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Change in the cells that express connective tissue growth factor in acute Coxsackievirus-induced myocardial fibrosis in mouse

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Abstract

Cardiac fibrosis and inflammation are major pathologic conditions that result from viral myocarditis. Connective tissue growth factor (CTGF) stimulates fibroblast proliferation and induces production of extracellular matrix molecules. We studied the correlation between CTGF and cardiac fibrosis in an acute Coxsackievirus B3 (CVB3) myocarditis animal model. Eight-week-old BALB/c mice were infected intraperitoneally with 10^4 plaque forming units (PFU) of CVB3. Myocardial inflammation peaked on day 7 and decreased markedly by day 14 post-infection (pi); cardiac fibrosis was noted from day 7 and peaked on day 14. By contrast, CTGF was weakly expressed by the interstitial cells in uninfected control hearts and also in the hearts of day 3 pi. CTGF expression measured by real-time PCR was elevated on day 3 and peaked on day 7 pi. TGF-β expression peaked at day 7 pi. The cell type of CTGF expression changed from interstitial cells to myocytes after virus infection. On day 7, CTGF was strongly expressed by myocytes and inflammatory cells surrounding calcified necrotic areas. In addition, cardiac myocytes expressed CTGF on day 14. Our results, based on an acute CVB3 model of myocarditis, provide evidence that CTGF may mediate the development of fibrosis after viral myocarditis, and that the cells expressed CTGF changes during the course of viral myocarditis.

Keywords: Connective tissue growth factor; Fibrosis; Viral myocarditis; Coxsackievirus

1. Introduction

Human pathogenic Coxsackievirus B3 (CVB3) is considered as the most frequent viral cause of myocarditis (Rey et al., 2001). Either complete healing or transition to a progressive disease that may result in dilated cardiomyopathy (DCM) follows acute CVB3 myocarditis (Martino et al., 1994).

Acute myocarditis is an inflammatory disease of the myocardium and results in necrosis of adjacent myocytes due to infiltration of mononuclear cells. Histologically, DCM is characterized by extensive interstitial fibrosis, myocyte hypertrophy and focal replacement fibrosis without active inflammation. Recently, CTGF has been shown to be involved in such cases of myocardial fibrosis; however, the role that CTGF plays in myocardial fibrosis, after CVB3 infection, has not been established CTGF was originally isolated from human umbilical vein endothelial cells; it is a 38 kD protein that contains 38 conserved cysteine residues and a heparin binding domain. It is a member of the CCN protein family, which includes *cyr61*, *nov* and CTGF (also termed fisp-12 in mouse) (Brigstock, 1999). CTGF also belongs to the insulin-like growth binding protein-related protein superfamily, and is sometimes referred to as IGFBP-rP2 (Kim et al., 1997). At the cellular level, CTGF induces extracellular matrix production and proliferation of connective tissue (Frazier et al., 1996; Igarashi et al., 1993). Overexpression of CTGF has been observed during wound repair and in fibrotic disorders of the skin, kidney, liver, lung and pancreas; (Igarashi et al., 1996; Ito et al., 1998; Lasky et al., 1998; Paradis et al., 1999; di Mola et al., 1999) Since these disorders usually exhibit excessive connective tissue and ECM formation, investigators have suggested that CTGF is involved in the development of fibrosis (Mori et al., 1999; Grotendorst et al., 1996). Transforming growth factor-β (TGF-β) up-regulates CTGF expression of fibroblasts *in vitro* (Grotendorst et al.,

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1996). Moreover, anti-CTGF antibodies inhibit secretion of collagen from TGF-β-stimulated fibroblasts (Duncan et al., 1999). Although there is considerable evidence demonstrating that TGF-β has a role in the pathogenesis of myocardial remodeling in heart failure, the role of CTGF in heart failure has not been ascertained to date. In the infarct zone of a rat model, CTGF mRNA expression is up-regulated in both cardiac myocytes and interstitial cells (Chen et al., 2000; Dean et al., 2005). This CTGF expression observed in cardiac fibroblasts and myocytes is known to be induced by TGF-β; in addition TGF-β also increases the production of fibronectin, collagen and plasminogen activator inhibitor-1 (Chen et al., 2000). However, the mechanism by which CTGF mediates myocardial collagen remodeling *in vivo*, and its relevance to inflammatory heart disease remains unclear (Gluck et al., 2001; Grun et al., 2005).

Since there are no reports of CTGF expression in a myocarditis model, we investigated the regulation of myocardial CTGF expression in a CVB3 myocarditis model to determine the time course and cell types associated with CTGF expression in an animal model of CVB3 myocarditis as well as the relationship between CTGF expression and the degree of fibrosis. Our results provide evidence that CTGF plays an important role in the development of fibrosis after viral myocarditis, and that the type of cell that expresses CTGF changes during the course of viral myocarditis.

