ORGINAL ARTICLE
Iran J Allergy Asthma Immunol
March 2009; 8(1): 1-9

Coxsackievirus B3 Infection Induced Viral Myocarditis by Regulating the Expression Pattern of Chemokines in Cardiac Myocytes

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Received: 10 April 2008; Received in revised form: 15 July 2008; Accepted: 27 July 2008

ABSTRACT

Viral myocarditis is a common cardiovascular disease, which has greatly threatened human health. However, up to now, the pathogenesis of viral myocarditis has been unclear, which leads to the lack of its effective treatments.

To investigate the role of chemokines in pathogenesis of viral myocarditis, mRNA expression for a panel of 19 chemokines detected by RT-PCR in myocardial tissue of BALB/c mice that were inoculated intraperitoneally with coxsackievirus B3. Moreover primary cultured cardiac myocytes were infected with coxsackievirus B3 following extraction of RNA, from myocytes the expression of 19 chemokines was detected by by RT-PCR.

Our results showed that there was much difference in the expression pattern of chemokines in myocardial tissue between infected mice with viral myocarditis and uninfected control mice. The expression of chemokines was varied significantly in clusters in myocardium post coxsackievirus B3 infection. There were also complexity and imbalance in the change of the expression of chemokines. In the meantime, Coxsackievirus B3 infection also influenced the expression pattern of chemokines in cardiac myocytes *in vitro*. However the expression of monocyte chemoattractant protein-1 alone was upregulated in cardiac myocytes post coxsackievirus B3 infection in the 19 detected chemokines.

The chemokine expression pattern changed in complexity and imbalance manner both in myocardium and in primary cultured cardiac myocytes after coxsackievirus B3 infection. Coxsackievirus B3 infection may start viral myocarditis by regulating the expression pattern of chemokines in cardiac myocytes. MCP-1 may be one of key chemokines in the initial stage of viral myocarditis.

Key word: Chemokine; Expression profile; Pathogenesis; Viral myocarditis

INTRODUCTION

Viral myocarditis is a common cardiovascular disease, which has become one of the main causes of sudden unknown death events in teenagers. Moreover chronic viral myocarditis often results in dilated cardiomyopathy.[1] However, up to now, the pathogenesis of viral myocarditis has been unclear, which leads to the inadequacy of its effective treatments. [2] It is known that coxsackievirus B3(CVB3) is an important

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pathogen of viral myocarditis, which accounts for more than fifty percent viral myocarditis cases. Previous studies showed that CVB3 not only could injure directly cardiac myocytes by Pro2A,[3] but also induce indirect injury by non-specific and specific immune responses.[4] Immunological injury in viral myocarditis is associated with the infiltration of a great number of inflammatory cells that is one of characteristic pathological changes in viral myocarditis.[5,6]

Generally, the infiltration of inflammatory cells is a complex and multiple-stepped process involving many cells and molecules.[7]Induced by inflammatory signals and cell-adhesion molecules, various inflammatory cells accumulate at the site of infection.[8] It has been confirmed that chemokines can provide migrating signal, activate cells and initiate the trafficking of the inflammatory cells.[9] More interestingly, several diseases are known to be associated with inappropriate activation of the chemokine network, including cardiovascular disease, allergic inflammatory disease, transplantation, neuroinflammation, cancer and HIV-associated disease.[10] Considering the character of pathological change in myocardium of viral myocarditis, we hypothesized that chemokines could be involved in the pathogenesis of viral myocarditis. However, it is unknown whether CVB3 can induce the expression of chemokine(s) in viral myocarditis. In the present study, the expression of 19 characteristic chemokines in four chemokine subfamilies was detected by RT-PCR in mouse models of viral myocarditis induced by CVB3 *in vivo* and *in vitro*. The results of this study will help understand the molecular pathogenesis of viral myocarditis and provide a potential target for prevention and therapy of viral myocarditis.

