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The mouse CCR2 gene is regulated by two promoters that are responsive to plasma cholesterol and peroxisome proliferator-activated receptor γ ligands

Yiming Chen, Simone R. Green, Jessica Ho, Andrew Li, Felizidad Almazan, Oswald Quehenberger *

Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0682, USA

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Abstract

We have previously shown that the expression of monocyte CCR2, the receptor for monocyte chemoattractant protein-1, is induced by plasma cholesterol. The present study examines the mechanisms that regulate monocyte CCR2 expression in hypercholesterolemia using a mouse model. Our data demonstrate that in the mouse, CCR2 expression in circulating monocytes is controlled by two promoters P1 and P2. The two distinct transcripts, which encode the same protein, are produced by alternative splicing in the 5′-untranslated region. Both promoters are constitutively active, but only P2 is stimulated by cholesterol. However, both promoters are repressed by peroxisome proliferator-activated receptor γ.

Keywords: CCR2; Chemokines; Promoter; Gene expression; Cholesterol; PPARγ; Atherosclerosis

Complications of atherosclerosis are the leading cause of mortality and morbidity in Western societies. The early lesions in human and mouse models of atherosclerosis consist primarily of lipid-laden macrophages called foam cells. Previous studies have shown that most of these foam cells originate from monocytes after their recruitment from the circulation into the subendothelial space [1,2]. The migration of monocytes into the arterial wall is stimulated by chemotactic molecules and the critical involvement of monocyte chemoattractant protein-1 (MCP-1) in monocyte infiltration and atherosclerotic plaque development is well established [3–6]. The biological activities of MCP-1 are mediated by CCR2, a seven transmembrane-domain G protein-coupled receptor, expressed primarily on monocytes and T lymphocytes [7].

Previously, we have reported that CCR2 expression was elevated in monocytes from hypercholesterolemic patients [8]. Further, we showed that these monocytes were hyper-responsive to chemotactic stimuli and we have argued that this may provide a mechanistic basis for their excessive trafficking into the arterial wall, aiding the development of atherosclerosis [9]. Although the detailed molecular mechanisms remained to be defined, cholesterol derived from plasma low density lipoprotein (LDL) appears to be a strong inducer of CCR2 expression.

The present study was designed to examine the mechanisms that regulate monocyte CCR2 expression in hypercholesterolemia. Our data demonstrate that in contrast to the human CCR2 gene, the mouse CCR2 gene is under the control of two distinct promoters producing two differential 5′ UTR splice variants encoding the same protein. Both promoters are constitutively active and repressed by rosiglitazone, a ligand for

* Corresponding author. Fax: +1 858 534 2005.
E-mail address: oquehenberger@ucsd.edu (O. Quehenberger).

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peroxisome proliferator-activated receptor γ (PPARγ), but only one promoter is stimulated by cholesterol.

Materials and methods

Animals and isolation of circulating monocytes. LDL receptor-deficient (LDLR KO) mice on a C57BL/6 background (The Jackson Laboratory) were made hypercholesterolemic by feeding a western diet containing 1.25% cholesterol for 4 weeks, which increased plasma cholesterol levels to about 1800 ± 210 mg/dl. The control group received normal chow and had plasma cholesterol levels of 220 ± 35 mg/dl. To study the effects of PPARγ on promoter activity, the hypercholesterolemic mice were treated with rosiglitazone for 4 weeks at a concentration of 20 mg/kg body weight/day.

Circulating mouse monocytes were isolated by centrifugation through Histopaque 1077 (Sigma) and further purified by plating in autologous serum, as described previously (purity > 85%) [10].

Primer extension and rapid amplification of cDNA 5′-ends (5′-RACE). Total RNA was isolated from mouse monocytes using RNeasy Mini kit (Qiagen). For primer extension analysis, the primer CCR2StC (ATATTATTGTCTTCCATTTCC) was end-labeled with [γ-^{32}P]ATP (Amersham) and used in the extension reaction. The resultant DNA was analyzed on a polyacrylamide gel.

