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COMMENTARY

CCN3: Doctor Jekyll and Mister Hyde

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Abstract CCN proteins are key regulators of signaling pathways that are essential for the control of normal life, from birth to death. As such, they make use of their unique mosaic structure to interact with several other regulatory proteins and ligands that control the fate of living cells. The various functions attributed to the CCN proteins may sometimes appear contradictory, but this situation reflects the complexity of the multimolecular scaffolds in which CCN proteins are engaged and the critical impact of the microenvironment that dictates the bioavailability of the elementary building blocks. CCN3 is one of the best examples of a CCN protein showing biological properties which may at first glance appear opposite or contradictory. Indeed, CCN3 acts both as a tumor suppressor and is associated with higher metastatic potential. Furthermore, the physical interaction of CCN3 with VEGF and its potential antiangionenic activity in glioma cells are in apparent contradiction with its proangiogenic activity in rabbit cornea. In this communication, I am revisiting the observations that led us to these apparent contradictions. After pointing out how the methodologies that were employed might have contributed to the confusion, I briefly discuss the dual biological activities of CCN3 in the context of tumor cell engineering and survival prognosis.

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Introduction

The CCN family of proteins contains six members designated CCN1 through CCN6. All these proteins share a common mosaic structure made up of the assembly of four modules. These modules share partial identity with four other large groups of regulatory proteins: IGF binding proteins (IGFBP); von Willebrand factor (VWC); thrombospondin type-1 (TSP1) and a group of growth factors and matrix proteins that contain a cysteine knot (CT). For a detailed review of the structural features of the CCN proteins, see the recent review by Holbourn et al. (2008). Apart from CCN5, which lacks a CT module, all CCN proteins share a closely related primary structure which includes a series of 38 cysteine residues that are strictly conserved in position and number except in CCN6 which is missing four cysteine residues in domain II (VWC; Holbourn et al. 2008).

CCN proteins are key signaling molecules which act both in the extracellular matrix, where they are secreted, and inside cells, where they can be detected in the cytoplasm and the nucleus. The pathways in which they were found to play essential roles include: regulation of adhesion, mitogenesis, migration and chemotaxis, cell survival, differentiation, angiogenesis, chondrogenesis, tumourigenesis and wound healing. They have also been implicated in many human diseases [for some comprehensive reviews see (Perbal 2001; Perbal and Takigawa 2005; Leask and Abraham 2006].

Results

Dr. Jekyll

CCN3 is the first example of a CCN protein with antiproliferative and tumor suppressor activities

The gene encoding CCN3 was originally identified in myeloblastosis associated virus (MAV) - induced nephroblastoma in chickens (Joliot et al. 1992; Perbal 1994). These avian tumors constitute a unique model of the Wilms' tumor in children (Perbal 1994) as they show all histological features of the human counterpart.

In one tumor, the MAV proviral genome was integrated into a cellular gene whose five exons encoded a putative 32 KDa protein in normal cells. The chimeric gene that resulted from the insertion of MAV encoded large amounts of an amino-truncated protein that proved to induce cellular transformation when expressed in normal primary chicken fibroblasts (CEF; Joliot et al. 1992). Interestingly, high levels of the full length normal protein were also detected in other MAV-induced nephroblastomas, hence the initial designations of this gene as nov (for nephroblastoma overexpressed) and novH for the human gene (Perbal 2006a).

Because the expression of the recombinant full length CCN3(NOV) protein induced growth arrest in CEF, the high levels of CCN3 found in the tumors was raising an apparent contradiction: how high levels of a protein with a negative effect on cell growth could be detected in well-developed aggressive avian tumors?

Our starting premise was that high levels of CCN3 matched the heterotypic differentiation of the blastemal cells into cartilage, bone, and muscle tissues as observed in these tumors (Chevalier et al. 1998). Indeed, we had obtained evidence suggesting that CCN3 was associated with growth arrest and differentiation of cartilage, bone and muscle in normal conditions (Perbal 2001).

Evidence accumulated rapidly in favor of CCN3 acting as antiproliferative and a tumor suppressor.

Glioma cells in which we forced the expression of CCN3 were growing at a much lower rate than their parental counterpart (Gupta et al. 2001). The expression of CCN3 into these cells considerably reduced their ability to induce tumors when injected into nude mice. Likewise, the ectopic expression of CCN3 in Ewing's cells reduced both their growth and tumorigenicity (Lin et al. 2003) In fact, cells engineered to produce CCN3 gave rise to abortive tumors that eventually regressed (Lin et al. 2003)

We had proposed that CCN3 impaired the development of the vasculature required for efficient development of the explanted tumor cells and that CCN3 might be used as an anti-cancer agent (Gupta et al. 2001).