MATERIALS AND METHODS

Mice and Virus

Four-week-old male BALB/c mice and neonatal mice were purchased from Experimental Animal Center of Fudan University. CVB3 (Nancy strain) was propagated in HeLa cells and purified by a method previously described by Michael et al.[11] Virus titer was routinely determined prior to infection by 50% tissue culture infectious dose ($TCID_{50}$) assay of HeLa cell monolayers according to previously published procedures.[12] Then the aliquots were stored at -70°C.

Preparation of Murine Cardiac Myocytes

Hearts were removed aseptically from neonatal mice within 72 hours of birth. Single cell suspensions of myocytes were prepared using a modified method of Huber et al.[13] Briefly, the hearts were minced finely and subjected to stepwise enzymatic digestion with 0.25% pancreatin. The dissociated cells were washed with complete basal Eagle's medium and depleted of endothelial cells and fibroblasts by two sequential 1-hour absorptions to plastic flasks at 37˚C. The nonadherent myocytes were removed, washed once, resuspended in complete basal medium and dispensed into tissue culture wells. After a period of 48 hours which was required for the myocytes to firmly attach to plastic, the cells were used as described below. According to observation on shape and beat of the obtained cells, more than 95% cells were identified to be cardiac myocytes.

Murine Model of CVB3 Infection *in vitro*

Cardiac myocytes were infected with 10 $TCID_{50}$ CVB3. After 2 hours, total RNA was extracted with TRIzol reagent. The expression of 19 chemokines was detected by RT-PCR. The amplicons were electrophoresed in 1.5% agarose gel and the bands were scanned.

Murine Model of CVB3 Myocarditis *in vivo*

Thirty four-week-old male BALB/c mice were inoculated intraperitoneally with 10^5 $TCID_{50}$ CVB3 in 100µl basal Eagle's medium, which constituted the murine model of CVB3 myocarditis *in vivo*, 30 mice were also inoculated with 100µl basal Eagle's medium which served as uninfected controls. Hearts and blood were aseptically collected from mice after 7 days post-infection. The collected hearts were washed in sterile basal Eagle's medium and portions of the hearts were snap-frozen in liquid nitrogen and stored at -70 °C for RNA extraction and PCR analysis. The remaining parts of the collected hearts were fixed in formal-buffered saline for histological studies. The sera isolated were used for detection the levels of creatine phosphokinase-MB (CK-MB).

RNA Extraction and Reverse Transcription

Total RNA was extracted from cultured myocytes in vitro by Trizol reagent (Biobasic Inc, Canada) according to the manufacturers' instruction. The RNA concentration was estimated by measurement of absorbance at 260 and 280 nm. The integrity of the RNA sample was assessed by resolution on a 1% formaldehyde-agarose gel and ethidium bromide staining. Each RNA sample was aliquoted and stored at -70˚C until it was used in RT-PCR experiment.

To synthesize the first-strand cDNA, approximately 1

μg of total RNA and 1 μl of oligo(dT) (0.5 μg/μl) were added into a microtube and sterile diethylpyrocarbonate-treated water was added to a total volume of 12 μl. The reaction tube was heated at 70˚C for 5 min and then chilled on ice. One μl of 10 mM mixed dinucleoside triphosphate, 4 μl of 5 × first-strand buffer, 2 μl of 0.5 M dithiothreitol and 1 μl of Moloney murine leukemia virus reverse transcriptase (M-MuLV RT) were added. The reaction mixture was incubated at 37˚C for 60 min. The reaction was terminated by incubation at 70˚C for 10 min, and the tube was transferred onto ice.