GeneRacer kit (Invitrogen) was used in the 5′-RACE reaction according to the manufacturer's instructions. Nested PCRs were performed using the GeneRacer 5′ primer (provided in the kit) together with the CCR2 gene-specific primer R2_5Prim (5′ACCAGGGAGTA GAGTGGAGGCAGG3′) in the primary PCR. For the secondary PCR, the GeneRacer 5′ nested primer (provided in the kit) was used with the CCR2 gene-specific primers R2_5Nest (5′CATCATCGTAG TCATACGGTGTGGTGG3′) for promoter P1 and R2_7K2 (5′GCA TGGCATTTACTGGTGCAGACACAC3′) for promoter P2. The CCR2 gene-specific primers were designed based on the mRNA sequence in the NCBI database (GenBank Accession No. NM_009915). The PCR products were analyzed by gel electrophoresis and sequencing.

Construction of reporter plasmids and luciferase assay. Mouse genomic DNA was isolated from mouse liver cells using Qiagen genomic-tip (Qiagen). The 5′-flanking regions corresponding to P1 and P2 were produced by PCR using the primers R2-3K (5′TCTTCCC AATGACAAGC3′) and R2-5Kr (5′ATACTCGAGATGGCTCCTT TATGATGG3′) for the P1 promoter, and R2-5K (5′GTGTGAGAT GAAGGTGG3′) and R2-7Kr (5′ATACTCGAGTTGCATGGCAT TTACTGG3′) for the P2 promoter. The PCR products were subcloned into the reporter vector pBNXH containing the firefly luciferase gene (provided by Dr. C. Glass, UCSD). Cells were seeded into 12-well plates (2.0×10^5 cells/well) and transfected with 0.5 μg of reporter plasmids using FuGENE 6 Transfection Reagent (Roche Applied Science). After 48 h, the cells were lysed and used for luciferase assays (Promega). All experiments were carried out in triplicate and the data were normalized to cell protein.

Real-time and semi quantitative RT-PCR. Total RNA was isolated from circulating mouse monocytes using RNeasy columns (Qiagen) and subjected to DNase I treatment (Promega). The CCR2 primers and probe for real-time PCR were designed using the Primer Express software version 1.5. Quantification of mRNA was performed using the ABI Prism7000 (Applied Biosystems). Gene expression of the GAPDH was used for normalization.

For semi-quantitative PCR the 5′ splice variants were amplified for 30 cycles with sense primers specific for the P1 promoter product (R2-UT5K, 5′ATCATAAAGGAGCCATACC3′) or the P2 product (R2-UT7K, 5′TATATGTGTGTGTCTGC3′) and antisense primer (R2-530, 5′TAACCAATGTGATAGAGCC3′). The amplified DNA was analyzed by gel electrophoresis and quantitated by densitometric scanning (ImageQuant). The identity of the product was confirmed by sequencing. As an internal standard, GAPDH was amplified and analyzed under identical conditions using primers as described [10].

Results

Identification of transcription initiation sites in the mouse CCR2 gene

To characterize the transcription start site of the mouse CCR2 gene, we carried out a primer extension analysis. The result showed a major band in addition to several weak bands, suggesting that multiple transcription start sites may exist (Fig. 1A). To confirm this, we performed a 5′-RACE analysis using mRNA isolated from circulating mouse monocytes. Nested PCR carried out with R2_5prim/R2_5Nest and adapter primers revealed three bands (Fig. 1B, left panel). Sequencing analysis of the products demonstrated that the top band represented amplification from full length mRNA. The lower bands corresponded to short messengers derived from the same promoter, probably resulting from mRNA degradation. To amplify products of a potentially weaker second promoter, we used another set of primers, R2_5prim/R2_7K2, with increased cycle numbers and obtained several products (Fig. 1B, right panel). Sequence analysis of the lowest band revealed an additional transcription start site, while the other bands resulted from non-specific priming.

