochondral ossification genetic factor, promotes endochondral ossification by acting upon chondrocytes, osteoblasts, endothelial cells, and osteoclasts (Takigawa 2003). In fact, CCN2 is involved in many events during the development and differentiation of chondrocytes. Particularly, CCN2 is expressed dominantly in hypertrophic chondrocytes. After being produced, CCN2 is transported and infiltrates into sites where it may interact with major growth factors for cartilage (Kubota and Takigawa 2007a, b, 2011; Kawata et al. 2012). In addition, although gene expression of CCN2 peaks in pre-hypertrophic and hypertrophic zones of cartilage, lower expression is observed in the calcifying tissues of normal embryonic mice but not in those of *chfa-1* deficient mice, indicating that osteochondrogenic lineage cells express at least low-level expression of CCN2 (Yamaai et al. 2005). Therefore, CCN2 may interact with these major growth factors for cartilage as well.

Among members of the TGF- β superfamily, TGF- β 1 was shown to physically bind CCN2 (Abreu et al. 2002). In this

Fig. 3 Binding of human recombinant CCN2 proteins to PTHrP and VEGF proteins analyzed by using SPR. CCN2 was immobilized on a C1 chip via amine coupling. Indicated concentrations of proteins were perfused over the biosensor surface, and injection started at time point 120 s with 60 s time intervals between different dilutions to record the

sensorgram. **a** SPR sensorgram was recorded for the interaction between CCN2 and PTHrP (100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM). **b** SPR sensorgram was recorded for the interaction between CCN2 and VEGF (100 nM, 33 nM, 11 nM, 3 nM, 1 nM). Data are representative of results from at least 3 different assays

study, we showed that TGF- β 3 also bound to CCN2. The binding affinity of CCN2 for TGF- β 3 (260 nM) was lower than that for TGF- β 1 (64.7 nM) and much lower than that for BMP-2, which we reported previously (Table 2; 0.77 nM). However, to conclude their physiological significance, further investigation on tissue concentrations of CCN2 and these growth factors and their co-localization *in vivo* is needed. In addition to TGF- β 3, we showed for the first time that GDF-5, also known as cartilage-derived morphogenetic protein-1 (CDMP-1), bound to CCN2 with a significantly high affinity. The K_d values for the interaction between GDF-5 and its receptors, BMPRIA, BMPRIB and BMPRII (Schwaerzer et al. 2012), are close to the value for the binding between GDF-5 and CCN2, although the affinity is one order of magnitude lower than in the case of the binding between CCN2 and BMP-2, FGF-1 or FGF-2 (Table 2). The temporal and spatial expression pattern of GDF-5 is mostly restricted to the developing appendicular skeleton and is predominantly found at the stage of pre-cartilaginous mesenchymal condensation and throughout the cartilaginous cores of the developing long bones (Chang et al. 1994; Luyten 1997). In addition, Coleman et al. (2013) recently showed that GDF-5 enhances not only chondrogenesis but also hypertrophy of chondrocytes *in vivo*. It is therefore reasonable to think that CCN2 complements GDF-5 actions during the entire process of chondrogenesis.

It was reported that PDGFs and their receptors are expressed during fracture healing (Fujii et al. 1999), which is achieved by endochondral ossification. PDGF-BB stimulates the proliferation and differentiation of chondrocytes cultured from rat rib growth-plates (Wroblewski and Edwall 1992). This growth factor is also reported to stimulate the proliferation of resting chondrocytes but to arrest the cells in a pre-maturation stage (Kieswetter et al. 1997). Moreover, it is well known that PDGF is abundant in platelets; and we previously reported that platelets are also rich in CCN2 (Kubota et al. 2004). These facts led us to speculate that CCN2 binds to PDGF. Our results showed that CCN2 bound to PDGF-BB with a K_d value of 230 nM. Although this binding affinity is lower than that between PDGF-BB and its receptor β -PDGFR (K_d =1.2 nM; Heidaran et al. 1990), it is reported that α 2-macroglobulin binds to PDGF-BB with an affinity (K_d =370 nM; Crookston et al. 1994) similar to that between PDGF-BB

and CCN2 and inhibits the binding of PDGF-BB to PDGFR (Raines et al. 1984). Therefore, these results suggest a possible physiological significance of CCN2/PDGF interaction, such as modification by CCN2 of the PDGF action on chondrocytes via their binding and co-operation between CCN2 and PDGF-BB via their binding in bone-fracture healing and other wound healing, since CCN2 is expressed during bone-fracture healing (Nakata et al. 2002) and wound healing of skin (Takigawa 2003). However, further investigation such as examining the co-localization with CCN2 and PDGF and its receptors is needed to conclude functional relevance.