External Standard for Real-time RT-PCR

To obtain an external standard for quantitative PCR assay, the monocyte chemoattractant protein-1(MCP-1) cDNA was cloned into plasmid vector and the sequence was detected. The PCR products of MCP-1 were generated from mice hearts with 1.5 U of recombinant Taq DNA polymerase (Takara, Kyoto, Japan) and 10 pmol of each of the two primers, 5'-CGGAATTCGCCACCATG CAGGTCCCTGTCAT-3' (sense) and 5'-GCTCTAGAC TAGTTCACTGTCACACT-3' (antisense). The PCR products were purified and directly inserted into the plasmid pVAX1. The recombinant plasmids were then used to transform Escherichia coli DH5α competent cells. The plasmids of individual resistant colonies were isolated, and the inserts were sequenced. The plasmid DNA was quantitated by measurement of absorbance at 260 nm and used as external standard for quantitative RT-PCR assay. After quantitation, 5.51×10, 5.51×10^2, 5.51×10^3, 5.51×10^4 and 5.51×10^5 copies of the MCP-1 fragment were measured for standard curve and analysis of stability of external standard for real-time RT-PCR. The R^2 of the standard curve was 0.994. The CVs of the inter-day and intra-day experiments were less than 10 percent.

Quantitative Measurement of Chemokine mRNA by Real-time RT-PCR

The quantitative analysis of chemokine mRNA was done using a LightCycler™ (Roche Diagnostic Company, Mannheim, Germany) with external standard of MCP-1. Glycerol 3-phosphate dehydrogenase (GAPDH) for Housekeeper gene was used as internal standard, and relative quantity of chemokine was represented by the ratio of the expression quantity of chemokine to that of GAPDH. The reaction mixture consisted of 2 μl of LightCycler™ DNA Master SYBR Green I. The cycling program had a 10 min initial denaturation at 95˚C and then entered a cycle of an instant 95˚C denaturation, 10 s of annealing at 60˚C and 12 s of extension at 72˚C with transition rate of 20˚C/s between temperature plateaus for a total of 40 cycles. Quantification data were analyzed using the LightCycler analysis software version 3.

Histological Studies

For histological analysis, hearts were fixed in 10% neutral buffered formalin and sectioned into 5-mm slices. Sections were stained with hematoxylin and eosin (H&E).

Two investigators who were blinded to the experimental treatment analyzed myocardial necrosis and infiltration of inflammatory cells in sections.

Statistical Analysis

The results were analyzed using two-tailed independent Student's t-test. The level of statistical significance was set at $P<0.05$.

RESULTS

Expression Profile of Chemokines in Myocardial Tissue of Mice with Viral Myocarditis *in vivo*

The chemokine superfamily is divided into four subfamilies (CXC, CC, C, CX3C) based on the arrangement of their amino terminal cysteine residues. The expression of 19 chemokines (summarized in Table 1) was detected in myocardial tissue of BALB/c mice. The results showed that 13 chemokines including macrophage inflammatory protein-2 (MIP-2), monokine induced by γ-interferon (MIG), interferon-γ inducible protein-10(IP-10), stromal cell derived factor-1 (SDF-1) (CXC family), macrophage inflammatory protein-1β (MIP-1β), MCP-1, monocyte chemoattractant protein-2 (MCP-2), monocyte chemoattractant protein-3(MCP-3), monocyte chemoattractant protein-5 (MCP-5), macrophage derived chemokine (MDC), regulated on activation normal T cell (RANTES) (CC family), lymphotactin(LTN)(C family) and fractalkine (FKN) (CX3C family) were detectable in the myocardial tissue of mice with viral myocarditis, whereas only 10 chemokines including SDF-1, MIP-1β, MCP-1, MCP-2, MCP-3, MCP-5, MDC, RANTES, FKN, LTN were found in normal control. Six chemokines including B lymphocyte chemoattractant (BLC), breast and kidney chemokine (BRAK), macrophage inflammatory protein-1α (MIP-1α), eotaxin (Eot), liver-and activation-related chemokine (LARC) and ALP were not detected either in viral myocarditis group or in uninfected control(Figure 1).

Table 1. The Chemokines and Overview of the primers used in this study.

