ELSEVIER

Bone 38 (2006) 671–677

BONE

www.elsevier.com/locate/bone

Expression and regulation of CCN genes in murine osteoblasts

Muriel S. Parisi [a], Elizabetta Gazzerro [a,b], Sheila Rydziel [a], Ernesto Canalis [a,b,*]

[a] *Department of Research, Saint Francis Hospital and Medical Center, Hartford, CT 06105, USA*
[b] *The University of Connecticut School of Medicine, Farmington, CT 06030, USA*

Received 30 June 2005; revised 20 September 2005; accepted 11 October 2005
Available online 28 November 2005

Abstract

Members of the CCN family of genes include cysteine-rich 61 (CYR61), connective tissue growth factor (CTGF), nephroblastoma overexpressed (NOV), and Wnt-induced secreted proteins (WISP) 1, 2 and 3. CCN proteins play a role in cell differentiation and function, but their expression and function in skeletal tissue is partially understood. We examined the expression and regulation of CCN genes in primary cultures of murine osteoblasts treated with transforming growth factor β (TGFβ), bone morphogenetic protein (BMP)-2, or cortisol. Northern blot analysis revealed the presence of CYR61, CTGF, NOV, and WISP 1 and 2 transcripts in murine osteoblasts, but not WISP 3 transcripts. Northern and Western blot analyses revealed that TGF β, BMP-2, and cortisol increased CYR61 and CTGF mRNA and protein levels. TGF β decreased NOV and increased WISP 2 mRNA and protein levels, and TGF β and BMP-2 increased, whereas cortisol decreased WISP 1 mRNA and protein levels. Nuclear run-on assays revealed that TGF β, BMP-2 and cortisol enhanced CYR61 and CTGF transcription, TGF β and BMP-2 induced and cortisol suppressed WISP 1, and TGF β induced WISP 2 transcription. Suppression of NOV transcription could not be detected due to low control levels. In conclusion, five of the six known CCN genes are expressed by osteoblasts and their transcription is regulated by TGF β, BMP-2 and cortisol.

Keywords: CCN genes; Transforming growth factor β; Bone morphogenetic proteins; Glucocorticoids; Bone formation

Introduction

Members of the CCN family of secreted, cysteine-rich regulatory proteins include cysteine-rich 61 (CYR61/CCN1), connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed (NOV/CCN3), and Wnt-induced secreted proteins (WISP) 1, 2, and 3 (CCN4, 5, and 6) [1]. CCN proteins are highly conserved cysteine rich (CR) proteins that share a common modular structure with four conserved domains, an insulin-like growth factor (IGF)-binding protein domain, a von Willebrand type C domain, a thrombospondin-1 domain, and a C-terminal domain, the latter absent in WISP 2 [2–4]. The architecture of CCN proteins makes them candidate molecules that can combine and interact with growth factors, in a manner analogous to that described for certain bone morphogenetic protein (BMP) antagonists, such as twisted gastrulation and chordin [5]. Like some BMP antagonists, CTGF interacts with BMPs and Wnt [6,7]. Interactions between CCN proteins and growth regulators are mediated by the CR or the C-terminal domain [1–3].

CCN proteins have important functions in cell proliferation and differentiation, and CYR61, CTGF, and NOV play a role in angiogenesis and cell adhesion [8–12]. CYR61 and CTGF induce chondrogenesis, and NOV interacts and activates the determinant of osteoblast cell differentiation, Notch [13–17]. Although a function for WISP 2 in skeletal tissue has not been reported, WISP 1 enhances the effect of BMP on osteogenesis and is found in areas of fracture repair, and WISP 3 mutations in humans lead to pseudo-rheumatoid dysplasia [18,19]. CTGF is expressed by osteoblasts and *ctgf* null mice exhibit impaired chondrocytic cell proliferation and angiogenesis [15,20]. These observations suggest that CCN gene products are important in bone and cartilage physiological events and repair.

* Corresponding author. Department of Research, Saint Francis Hospital and Medical Center, 114 Woodland Street, Hartford, CT 06105-1299, USA. Fax: +1 860 714 8053.
E-mail address: ecanalis@stfranciscare.org (E. Canalis).