Earlier, our group showed that IGF-I and IGF-II (Takigawa et al. 1997), as well as CCN2 (Nakanishi et al. 2000; Takigawa 2013), stimulate chondrocyte proliferation and proteoglycan synthesis. Nevertheless, no verified physical interaction was found between CCN2 and IGFs. Since CCN2 has an IGFBP-like module, some investigators had occasionally referred to CCN2 as IGFBP8 or IGFBPrP2 (Perbal and Takigawa 2005). However, other studies indicated that the name IGFBP8 or IGFBPrP2 is inappropriate for CCN2 (Vorwerk et al. 2002). In this light, it was also reported that although structurally similar, the aminoterminal domain of CCN3 is unable to replace the aminoterminal domain of IGFBP-3 in forming a high-affinity IGF-binding site (Yan et al. 2006). Therefore, the nomenclature of IGFBP-related proteins (which implies functional relationship to the classical IGFBPs) is inappropriate for CCN proteins, as proposed by Yan et al. (2006). Using transgenic mice overproducing CCN2 in cartilage, we previously observed that CCN2 stimulates the gene expression of IGF-I and IGF-II, as well as the production of IGF-R in chondrocytes, thus enhancing the IGF-IGFR pathway (Tomita et al. 2013). With reference to IGFs, it could be concluded that the regulatory role of CCN2 with respect to the gene expression of these growth factors is more significant than any physical interaction.

PTH/PTHrP signaling is important for chondrocyte differentiation. PTH and PTHrP stimulate chondrocyte differentiation and chondrogenesis of mesenchymal stem cells (Takigawa et al. 1980; Kim et al. 2008). However, in the late stage of the differentiation of growth-plate chondrocytes, PTHrP inhibits their terminal differentiation (Lee et al. 1996). In this study, we found that CCN2 did not bind to PTHrP (Table 1). Considering that CCN2 accelerates not only the maturation but also the hypertrophy of growth-plate chondrocytes (Takigawa 2013), at least physical interaction between CCN2 and PTHrP would not seem to be involved in the multi-functionality of CCN2. However, in light of the case of IGFs, further investigation including studies on regulation of gene expression is needed to discuss the functional relevance of interaction between them.

CCN2 is an angiogenic factor that is involved in angiogenesis in the final stage of endochondral ossification (Shimo

Table 2 Previously reported binding constants (K_d) measured by SPR for the binding between CCN2 and various growth factors

Growth factor	Dissociation Constant (K_d)	Reference
BMP-2	0.77 nM	Maeda et al. (2009)
FGF-1	3.98 nM	Abd El Kader et al. (2014)
FGF-2	5.5 nM	Nishida et al. (2011)
OPG	24.5 nM	Aoyama et al. (2015)

et al. 1998; Shimo et al. 1999; Kubota and Takigawa 2007a, b). Similarly, as an angiogenetic factor, VEGF is essential for establishing epiphyseal vascularization (Maes et al. 2004). Importantly, both genes are expressed in hypertrophic chondrocytes. CCN2 stimulates the expression of VEGF in chondrocytes (Nishida et al. 2009). Given that the dissociation constant between CCN2 and VEGF is 4.8 nM, and together with Inoki's findings (2002), we here confirmed that CCN2 could physically interact with VEGF. Therefore, we could conclude that CCN2 not only stimulates the production of VEGF, but also interacts with VEGF and collaboratively stimulates angiogenesis in the final stage of endochondral ossification.

Collectively with our previously reported data about physical interactions of CCN2 with BMP-2, FGF-1, FGF-2, and OPG (Table 2), our present findings indicate that CCN2 promoted chondrogenesis and chondrocyte proliferation, differentiation and hypertrophy by enhancing the actions of certain growth factors via physical interaction with them, as well as by stimulating the gene expression of other growth factors, or both, resulting in enhancement of the entire process of endochondral ossification. Our present data may help to develop a new research avenue for studying the modification/enhancement of chondrocyte differentiation and endochondral ossification, thus leading to a better understanding of the molecular actions of CCN2 and other CCN proteins.

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