Official name	Common synonyms	Primer sequences
CXCL2	MIP-2	sense 5'-CACTTCAGCCTAGCGCCAT-3'
		antisense 5'-AGGTCAGTTAGCCTTGCCT-3'
CXCL9	MIG	sense 5'-CTCAGCTCTGCCATGAAG-3'
		antisense 5'-TCTTATGTAGTCTTCCTTG-3'
CXCL10	IP-10	sense 5'-TCAGCACCATGAACCCAAGT-3'
		antisense 5'-GCGTGGCTTCTCTCCAGTT-3'
CXCL12	SDF-1	sense 5'-CGGAATTCGCCACCATGGACGCCAAGGTCG-3'
		antisense 5'-GCTCTAGAGCCTTGTTTAAGCTTTCTCCAGG-3'
CXCL13	BLC	sense 5'-GAATGAGGCTCAGCACAGC-3'
		antisense 5'-CTTCAGGCAGCTCTTCTCTT-3'
CXCL14	BRAK	sense 5'-TCCGGCCAGCATGAGGCT-3'
		antisense 5'-ATGATCGTCCACCCTATTCT-3'
CCL2	MCP-1	sense 5'-CGGAATTCGCCACCATGCAGGTCCCTGTCAT-3'
		antisense 5'-GCTCTAGACTAGTTCACTGTCACACT-3'
CCL3	MIP-1α	sense 5'-TCACCTGCTCAACATCATG-3'
		antisense 5'-TCAGGCATTCAGTTCCAGGT-3'
CCL4	MIP-1β	sense 5'-AGCAACACCATGAAGCTCT-3'
		antisense 5'-AGCTGCTCAGTTCAACTC-3'
CCL5	RANTES	sense 5'-CGGGATCCGCCACCATGAAGATCTCTGCAG-3'
		antisense 5'-CGGAATTCTCTATCCTAGCTCATCT-3'
CCL7	MCP-3	sense 5'-CCATGAGGATCTCTGCCAC-3'
		antisense 5'-TTCTGTTCAGGCACATTTCTT-3'
CCL8	MCP-2	sense 5'-AACATGAAGATCTACGCAGT-3'
		antisense 5'-AGGTTCAAGGCTGCAGAATT-3'
CCL11	Eot	sense 5'-ACCATGCAGAGCTCCACAG-3'
		antisense 5'-GTGTCAAGAGAGGAGGTTGT-3'
CCL12	MCP-5	sense 5'-GACCTCAACATGAAGATTTC-3'
		antisense 5'-ACTCTCAGCCTAGACATG-3'
CCL20	LARC	sense 5'-AATGGCCTGCGGTGGCAAG-3'
		antisense 5'-TTACATCTTCTTGACTCTTAG-3'
CCL22	MDC	sense 5'-CATCATGGCTACCCTGCGT-3'
		antisense 5'-CCTAGGACAGTTTATGGAGT-3'
CCL27	ALP	sense 5'-GTGAGCATGATGGAGGGGCT-3'
		antisense 5'-TCGTCTAATGTTGCTTTATTAG-3'
CX3CL1	FKN	sense 5'-CGGAATTCGCCGCCATGGCTCCCTCGCC-3'
		antisense 5'-GCTCTAGATCAGGAGCTAGATCCAGATTG-3'
XCL1	LTN	sense 5'-GACCTCAGCCATGAGACT-3'
		antisense 5'-GTTACCCAGTCAGGGTTAT-3'
	GAPDH	sense 5'-CTGCACCACCAACTGCTTAG-3'
		antisense 5'-GTCTGGGATGGAAATTGTGA-3'

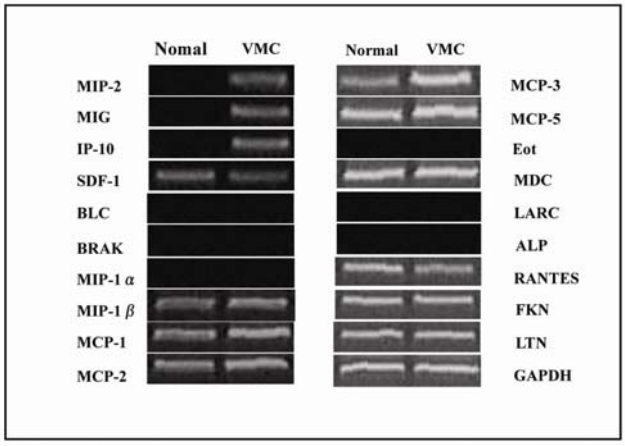

Figure 1. Expression profile of chemokines in myocardial tissue of mice. Male BALB/c mice were inoculated intraperitoneally with CVB3 or MEM Eagle medium. After 7 days post infection, hearts were removed and total RNA of myocardial tissue was extracted. The expression of chemokines was detected by RT-PCR. The amplicons were electrophoresed in 1.5% agarose.