The potential anti-angiogenic effect of CCN3 agrees with evidence supporting a physical interaction between CCN3 and VEGF (Perbal 2006b, and unpublished results), but apparently contrasts with the observation that CCN3 induces neovascularization when implanted in rat cornea (Lin et al. 2003).

In the case of chronic myeloid leukemia (CML), the expression of CCN3 was high in patients who recovered after treatment with imatinib, whereas it was considerably reduced in patients in the acute phase of tumor development and patients with relapse after drug treatment (McCallum et al. 2006).

Recent results indicated that CCN3 can restore regulatory processes in CML cells by inhibiting proliferation and inducing apoptosis (S. Irvine, personal communication). It was previously reported that the expression of CCN3 resulted in a higher number of apoptotic cells in Ewing tumor cell cultures (Benini et al. 2005). However, melanocytes transfected with an adenoviral construct driving the expression of CCN3 were growth-inhibited but did not show an increase of apoptosis, as measured by caspase 3 levels (Fukunaga-Kalabis et al. 2006). Therefore, these observations suggested that the apoptotic effects of CCN3 might be cell type specific.

Interestingly, the expression of the cell cycle inhibitor protein p21 was found to be increased in response to CCN expression in gliomas (Bleau et al. 2007) in melanocytes (Fukunaga-Kalabis et al. 2006) and Kusa cells (Katsuki et al. 2008) reinforcing the idea that the antiproliferative activity of CCN3 was independent of apoptosis.

Early analysis of CCN3 expression in renal cell carcinoma (Glukhova et al. 2001) and prostate cancer cells (Maillard et al. 2001) had led to the conclusion that an elevated expression of CCN3 was associated with a high proliferative rate for these tumor cells. In light of a report that also associated high expression of CCN3 to active cellular proliferation (Liu et al. 1999), an apparently paradoxical situation developed.

However, the data that associated CCN3 to stimulation of cell growth were based on the direct or indirect measurement of cells in the S phase, not on cell counts.

Our recent results (Bleau et al. 2007) established that the negative effect of CCN3 on cell growth resulted from a "slow down" of the S-G2 transition with a relative reduction in the number of cells undergoing a complete cell cycle, and a temporary increase of cells in the S phase (Fig. 1).

Given these results, the previous observations that associated high CCN3 expression with increased cell multiplication, in fact reflected the increase in the number of cells in the S phase that resulted from the antiproliferative activity of CCN3.

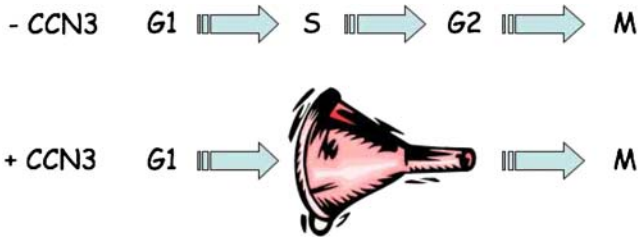

Fig. 1 Effect of CCN3 on cell cycle progression. The G59 glioma cells (CCN3 negative) and their G540 transfected derivative (CCN3 positive) were synchronized by release of an aphidocolin block. The distribution of the growing cells in the four different phases of the cell cycle was established by FACS analysis. See Bleau et al. (2007) for experimental details

In conclusion, claims that CCN3 could stimulate or be associated with increased cell proliferation were misguided by our lack of knowledge regarding the biological effects of CCN3 on the cell cycle.

Mr. Hyde

CCN3 expression is associated with metastatic potential

Despite its antiproliferative activity on tumor cells, CCN3 was also found to increase the migration and invasion of Ewing's tumor cells (Benini et al. 2005). Motility assays performed using Transwell chambers with 8 μm pore size, established that CCN3 expressing cells migrated three to four times faster than the parental cells which did not express detectable amounts of CCN3. Also, when invasion capacity was assessed in Matrigel invasion assay, cells expressing high levels of CCN3 migrated twice as much as the parental cells (Benini et al. 2005).

These observations were consistent with the association that we had previously established between CCN3 expression and worse patient outcome (Manara et al. 2002). Indeed, the increase of mobility and invasion conferred by CCN3 on the transfected cells accounted for the higher metastatic potential of Ewing cells positive for CCN3 in the primary tumor (Manara et al. 2002). More recently, quantitative PCR measurement of CCN3 in patients confirmed that the expression of CCN3 was associated with poor prognosis (Perbal et al., submitted)

Similar conclusions were reached in the cases of osteosarcomas. In these tumors, we could establish that, not only was an elevated expression of CCN3 associated with a higher risk of developing metastasis, it also matched increased levels of MRD4 protein in these cells. While the expression of CCN1 and CCN2 was correlated to the expression of ostogenic differentiation markers, and showed no prognostic value in osteosarcomas, the levels of CCN3 in these tumors were independent of differentiation markers expression (Perbal et al. 2008).

