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ARTICLE

Expression of Connective Tissue Growth Factor (CTGF/CCN2) in a Mouse Model of Rhabdomyosarcomagenesis

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Connective tissue growth factor (CTGF/CCN2) is a cysteine-rich matricellular protein that belongs to the CCN (CYR61, CTGF, NOV) protein family. It is highly expressed by human rhabdomyosarcoma cells and sustains their survival. In this study we investigated CCN2 expression in a mouse model of spontaneous rhabdomyosarcomagenesis that combines HER-2/neu oncogene activation and p53 oncosuppressor gene inactivation (BALB-p53neu mice). Murine rhabdomyosarcoma cells showed a 4-26 fold increase in CCN2 mRNA expression regarding to normal thigh muscle. Moreover, they expressed CCN2 protein at levels comparable to human rhabdomyosarcoma cells. Therefore BALB-p53neu mice might be useful for the evaluation of the role played by CCN2 in rhabdomyosarcoma *in vivo*. (Pathology Oncology Research Vol 13, No 4, 336–339)

Key words: rhabdomyosarcoma, mouse model, CCN proteins, CCN2/CTGF

Introduction

Connective tissue growth factor (CTGF or CCN2) belongs to the CCN family of proteins that comprises other five members: CCN1/CYR61 (cysteine-rich protein 61), CCN3/NOV (nephroblastoma overexpressed gene), CCN4/WISP-1 (Wnt-induced secreted protein-1), CCN5/WISP-2 and CCN6/WISP-3.[2] They are secreted, cysteine-rich, matricellular proteins that share a common modular structure of four domains: insulin-like growth factor binding domain, von Willebrand type C domain, thrombospondin-1 domain and cystine knot domain (with the exception of CCN5 that lacks the last one).[2] CCN2 has multiple biological functions, dependent on the cell type and the cellular context. It regulates cell proliferation, survival, migration and differentiation, and has a role in angiogenesis, wound healing, fibrosis, chondrogenesis and osteogenesis.[8] Moreover, all CCN proteins are emerging as key regulators of tumorigenesis, involved in the regulation of tumor cell growth and tumor-stroma interactions, thus being promising targets for cancer therapy.[9]

We have previously found that CCN2 might be a therapeutic target for human rhabdomyosarcoma, the most common soft tissue sarcoma of skeletal muscle origin in childhood. CCN2 was found highly expressed by human rhabdomyosarcoma cell lines and tumor specimens of embryonal and alveolar histotypes. Moreover, functional studies have indicated that it sustained human rhabdomyosarcoma cell survival and myogenic differentiation in an autocrine way.[3] In this study we took advantage of a mouse model of rhabdomyosarcomagenesis established in our laboratory through the combination of HER-2/neu oncogene activation and p53 oncosuppressor gene inactivation (BALB-p53neu mice).[6] We investigated whether murine rhabdomyosarcoma cells expressed CCN2 like human rhabdomyosarcoma cells making BALB-p53neu mice a suitable preclinical model for the study of the role played by CCN2 in rhabdomyosarcoma *in vivo*.

Materials and methods

Mice and cell lines

BALB/c mice transgenic for the activated rat HER-2/neu oncogene under the control of the MMTV-LTR promoter and with a knockout allele for the p53 oncosuppressor gene,

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referred to as BALB-p53neu mice, were obtained in our animal facility through the cross of BALB/c $p53^{+/-}$ mice (BALB/cJ-*Trp53*^{tm1Tyj} mice, purchased from The Jackson Laboratory, Bar Harbor, MI) with BALB/c HER-2/neu transgenic mice, referred to as BALB-NeuT mice.[6] Experiments were authorized by the local animal use and care committee. All BALB-p53neu male mice develop rhabdomyosarcomas exclusively in the retrovesical region. Five murine rhabdomyosarcoma cell lines (called RMSp53neu-1, -2, -3, -4 and -5) were derived from BALB-p53neu primary rhabdomyosarcomas. Cell lines and tumors express HER-2/neu antigen and are p53 null because of the loss of the remaining p53 allele.[6] C2C12 mouse myoblasts were purchased from the American Type Culture Collection (Manassas, VA). Cells were routinely cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum in a 7% CO_2 humidified atmosphere at 37°C.