Quantitative Analysis of Chemokines in Myocardial Tissue of Mice with Viral Myocarditis *in vivo*

Because the expression quantity of chemokines is associated with their biological function, the expression of positive chemokines was also detected quantitatively by real-time RT-PCR. Among the constitutively expressed chemokines, seven of them including MIP-1β, MCP-1, MCP-2, MCP-3, MCP-5, MDC and LTN should up-regulated expression, which were 2.2,1.9,1.4,3.6,1.5,1.5 and 1.1 folds respectively higher than that of uninfected control (120%, 92%, 37%, 255%, 54%, 47% and 15% *vs.* uninfected control)(P<0.01). RANTES showed down-regulated expression, which was reduced 16% *vs.* control (P<0.05).

There was no significant difference between viral myocarditis and control in expression of FKN and SDF-1 (P>0.05). MIP-2, MIG and IP-10 were inducible in viral myocarditis group, whereas there were not detected in uninfected control (Figures 2 and 4). Then the chemokines were ranked by the quantity of expression in myocardial tissue of uninfected control mice, the sequences from high expression to low expression were RANTES, SDF-1, MCP-5, MCP-2, MDC, LTN, MCP-1, FKN, MCP-3 and MIP-1β, whereas in myocardial tissue of mice with viral myocarditis, the sequences were MCP-3, IP-10, MCP-1, MCP-5, MDC, MCP-2, MIG, MIP-1β, MIP-2, SDF-1, LTN, RANTES and FKN (Figures 3 and 4).

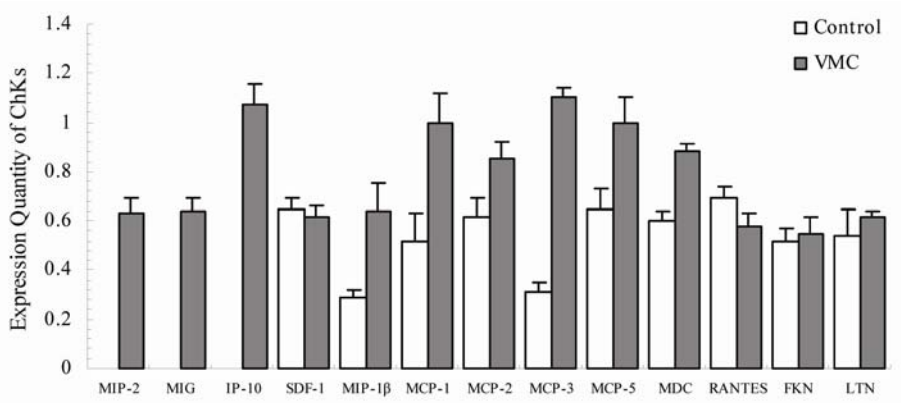

Figure 2. Expression quantity of chemokines in myocardial tissue of mice. The expression of chemokines was detected quantitatively by real-time RT-PCR. The expression quantity of chemokines(ChKs) was represented by the ratio of the expression quantity of chemokines to the expression quantity of GAPDH.