Genomic organization of the mouse CCR2 gene

To determine the genomic organization of the CCR2 gene, the sequences of the RACE products were aligned with that of the genomic DNA from the NCBI database. The mouse CCR2 gene contains four exons and two introns extending over a region of about 6.4 kb (Fig. 2). The exon/intron boundaries conformed to the splice donor/acceptor consensus sequence. The two isoforms of transcripts are different only in the untranslated region but are identical in the coding region. One transcript contains three exons separated by two introns, while the other contains three exons but only one intron (Fig. 2).

Functional analysis of the P1 and P2 promoters

The identification of two transcription initiation sites suggested that the expression of the mouse CCR2 gene might be under the control of two distinct promoters. To verify this hypothesis, we amplified by PCR the fragments containing about 1.8 kb of the 5′ flanking regions immediately upstream of each transcription start site and the adjacent exons 1 or 2, using genomic DNA as the template. The fragments were subcloned into the reporter vector pBNXH, which contains the promoterless firefly luciferase gene, and the resulting plasmids

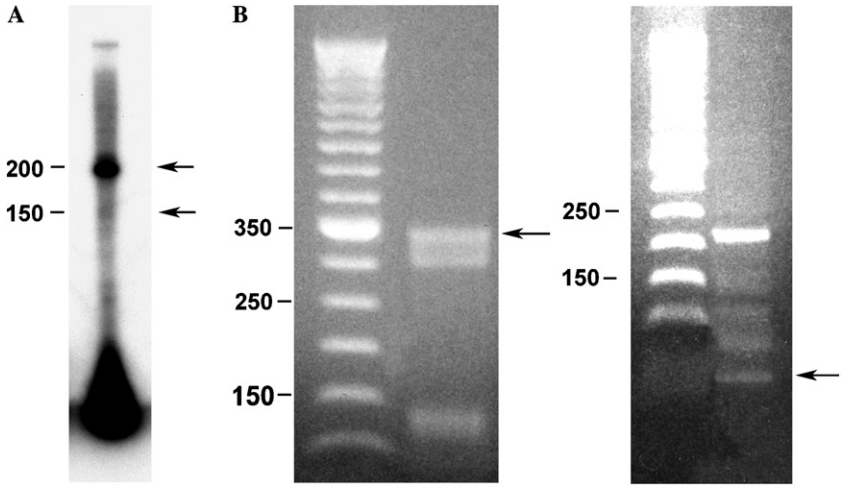

Fig. 1. Identification of two transcription initiation sites in the mouse CCR2 gene. (A) Primer extension revealed one major band and one minor band (arrows). (B) 5′ RACE showed the transcripts amplified from the primer R2_5prim/R2_5Nest (left panel, arrow) and the primer R2_5prim/R2_7K2 (right panel, arrow). (C) The sequence of exons 1, 2, and 3, and the partial sequence of exon 4 are shown. The sequence of the primer used in the primer extension is double-underlined. The sequence of R2_5prim is underlined; R2_5Nest is dot-underlined; and R2-7k2 is dash-underlined. The translation start codon is shaded.