Real-time PCR

RNA was extracted from cultured cells, normal thigh skeletal muscle, retrovesical muscle tissue and primary rhabdomyosarcomas by Trizol reagent (Invitrogen, Milan, Italy) and reverse transcribed as reported.[3] Mouse CCN2 expression was analyzed by quantitative real-time PCR using ABI Prism 5700 sequence detection system (Applera, Milan, Italy). Real-time PCR was performed using SYBR Green PCR Master Mix Reagents (Applera). Mouse CCN2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer pairs were designed using Primer Express Software version 2.0: CCN2: forward, 5'-CCCACACAAGGGC-CTCTTCT-3'; reverse, 5'-ACCGACCCACCGAAGACAC-3'; GAPDH: forward, 5'-GCTCACTGGCATGGCCTTC-3'; reverse, 5'-CCTTCTTGA TGTCATCATACTTGGC-3'. CCN2 mRNA expression levels were determined relative to normal thigh muscle of BALB-p53neu mice using the ΔΔCt method and GAPDH as endogenous reference gene (for a more detailed protocol refer to Palladini et al.[7]).

Western blot

Rhabdomyosarcoma cells were lysed in situ with 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 150 mM NaCl plus phosphatase and protease inhibitors (all reagents were purchased from Sigma, Milan, Italy) for 30 min on ice. Nuclei were removed by centrifugation at 12,000 x g at 4°C for 15 min and protein concentration in the supernatants was determined by DC Protein Assay (Bio-Rad, Milan, Italy). Proteins were separated on a 12% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad) and processed as described.[3] 40 μg of total cellular protein were loaded for evaluation of CCN2 expression. Actin (10 μg total cellular proteins in each sample) was used as housekeeping protein for sample normalization. Goat anti-CTGF polyclonal antibody (L-20, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-actin antibody (Sigma) were used as primary antibodies. Protein presence was detected through the incubation with the respective horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology) followed by a colorimetric reaction (Opti-4CN Substrate kit, Bio-Rad). Western blots were analyzed with TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). Background was subtracted with the "image rectangle" method. CCN2 protein expression was determined dividing CCN2 band volumes by actin band volumes for each sample. Molecular sizes of CCN2 proteins were calculated according to the run of precision plus protein standards (Bio-Rad). The standard curve was obtained applying the "first order Lagrange" equation.

Results

Expression of CCN2 by murine rhabdomyosarcoma cells

As mouse model of rhabdomyosarcomagenesis we used male BALB-p53neu mice that spontaneously develop rhabdomyosarcomas in the genitourinary region (retrovesical tissue) around 11-21 weeks of age (100% incidence) due to HER-2/neu oncogene activation coupled with the inactivation of one allele of p53 oncosuppressor gene.[6] We investigated CCN2 expression by real-time PCR in five murine rhabdomyosarcoma cell lines, four primary tumors and normal thigh muscle of BALB-p53neu mice. All rhabdomyosarcoma cell lines and tumors expressed CCN2, with an expression of 4 to 26-fold higher compared to that of normal thigh muscle (*Fig. 1*). CCN2 expression was not

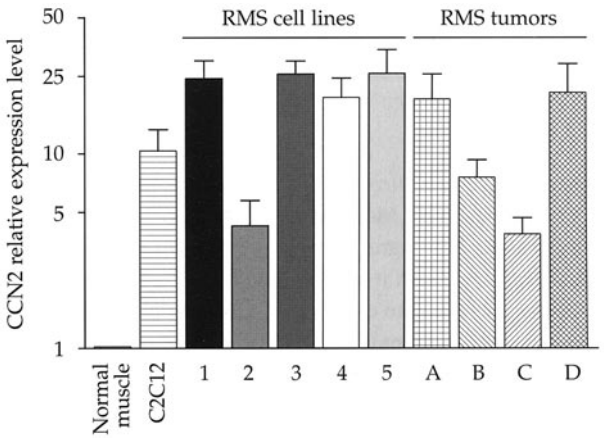

Figure 1. *Real-time PCR analysis of CCN2 expression by murine rhabdomyosarcoma (RMS) cell lines and primary tumors. Fold increase with respect to CCN2 expression by normal thigh muscle of BALB-p53neu mice is shown on the Y axis. RMSp53neu-2 cell line was obtained from tumor B, while the other cell lines and tumors are unmatched samples. C2C12 are murine proliferating myoblasts.*