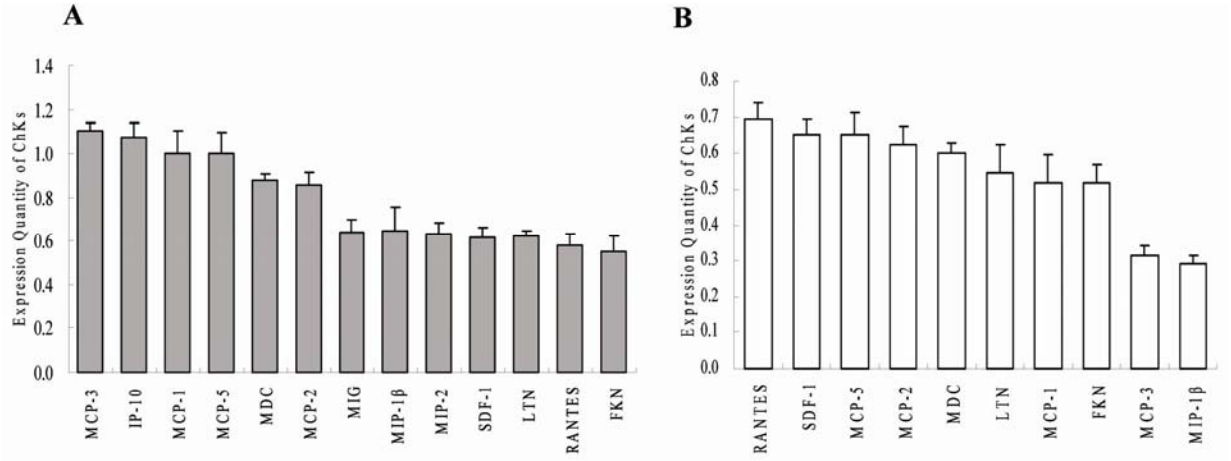

Figure 3. Expression of chemokines in myocardial tissue of mice. The expression quantity of chemokines(ChKs) from high to low expression was ranked in mice with viral myocarditis(A) and normal control mice(B).

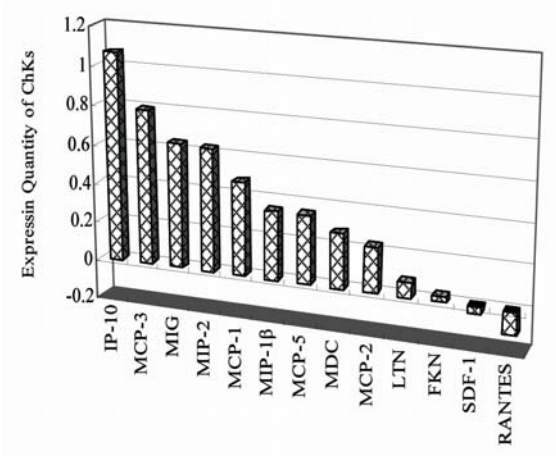

Figure 4. Changed expression quantity of chemokines in myocardial tissue of mice with viral myocarditis. Changed quantity of chemokines(ChKs) was obtained by the expression quantity of viral myocarditis substracted from the expression quantity of normal.

Expression of Chemokines in Cultured Cardiac Myocytes with CVB3 Infection *in vitro*

To investigate regulation of CVB3 infection in the cardiac myocytes, the expression of 19 chemokines was also detected by RT-PCR in primary cultured cardiac myocytes infected with CVB3. The results are presented in figure 5 and table 2. 10 chemokines including MIP-2, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, MCP-3, MCP-5, LARC and MDC were detectable in uninfected cardiac myocytes, whereas only 7 chemokines including MIP-2, IP-10, MCP-1, MIP-1β, RANTES, MCP-3, MDC were found in CVB3-infected cardiac myocytes. 9 chemokines (MIG, SDF-1, BLC, BRAK, MCP-2, Eot, ALP, FKN and LTN) were detected neither in infected cardiac myocytes nor in uninfected control. IP-10, MIP-1α, RANTES, MCP-5 and LARC showed down-regulated expression in infected cardiac myocytes compared with uninfected cardiac myocytes. There was no significant difference between CVB3-infected cardiac myocytes and uninfected cardiac myocytes in expression of 4 chemokines (MIP-2, MIP-1β, MCP-3 and MDC).

Table 2. Expression of chemokines in primary cultured cardiac myocytes.