8756-3282/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2005.10.005

The structure, expression and known function of CCN gene products imply that they may act as local regulators of osteoblast cell differentiation and function. A systematic study of CCN gene expression in osteoblasts should provide additional information toward our understanding of the role played by CCN genes in skeletal tissue. The aim of this study was to evaluate the expression of CCN genes in murine osteoblasts, and study their regulation by TGF β, BMP-2 or cortisol, agents known to regulate osteoblast differentiation and function [21–23].

Materials and methods

Culture technique

Parietal bones were obtained from 2 to 3 day old newborn CD1 albino mice. This project was approved by the Institutional Animal Care and Use Committee of Saint Francis Hospital and Medical Center. Cells were obtained by five sequential digestions of the parietal bone using bacterial collagenase (CLS II, Worthington Biochemical, Freehold, NJ) [24]. Cell populations harvested from the third to the fifth digestions were cultured as a pool, and were previously shown to have osteoblastic characteristics [24]. Osteoblastic cells (Ob cells) were plated at a density of 5000 to 7000 cells/cm^2, and cultured in a humidified 5% CO_2 incubator at 37°C until reaching confluence (about 50,000 cells/cm^2). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids and 10% fetal bovine serum (FBS, from Atlanta Biologicals, Lawrenceville, GA). Ob cells were grown to confluence, transferred to serum-free medium for 20 to 24 h and exposed to test or control medium in the absence of serum for 2 to 48 h, as indicated in the text and legends. Medium was replaced after 24 h. For nuclear run-on assays, Ob cells were grown to subconfluence, trypsinized, replated, and grown to confluence when they were serum-deprived and exposed to test or control solutions for 2 or 6 h. Cortisol (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and diluted 1:10,000, recombinant human TGF β 1 (a gift from Genentech, Inc., South San Francisco, CA), and BMP-2 (a gift from Wyeth Research, Collegeville, PA) were dissolved in DMEM.

Northern blot analysis

Total cellular RNA was isolated using an RNeasy kit (Qiagen, Chatsworth, CA), quantitated by spectrometry, loaded on a formaldehyde agarose gel, blotted onto Gene Screen Plus charged nylon (Perkin Elmer Life Science, Boston, MA), and sequentially hybridized with a 579 base pair (bp) fragment of murine CYR61 cDNA (American Type Culture Collection (ATCC), Manassas, VA), a 1.6 kilobase (kb) fragment of murine CTGF cDNA (Rolf-Peter Ryseck, Princeton, NJ), a 1.0 kb fragment of murine NOV cDNA, a 1.6-kb fragment of murine WISP 1 cDNA, and a 1.1 kb fragment of murine WISP 2 cDNA (all three from ATCC), a 1.2 kb fragment of human WISP 3 cDNA (Genentech), and a 752-bp fragment of murine 18S cDNA (ATCC), all labeled with 5 μCi [α-^{32}P] deoxycytidine triphosphate (Perkin Elmer Life Science). Hybridizations were performed at 42°C for 24–72 h, and post-hybridization washes were carried out at 65°C in 1x saline-sodium citrate (SSC) for 30 min. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film, employing Cronex Lightning Plus intensifying screens (Perkin Elmer Life Science). Relative hybridization levels were determined by densitometry. Northern analyses shown are representative of three or more cultures, except for CTGF expression (n = 2), since it confirms previously published data from this laboratory in rodent Ob cells [20].

Nuclear run-on assay

To examine changes in the rate of transcription, nuclei were isolated by Dounce homogenization in a Tris buffer containing 0.5% IGEPAL CA-630 (Sigma Chemical Co.). Nascent transcripts were labeled by incubation of nuclei in a reaction buffer containing 500 μM each of adenosine, cytidine, and guanosine triphosphates, 150 units RNasin (Promega, Madison, WI), and 250 μCi [α^{32}P]-uridine triphosphate (UTP) (300°Ci/mmol, Perkin Elmer Life Science), followed by RNA extraction [25]. Equal amounts (1μg) of linearized restriction fragments of CYR61, CTGF, NOV, WISP 1, WISP 2, WISP 3 and linearized plasmid pcDNA 3.1 were immobilized onto GeneScreen Plus by slot blotting (Perkin Elmer Life Science). Equal counts per minute of [^{32}P]-RNA from each sample were hybridized to cDNAs at 42°C for 72 h and washed in 1× SSC at 65°C for 20 min. Hybridized cDNAs were visualized by autoradiography.