In the case of melanomas, CCN3 protein levels were significantly higher in cells from stage IIIB-C patients with short survival than in melanoma cells from patients with long survival (Vallacchi et al. 2008). The role of CCN3 in the establishment of visceral metastases was supported by the observation that SCID mice injected with cells stably expressing CCN3 developed a much higher number of hepatic metastatic metastases than the mice injected with CCN3 negative tumor cells (Vallacchi et al. 2008).

Discussion

The observations reported above clearly demonstrate the need to clarify precisely the experimental conditions and the biological systems used to assess the biological properties of CCN proteins.

According to the literature, it is obvious that we must distinguish between two fundamentally different situations regarding the consequences of (1) forced expression (high or low) of a CCN protein in a cell in which there is no detectable production of that protein, and (2) endogenous production of the same CCN protein.

In both cases, one can predict that, based on the multimodular structure of the CCN proteins and their ability to interact with several partners, the biological properties of CCN proteins will depend upon the local context; i.e., the bioavailability of the various partners at a precise time and a precise location in the organism. This aspect has already been discussed (Perbal 2001) and there is an increasing body of evidence supporting that model.

On the contrary, so far not much has been done to compare the biological properties of CCN proteins produced endogenously with those of the same proteins produced by genetic engineering. Along the same line, addition of a particular recombinant CCN protein to cells that do not produce it may result in biological effects that are distinct from those which would result from the production of the protein by these cells. Several reasons may account for these potential differences. For example, it is quite conceivable that the endogenous CCN proteins expressed naturally by cells are modified, at a post-translational level, by these cells and might combine to other chaperone or transporters that may eventually affect their biological properties through addressing or by favoring interactions with multiprotein complexes at a higher organizational level.

There is no doubt that highly organized macromolecular complexes must be the basis for efficient coordinated signaling networks. How CCN proteins integrate such complexes is a very timely and challenging question, since answers that will be drawn from such studies will shed new light on the biological properties of CCN proteins.

Strategies aimed at stimulating or inhibiting expression of CCN proteins in cells that do not produce them may be a way to tackle these problems as long as the biological systems in which we perform these manipulations are compatible with the expression of CCN proteins. In other words, expressing a CCN protein in a context where it is not able to combine with its natural partners or where it will physically interact with proteins that are not in its natural environment in vivo, might lead to effects that are not relevant to the biology of these proteins.

The numbers of conflicting observations that are assigned to different "micro-environments", or various "biological contexts," may in fact result from the different experimental protocols that were used.

These two parameters are important, but they must be considered in physiological situations.

Strategies based on the ectopic addition of recombinant CCN proteins may also lead to misinterpretations, since exogenous proteins may not be channeled properly to their biological targets, either at the level of the cell membrane, extracellular matrix, or inside the cells.

These considerations might help us understand why the same CCN protein shows quite distinct properties, such as the pro- and anti-angiogenic activity of CCN3, when added exogenously or expressed from inside the cell. They can also provide explanations for the dual biological properties of CCN3 versus tumor cells.

As described above, the CCN3 protein shows growth inhibitory and tumor suppressor functions, whereas expression of CCN3 is associated with higher risk of developing metastases.

It is conceivable that the exogenous protein interacts with outbound signaling pathways so as to reduce the progression in the cell cycle, whereas production of CCN3 by tumor cells might affect the sub cellular distribution and processing of CCN3. Along this line, the negative effects of the secreted CCN3 protein would be counter-balanced by the production and subcellular distribution of intracellular CCN3 proteins that would interact with "inbound" signaling pathways. Disturbance of these signaling pathways may account, at least in part, for the association between CCN3 expression and bad prognosis

The detection of nuclear CCN proteins in tumor cells is well-documented (Perbal 2004), and there is an increasing body of evidence in favor of nuclear CCN proteins involved in cell proliferation. In the case of CCN3, accumulation of CCN3 in the nucleus of tumor cells (Perbal 1999) is believed to repress the expression of tumor suppressor genes thereby contributing to the loss of negative signals controlling proliferation (Planque et al. 2006).

Understanding at a molecular level how the CCN proteins become part of multimolecular regulatory complexes and exert their functions in extra-cellular and intracellular signaling is a main challenge that future studies will need to address.

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