Chemokine	Uninfected myocytes	Infected myocytes
CXCL2/MIP-2	+	+
CXCL9/MIG	-	-
CXCL10/IP-10	+	+
CXCL12/SDF-1	-	-
CXCL13/BLC	-	-
CXCL14/BRAK	-	-
CCL2/MCP-1	+	+
CCL3/MIP-1α	+	-
CCL4/MIP-1β	+	+
CCL5/RANTES	+	+
CCL7/MCP-3	+	+
CCL8/MCP-2	-	-
CCL11/Eot	-	-
CCL12/MCP-5	+	-
CCL20/LARC	+	-
CCL22/MDC	+	+
CCL27/ALP	-	-
CX3CL1/FKN	-	-
LTN	-	-
GAPDH	+	+

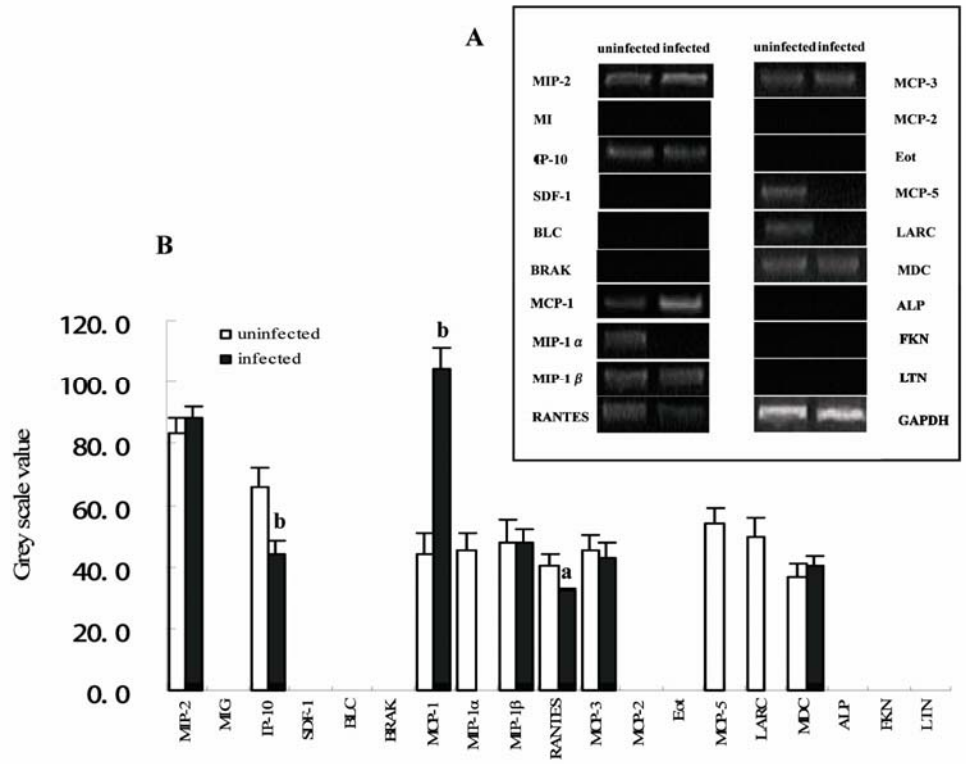

Figure 5. Expression of chemokines in primary cultured cardiac myocytes. Cardiac myocytes were separated by digestion with 0.08% trypsin and infected with CVB3. After 2 hours, total RNA was extracted with TRIzol reagent. The expression of 19 chemokines was detected by RT-PCR. The amplicons were electrophoresed in 1.5% agarose gel (A) and the bands were scanned (B).a *P* <0.05 vs. uninfected. b *P* <0.01 vs uninfected.

Only MCP-1 was up-regulated in infected cardiac myocytes. The expression of MIP-2 was the highest in quantity among the positive expressed chemokines in uninfected cardiac myocytes, whereas the expression of MCP-1 was the highest in quantity in CVB3-infected cardiac myocytes.

