A Novel Activator of C-C Chemokine, FROUNT, is Expressed With C-C Chemokine Receptor 2 and its Ligand in Failing Human Heart

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ABSTRACT

Background: A novel activator of C-C chemokines, FROUNT, directly binds C-C chemokine receptor (CCR) 2 and plays a central role in the chemokine system. Activation of the chemokine system appears to be involved in the pathogenesis of congestive heart failure (CHF). The purpose of this study was to determine whether FROUNT is expressed with CCR2 and its ligand (CCL2) in failing human heart.

Methods and Results: We examined endomyocardial biopsy tissues obtained from 71 patients with CHF (HF group) and 20 subjects without CHF (non-HF group). FROUNT, CCR2, and CCL2 mRNA levels were higher in the HF group than in the non-HF group ($P < .001$). FROUNT mRNA levels were positively correlated with CCR2 and CCL2 mRNA levels in the HF group. FROUNT and CCL2 signal was seen in the cytoplasm of cardiac myocytes in failing hearts. Levels of FROUNT mRNA were negatively correlated with left ventricular ejection fraction. FROUNT, CCR2, and CCL2 mRNA levels were higher in the severe HF subgroup than in the mild HF subgroup.

Conclusions: The expression of FOUNT-mediated CCL2/CCR2 may have important implications in the pathogenesis of CHF. The CCL2/CCR2 pathway via FROUNT may influence the clinical severity of CHF. (*J Cardiac Fail 2007;13:114−119*)

Key Words: Congestive heart failure, Dilated cardiomyopathy, In situ hybridization, Real-time RT-PCR.

Previous studies have shown that activation of inflammatory reaction may be involved in the progression of congestive heart failure (CHF) through mechanisms such as activated tumor necrosis factor-α cascade, activation of inducible nitric oxide synthesis, and an alteration in extracellular matrix in failing human heart.[1−3] Activation of leukocytes and monocytes/macrophages and migration of these cells from the circulation to areas of myocardial inflammation appear to be important factors in the immunologic response in CHF.[4,5] Chemokines represent a family of inflammatory cytokines that control chemotaxis of leukocyte subsets into inflamed tissues.[6] Chemokines are a family of small molecular mass proteins (8 to 16 kd), which are classified into subfamilies on the basis of their conservation of a 4-cysteine motif and their ability to cause the directed migration of leukocytes in vitro.[7] Raised levels of C-C chemokine receptor 2 (CCR2) and its ligand (CCL2), also called macrophage chemoattractant protein-1, may not only be a parameter of enhanced immune activation in CHF, but may also reflect an important pathogenic mechanism in this disease.[8]

A recent report has indicated that a novel clathrin heavy chain homology protein, FROUNT, is essential for linking of activated CCR2 to the PI(3)K-Rac-lamellipodium protrusion cascade during chemotaxis.[9] It may be hypothesized that FROUNT plays a central role in the CCL2/CCR2 pathway in chronic inflammatory diseases associated with macrophage infiltration, such as CHF. However, the precise mechanism underlying the connection between FROUNT expression and the CCL2/CCR2 pathway in failing human heart is uncertain. On the basis of these considerations, our aim was to determine whether the FROUNT-mediated

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CCL2/CCR2 pathway is activated in failing human heart, and whether this is related to clinical parameters in CHF.

Methods

Subjects

We examined myocardial tissues obtained from 71 patients with CHF by right ventricular endomyocardial biopsy. This biopsy was performed to identify the causal disease underlying left ventricular (LV) dysfunction. After the biopsy, the origin of LV dysfunction was diagnosed as primary dilated cardiomyopathy (DCM) in all of these patients. The clinical diagnosis of DCM was made according to the World Health Organization/International Society and Federation of Cardiology Task Force criteria.[10] Patients were excluded from the study if they had clinical signs of acute infection, severe renal failure, rheumatoid disease, or myocardial infarction within the previous 6 months or if they were suspected of having a malignant or primary wasting disorder. Left ventriculography was performed to determine LV ejection fraction (LVEF) and LV end-systolic volume index at the time of biopsy.

Nonfailing heart (non-HF) samples were obtained by endomyocardial biopsy from 20 subjects with suspected cardiac disorders such as myocardial deposition disease, hypertensive heart disease, and hypertrophic cardiomyopathy, on the basis of arrhythmia and echocardiographic changes such as premature beats and a slight increase in ventricular wall thickness. The resulting pathologic findings and close clinical examination failed to show any evidence of myocardial disease or functional abnormality, and these subjects were thus designated as a non-HF group. This study protocol was approved by our hospital ethics committee, and written informed consent was obtained from all subjects.