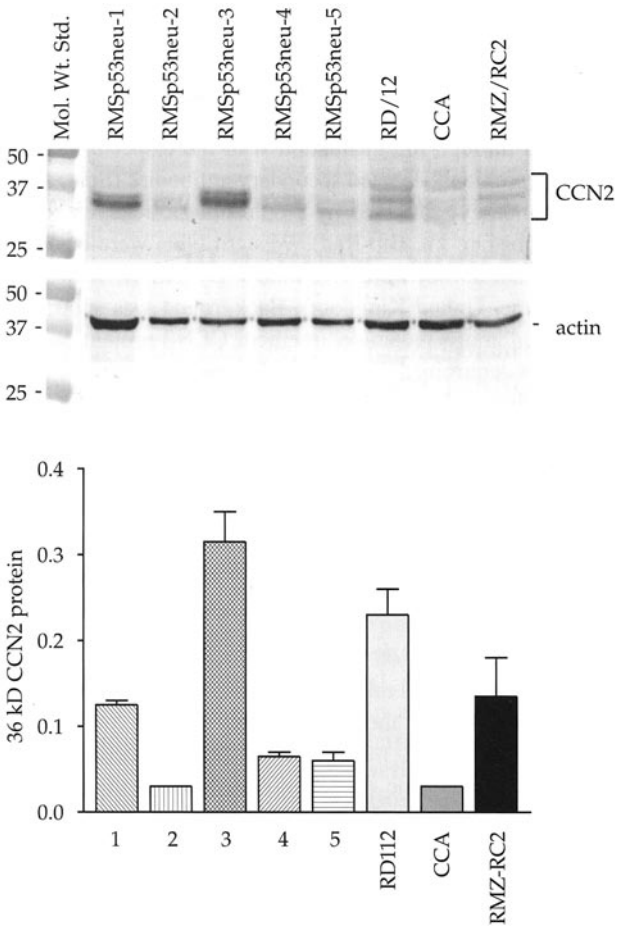

Figure 2. *CCN2 protein expression by murine and human rhabdomyosarcoma cell lines. RD/12, CCA and RMZ-RC2 are human rhabdomyosarcoma cell lines of the embryonal (RD/12, CCA) and the alveolar (RMZ-RC2) histotypes.[3] Upper panel: Western blot image. Mol. Wt., M_r in thousands. Lower panel: image analysis of CCN2 protein expression (36 kD isoform) performed with TotalLab software. CCN2 protein expression normalized to that of actin (mean ± SE from two independent Western blots) is shown*

induced by the *in vitro* culture as primary tumors already expressed CCN2. Moreover, RMSp53neu-2 cell line showed a CCN2 expression level quite similar to that of tumor B, from which it was derived (*Fig. 1*). C2C12 proliferating myoblasts also expressed CCN2 but at a lower level than rhabdomyosarcoma cell lines (with the only exception of RMSp53neu-2 cell line) (*Fig. 1*). Western blot analysis of CCN2 protein expression revealed that all of the murine rhabdomyosarcoma cell lines expressed 34-36 kD CCN2 isoforms although with differences in the expression levels (*Fig. 2*). Similarly, human rhabdomyosarcoma cell lines produced 34-36-38 kD CCN2 isoforms (*Fig. 2*). We previously documented the production of several CCN2 isoforms and fragments by RD/12 cells.[3] CCN2 is a 32-38 kD protein with 2-8 kD of N-linked sugars and a 30 kD core.[11] The mean level of CCN2 protein expression by murine rhabdomyosarcoma cell lines was comparable to human rhabdomyosarcoma cell lines (*Fig. 2*).