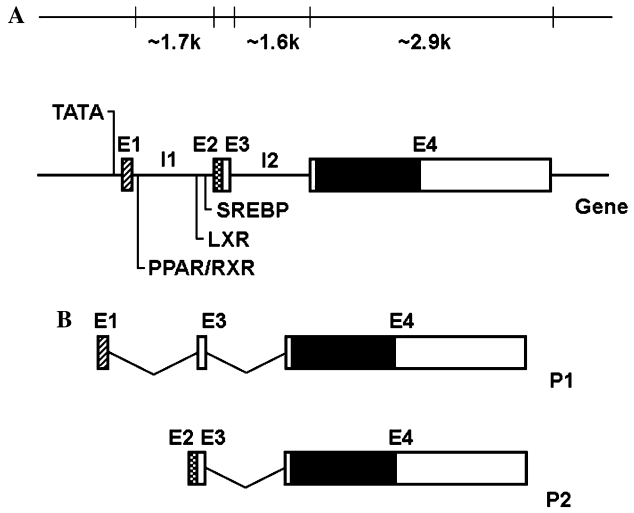

Fig. 2. Schematic diagrams of the genomic organization (A) and mRNAs (B) of the mouse CCR2 gene. E1, E2, E3, and E4 represent exons 1, 2, 3, and 4, respectively. I1 and I2 represent introns 1 and 2, respectively. The open reading frame is shown in solid black.

pBNXH-P1 and pBNXH-P2 were transiently transfected into human HEK293 and HMCB cells. Analysis of the promoter activity demonstrated that both P1 and

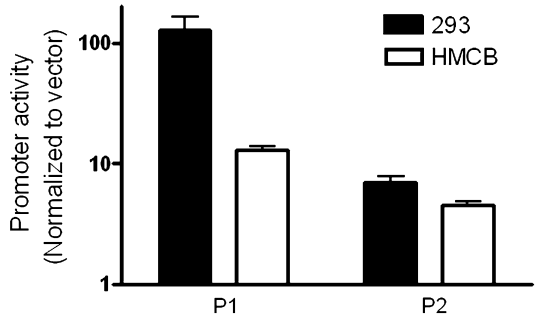

Fig. 3. The mouse CCR2 gene is activated by two promoters. Luciferase assay of the 5′-flanking regions of the mouse CCR2 gene corresponding to P1 and P2 shows that both promoters are functional in human HEK-293 and HMCB cells. The luciferase activity is calculated relative to that of the promoterless pBNXH vector. Data shown are means ± SD ($n = 3$).

P2 were constitutively active with P1 being the stronger promoter (Fig. 3).

Regulation of monocyte CCR2 promoter activity in hypercholesterolemic mice

In previous studies, we showed that the CCR2 expression was increased in monocytes isolated from hypercholesterolemic patients and that the CCR2

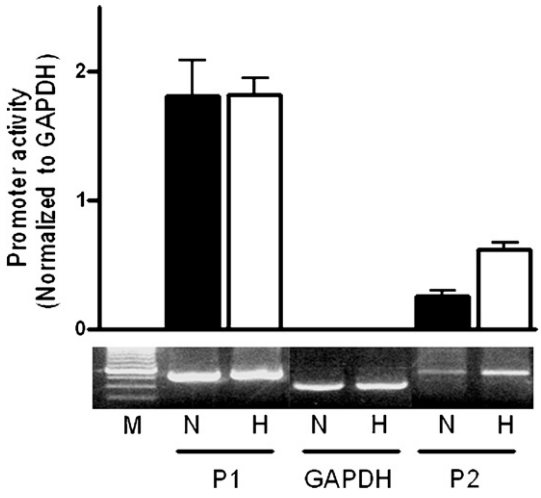

Fig. 4. The promoter P2 of the mouse gene is activated by plasma cholesterol. Monocytes were isolated from normal ($n = 4$) and hypercholesterolemic ($n = 4$) mice and the CCR2 promoter activity was determined by RT-PCR. The inset shows a representative agarose gel of the RT-PCR products and the scanned results are shown in the bar graph. N, normal chow; H, western diet; P1, P1 promoter; and P2, P2 promoter.

expression was induced by cholesterol derived from plasma LDL [8]. To examine which promoter was affected by cholesterol, we analyzed by PCR the transcripts that are specific for the P1 or P2 promoter. As shown in Fig. 4, there was no significant difference of P1 promoter activity in monocytes from hypercholesterolemic or control mice. In contrast, the activity of promoter P2 was significantly enhanced in monocytes from hypercholesterolemic mice compared with normal controls (Fig. 4).