Extraction of RNA

Total RNA was extracted from endomyocardial biopsy tissues by the acid guanidinium thiocyanate-phenol-chloroform method, and treated with DNase I (GIBCO BRL).[11]

Oligonucleotides of Primers and Probes

The following sequences for primers and probes were used for relative quantification of targeted gene expression for FROUNT (GenBank accession number AF498261): forward primer 5′-ACG CTC GTG TCA GAC AGG TTC-3′, reverse primer 5′-TCA CTG AGC ATC ATG GCT GG-3′ and probe 5′-AGC GAT TAC TGT GAG CGA GGC TGC TTT TC-3′, CCR2 (GenBank accession number NM_000647): forward primer 5′-GCG TTT AAT CAC ATT CGA GTG T-3′, reverse primer 5′-TGG CAA ATT AGG GAA CAA TAT G -3′ and probe 5′-AGT GCT TCG CAG ATG TCC TTG ATG C-3′, CCL2 (GenBank accession number Y18933): forward primer 5′-CAA GCA GAA GTG GGT TCA GGA T-3′, reverse primer 5′-AGT GAG TGT TCA AGT CTT CGG AGT T-3′ and probe 5′-CAT GGA CCA CCT GGA CAA GCA AAC C-3′. For all myocardial specimens, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified using TaqMan GAPDH control reagents as an internal control (PE Biosystem, Foster City, CA).

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction

We analyzed mRNA expression levels using a real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method as previously described.[1] The cDNA was synthesized and amplified from total RNA and human control RNA (PE Biosystem) by reverse transcription and PCR using a Taq Man EZ RT-PCR kit (PE Biosystem). A real-time quantitative PCR method was developed using real-time detection and 5′ nuclease assay by an ABI PRISM 7700 sequence detector (PE Biosystem). To improve the accuracy of real-time RT-PCR for quantification, amplifications were performed in triplicate for each RNA sample. To account for variations in input RNA and RT efficiency, FROUNT, CCR2. and CCL2 RNA levels were normalized to GAPDH expression in each sample. To account for PCR amplification of contaminating genomic DNA, a control without RT was included.

In Situ Hybridization

In situ hybridization was performed on paraffin sections to identify the cellular sources of FROUNT and CCL2 mRNA expression. Antisence oligonucleotide probes of FROUNT (5′-GAA AAG CAG CCT CGC TCA CAG TAA TCC CT-3′) and CCL2 (5′-CAT GGA CCA CCT GGA CAA GCA AAC C-3′) were used for in situ hybridization (GenBank accession number AF498261 and Y18933, respectively). The probe was labeled with a 3′-biotinylated tail (Britati tail). For every specimen, we used a 20-base poly-T oligonucleotide probe (Research Genetics) to examine the retention of mRNA in biopsy samples. Hybridization was performed with a MicroProbe staining system (Fisher Scientific). Tissue sections were placed on Probe ON Plus microscope slides (Fisher Scientific) and were rapidly dewaxed, cleared with alcohol, rehydrated with Tris-based buffer, and then digested with pepsin for 3 minutes at 105°C. The probes were applied in a formamide-free diluent, and the slides were heated to 105°C for 3 minutes, cooled to room temperature, and allowed to hybridize at 40°C for 30 minutes. The sections were then washed with 2X SSC buffer (300 mmol/L NaCl and 30 mmol/L trisodium citrate, pH 7.0) at 45°C and detected with alkaline phosphatase-conjugated streptavidin. After hybridization, the products were washed in AP chromogen buffer, pH 9.5 at room temperature, and then visualized with fast red. The slides were counterstained with hematoxylin, air dried, and then coverslipped for microscopic examination.

Statistical Analysis

All values are presented as mean ± SE. Kolmogorov-Smirnov analysis was performed to assess data distribution. An unpaired *t*-test was performed for normally distributed data, and nonparametric Mann-Whitney test was performed when this was not appropriate. Spearman correlation coefficients were used to examine the relationship between FROUNT, CCR2, and CCL2 levels and clinical parameters. A value of $P < .05$ was considered statistically significant.

Results

Baseline Characteristics

Baseline characteristics of the HF and non-HF groups are shown in Table 1. Data from LV geometry and pressure studies showed significant differences between the groups. There were no differences between the groups for the other baseline data.