2. Materials and methods

2.1. Virus and histological analysis

CVB3 was derived from the infectious cDNA copy of the cardiotropic H3 strain of CVB3 (Knowlton et al., 1996). Eight-week-old inbred female BALB/c mice were infected by intraperitoneal injection of 10^4 plaque-forming units (PFU) of CVB3. On days 3, 7 and 14 mice were euthanized and their serum and hearts were collected. The hearts were fixed in 10% formalin, embedded in paraffin and sections were stained with hematoxylin and eosin, and Masson's trichrome.

2.2. Analysis of CTGF, TGF-β and procollagen 1 gene expression

To quantify CTGF, TGF-β and procollagen 1 expression, real-time PCR using 5 ng DNA was performed as previously described (Sakamoto et al., 2001). For the mRNA quantification, complementary DNA (cDNA) was synthesized using 1 μg RNA through a reverse transcription reaction (Maxime RT premix kit, iNtRON Biotechnologies, Korea). Real-time PCR quantitative mRNA or DNA analyses were performed in an ABI Prism 7000 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) to quantify amplicons. The standard PCR conditions were 95 °C for 10 min, then 40 cycles at 95 °C (30 s), 55 °C (30 s), and 72 °C (30 s), followed by the standard denaturation curve. The sequences of primers were designed using primerexpress software (Applied Biosystems) with nucleotide sequences found in the GenBank database. The primer sequences:

(1) for the internal control, glyceraldehydes phosphate dehydrogenase (GAPDH): 5′-GCCAAGGATATCCATGACAACT-3′ and 5′-CTGCCTGGTCCAGCCACAGA-3′;
(2) for CTGF: 5′-CAAAGCAGCTGCAAATACCA-3′ and 5′-GGCCAAATGTGTCTTCCAGT-3′;
(3) for procollagen 1: 5′-ACGTCCTGGTGAAGTTGGTC-3′ and 5′-CAGGGAAGCCTCTTTCTCCT-3′;
(4) for TGF-β: 5′-ATACAGGGCTTTCGATTCAG-3′ and 5′-CAGCAGTTCTTCTCTGTGGA-3′.

PCR conditions for each target were conscientiously optimized with regard to primer concentration, absence of primer–dimer formation, and efficiency of amplification of target genes and the housekeeping gene control. SYBR Green PCR Master Mix (Applied Biosystems), 200 nm specific primers, and 2.5 ng cDNA were used in each reaction. The threshold for positivity of real-time PCR was determined based on negative controls. For mRNA analysis the calculations for determining the relative level of gene expression were made according to the instructions from the User's Bulletin (P/N 4303859) from Applied Biosystems, by reference to the GAPDH in the sample, using the cycle threshold (Ct) method. The mean Ct values from triplicate measurements were used to calculate the expression of the target gene with normalization to a housekeeping gene used as an internal control (GAPDH), using the 2–A¨ Ct formula, also according to the User's Bulletin. The levels of negative controls without RNA or DNA and without reverse transcriptase were also calculated.

2.3. In situ hybridization

A 1.1 kb fragment of human CTGF cDNA cloned into pcDNA3.1 (Invitrogen, USA) was used. Antisense and sense digoxigenin-UTP-labeled cRNA probes were synthesized by *in vitro* transcription with the relevant RNA polymerase (Boehringer, Mannheim, Germany). Paraffin-embedded sections (6 μm) of mouse hearts were deparaffinized in xylene, rehydrated and treated with 0.1% Triton X-100 in PBS. The sections were permeabilized with 20 μg/ml of proteinase K, acetylated with 0.1 M triethanolamine and acetic anhydride, and prehybridized for 1 h at 68 °C. Hybridization mixture (50% formamide, 2× SSC, 5% dextran sulfate, 150 μg/ml tRNA, 150 μg/ml denatured salmon sperm DNA, 5× Denhardt's solution) was then applied to the section, which was subsequently incubated overnight at 68 °C with a digoxigenin-labeled CTGF-specific riboprobe. After hybridization, the sections were washed with 4× SSC and 2× SSC (50% formamide) at 50 °C. Immunologic detection of digoxigenin-labeled transcripts was performed according the manufacturer's protocol (Boehringer Mannheim, Germany). Alkaline phosphatase was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride (BCIP/NBT; Sigma, USA) and levamisole at room temperature in the dark. Sections were dehydrated and coverslips applied. For the negative controls, *in situ* hybridization with a sense probe was performed.