DISCUSSION

Previous study showed that there was a strong association between several diseases with chemokines and chemokine receptors.[10] Considering the character of pathological changes in myocardium of viral myocarditis and the ability of chemokines to recruit potentially destructive leukocytes in tissues, we supposed that chemokines were involved in the pathogenesis of viral myocarditis. In this paper, we found that there was much difference in the expression pattern of chemokines in myocardial tissue between mice with viral myocarditis and uninfected control mice. The expression of chemokines in myocardial tissue of viral myocarditis varied significantly in clusters including not only one chemokine, but also many chemokines. Moreover there was imbalance and complexity in expression quantity of every chemokine in myocardial tissue of viral myocarditis, in which some chemokines were upregulated, while others were down-regulated. These results indicated that the chemokine expression in the myocardium of mice with viral myocarditis was regulated in complex ways, likely from CVB3, TNF-a, IL-1, IL-6, infiltrating inflammatory cells and so on.[14-17] Each of these factors alone or in combination could determine the expression pattern of chemokines in viral myocarditis.

Because multifactors and multicells may contribute to the change of chemokine expression pattern in myocardium of mice with viral myocarditis, further research is needed to study the regulation of CVB3 infection on the expression of chemokines in cardiac myocytes. This study indicated that 10 chemokines of the 19 detected chemokines including MIP-2, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, MCP-3, MCP-5, LARC and MDC were constitutively expressed in cardiac myocytes, and the other chemokines were not detected. To our surprise, when the cardiac myocytes were infected by CVB3, the

expression of 5 chemokines among the 10 chemokines was down-regulated, expression of 4 chemokine was not affected, only the expression of MCP-1 was up-regulated overtly. The results suggest that CVB3 infection induced different regulation on different chemokines in the cardiac myocytes. Most Interestingly, the expression change of chemokines in cardiac myocytes appeared after two hours post CVB3 infection, which happened much earlier than the infiltration of inflammatory cells in myocardium of mice after CVB3 infection.[5] It strongly suggests that CVB3 infection may start viral myocarditis by regulating the expression pattern of chemokines in cardiac myocytes, and that MCP-1 may be one of the key chemokines in the initial stage of viral myocarditis.

Previous studies have suggested that CVB3 influences hostgene responses by several mechanisms.[18,19] Subsequent to virus attachment to a target cell receptor, CVB3 RNA is released into the cell and acts as a template for translation of the viral polyprotein and replication of the viral genome. Viral receptors include the coxsackievirus and adenovirus receptors and the decay-accelerating factor coreceptor.[20,21]

The mitogen-activated protein kinases manipulate the signaling machinery regulating CVB3 replication and host gene responses, responding to diverse extracellular stimuli and transducing signals from the cell membrane to the nucleus.[22,23] Mitogen-activated protein kinases constitute a superfamily of highly related serine/threonine kinases. At least seven members of the mitogen-activated protein kinase family have been identified in mammals, of which ERK 1 and 2 regulate a wide range of cellular functions, including cell proliferation, transformation, differentiation, and notably cell survival and death.[24,25] Small GTP-binding protein Ras has been shown to activate the Raf/MEK/ERK cascade by binding to Raf and anchoring it at the cell membrane, where Raf is phosphorylated and activated by other kinases.[26] Recently,the ERK pathway has been implicated in the regulation of viral gene expression and replication for CVB3. [27] Our finding that CVB3 infection has different effects on the expression of different chemokines is worth noticing. Several mechanisms may account for the expression pattern of chemokines by CVB3 infection in cardiac myocytes. For example, interaction of CVB3 (through its structural protein[s]) with its receptor, replication of CVB3 virus, and signal transduction by the ERK1 and ERK2 pathways may all contribute to the regulation of chemokine expression in CVB3-infected cardiac myocytes.

In summary, the present study revealed the change of chemokine expression pattern in cardiac myocytes post CVB3 infection. The results from this study will contribute to the understanding of the mechanism of viral myocarditis and may be extended to other inflammation-associated infectious diseases.

ACKNOWLEDGMENTS

This work was supported by Henan Province Research Project Grant (0496060902), the Major State Basic Research Development Program of the People's Republic of China (2001CB510005) and the Key Scientific and Technological Program of the Ministry of Education.

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