Real time reverse transcription (RT) polymerase chain reaction (PCR)

To measure osteocalcin, alkaline phosphatase and runt related transcription factor (Runx)-2, 3 μg of RNA were reverse transcribed in the presence of 5′ CCCAGCACAACTCCTCCCTA 3′ primer for osteocalcin, 5′ ATTCGGG-CAGCGGTTACTGT 3′ for alkaline phosphatase, and 5′ CACGGG-CAGGGTCTTGTTG 3′ for Runx-2 and SuperScript III reverse transcriptase (Invitrogen) and amplified in the presence of 5′ CACTTACGGCGC-TACCTTGGGTAAG[FAM]G 3′ primer for osteocalcin, 5′ CACCATTCTT-CATGTTCTGGGAGATGG[FAM]G 3′ for alkaline phosphatase, and 5′ CACAGGCGACAGTCCCAACTTCCTG[FAM]G 3′ for Runx-2 and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 54°C for 45 cycles, (26). Gene copy number was estimated by comparison with a standard curve constructed using osteocalcin cDNA (ATCC, Manassas, VA), alkaline phosphatase cDNA (ATCC) or Runx-2 cDNA (Y. Ito, Kyoto, Japan) and corrected for GAPDH (ATCC) copy number. GAPDH was reversed transcribed using 5′ AGCTTCCCGTTCAGCTCTGG 3′ primer and amplified in the presence of 5′ CACGCTCTGGAAAGCTGTGGCG[FAM]G 3′, and copy number estimated by comparison with a standard curve. Reactions were conducted in a 96-well spectrofluorometric thermal iCycler (Biorad), and fluorescence was monitored during each PCR cycle at the annealing step [26].

Western blot analysis

Medium aliquots (1 ml) from Ob cell cultures were precipitated with 10% trichloroacetic acid, and the pellet suspended in Laemmli sample buffer to give a final concentration of 2% sodium dodecyl sulfate and fractionated by polyacrylamide gel electrophoresis on a 12% denaturing gel in the presence or absence of reducing agents [27]. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA), blocked with 2% bovine serum albumin (BSA), and exposed to a 1:100 dilution of goat antibody raised against human CYR61, human CTGF, human NOV, murine WISP 1, or murine WISP 2 (all antibodies from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, except for antibody against CTGF from S. Williams, Miami, FL) in 1% BSA overnight. Blots were exposed to a 1:10,000 dilution of rabbit anti-goat IgG antiserum conjugated to horseradish peroxidase and developed with a horseradish peroxidase chemiluminescence detection reagent. To identify immunoreactive proteins, recombinant murine CTGF peptide (S. Williams, Miami, FL), and cellular extracts from Tg, Lg, and A549 cells transfected with NOV, WISP 1 and 2 expression vectors, respectively (all from Santa Cruz Biotechnologies) were

Table 1
Expression of osteocalcin, alkaline phosphatase and Runx-2 mRNA in confluent cultures of Ob cells

Gene copy number/μg RNA			
Hours	Osteocalcin	Alkaline phosphatase	Runx-2
2	4.7 ± 0.4	650 ± 110	1700 ± 510
6	11.5 ± 3.6	1820 ± 490	2190 ± 210
24	17.5 ± 3.8	1390 ± 620	4020 ± 1390
48	22.8 ± 4.5	1470	2810 ± 550

Confluent Ob cells were serum deprived overnight, medium was changed and cells cultured for the times indicated. RNA was extracted and processed by real time RT-PCR. Values are means ± SEM; n = 3, except for alkaline phosphatase at 48 h where n = 2.