To investigate whether an increased expression of CCN2 might be related to tumor progression, we compared, by real-time PCR analysis, CCN2 expression of murine rhabdomyosarcoma cells with that of the site of origin of rhabdomyosarcoma (retrovesical muscle tissue) and of normal thigh muscle derived from 7-week-old male BALB-p53neu mice and from parental mice that harbor either single genetic alteration (BALB-NeuT mice, carrying the activated HER-2/neu oncogene and BALB-p53$^{+/-}$ mice with a knockout allele of the p53 oncosuppressor gene). Retrovesical muscle tissue gives rise to rhabdomyosarcomas only in BALB-p53neu mice and not in parental mice. Murine rhabdomyosarcoma cells showed a higher CCN2 expression compared with thigh muscle (*Fig. 3*). On the other hand, they showed an expression level similar to retrovesical muscle tissues of BALB-p53neu and BALB-NeuT mice (*Fig. 3*). Retrovesical muscle tissue expressed more CCN2 than thigh muscle (*Fig. 3*).

Discussion

We previously found that CCN2 might be a therapeutic target for human rhabdomyosarcoma, being highly expressed by human rhabdomyosarcoma cells and promoting their survival.[3] In this paper we found that CCN2 was also expressed both *in vivo* and *in vitro* by murine rhabdomyosarcomas developed in a mouse model of spontaneous rhabdomyosarcomagenesis based on HER-2/neu oncogene activation and p53 oncosuppressor gene inactiva-

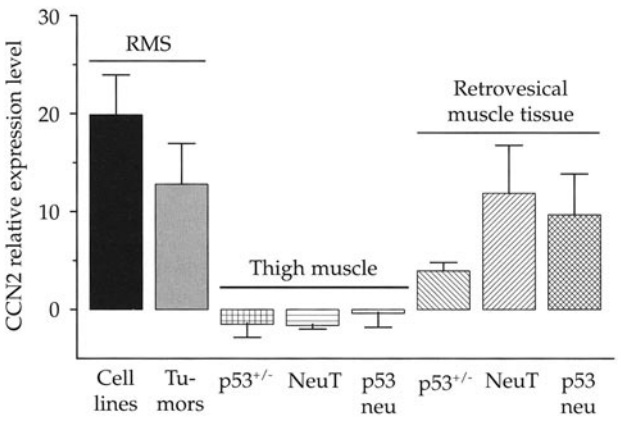

Figure 3. *Expression of CCN2 by murine rhabdomyosarcoma (RMS) cells, normal thigh muscle and retrovesical muscle tissue of 7-week-old BALB-p53$^{+/-}$, BALB-NeuT and BALB-p53neu male mice evaluated by real-time PCR analysis. Data were obtained from two mice. Fold change regarding to CCN2 expression of one BALB-p53neu muscle sample is shown on the Y axis. The differences between RMS cell lines and normal thigh muscles are statistically significant, $P<0.05$ at Student's t test.*

tion. Murine rhabdomyosarcoma cell lines resembled human rhabdomyosarcoma cell lines regarding morphology, defective myogenic differentiation, expression of insulin-like growth factor (IGF)-II, IGF-I receptor and IGF-II receptor.[6] Here we report that CCN2 expression is a shared feature of murine and human rhabdomyosarcomas.

In BALB-p53neu male mice retrovesical muscle tissue is specifically affected by the process of tumorigenesis whereas other skeletal muscles never develop rhabdomyosarcomas. The level of CCN2 expression by murine rhabdomyosarcoma cells was higher than that of normal thigh muscle but was comparable to preneoplastic retrovesical muscle tissue of BALB-p53neu mice and to normal retrovesical muscle tissue of BALB-NeuT mice. Therefore, retrovesical muscle tissue itself might be a high CCN2 expressor tissue type and CCN2 expression might support the process of retrovesical rhabdomyosarcomagenesis in BALB-p53neu mice.

Up to now the role of CCN2 in oncology has been evaluated *in vivo* in human tumor xenograft models[1,4,10,12] or in syngeneic models of experimental metastasis.[5] Transgenic mice that spontaneously develop CCN2-expressing tumors might allow to evaluate the efficacy of new therapeutic strategies since they reproduce the various stages of tumor progression, angiogenesis, tumor-stroma and tumor-immune system interactions. Therefore BALB-p53neu mice could be a useful tool to study the role played by CCN2 in rhabdomyosarcoma *in vivo*.

Acknowledgments

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