Regulation of P1 and P2 by the PPARγ ligand rosiglitazone

PPARγ is a member of the nuclear hormone receptor superfamily of ligand-inducible transcription factors, which plays a prominent role in several physiological processes including the inflammatory response associated with atherosclerosis [11]. Our previous studies showed that activation of PPARγ by its ligand rosiglitazone reduced CCR2 expression in THP-1 cells as well as in primary human and mouse monocytes [12]. We now extend this study to identify the CCR2 promoter that is under the control of PPARγ. LDL receptor-negative mice were put on a high fat diet to induce hypercholesterolemia. After subsequent treatment with rosiglitazone for 4 weeks, monocytes were isolated and CCR2 expression was quantitatively measured by real-time PCR. As shown in Fig. 5A, rosiglitazone reduced monocyte CCR2 mRNA levels about sevenfold. Similar results were also obtained with semi-quantitative PCR. Further analysis of the individual promoters showed that both P1 and P2 promoter activities were decreased about sixfold and fivefold, respectively (Fig. 5B). These results demonstrated that PPARγ inhibits CCR2 expression in mouse monocytes by regulating the activity of both promoters P1 and P2.

Discussion

Due to the ease with which knockout and transgenic animals can be created, mouse models are commonly used to study gene function in relation to various pathological disorders including atherosclerosis. It is, therefore, important to have a precise knowledge of the mechanisms of gene regulation and to understand the differences between human and mouse disease models. In the present study, we identified two promoters that direct the CCR2 expression, giving rise to two distinct transcripts in the mouse that differ only in the 5′ UTR but encode an identical protein. Noteworthy, while the open reading frames of chemokine receptors are usually intronless, the structure of the 5′ untranslated and promoter regions tended to be more complex. Multiple

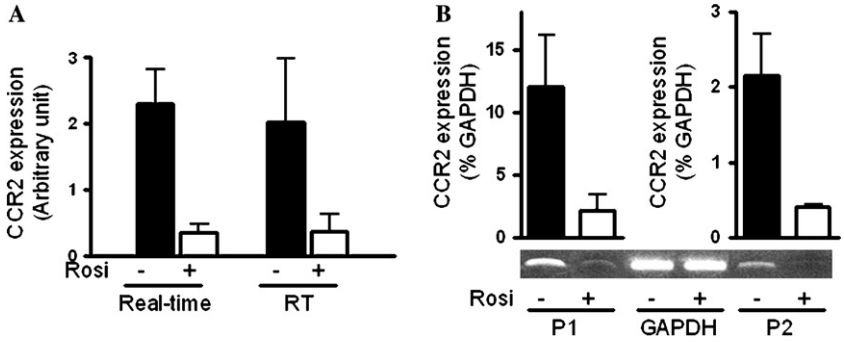

Fig. 5. The activity of both monocyte CCR2 promoters P1 and P2 is reduced by PPARγ ligands. Hypercholesterolemic mice were treated with rosiglitazone and monocyte CCR2 promoter activities were determined. (A) The total promoter activity of the monocyte CCR2 gene was analyzed by real-time PCR (real-time) and semi-quantitative RT-PCR (RT). (B) The activity of the individual promoters P1 and P2 was determined by semi-quantitative RT-PCR. The inset shows a representative agarose gel of the PCR products and the scanned results are shown in the bar graph. The promoter activities are normalized to that of GAPDH. The data are from two independent groups of rosiglitazone-treated mice ($n = 3$) and two control groups ($n = 3$).

promoters regulating the expression of various splice variants that encode the same protein have been reported for several chemokine receptors including CCR3, CCR5, CCR8, CXCR2, and CX3CR1 [13–17]. In contrast to the mouse, the promoter of the human CCR2 gene appears to be under the control of a single promoter and no 5′ UTR splice variants have been described [18].

Previously, we have shown that monocyte CCR2 expression is not steady but is finely regulated through a network of cytokines and other factors [19]. While pro-inflammatory cytokines rapidly reduce CCR2 expression, conditions of hypercholesterolemia, and more specifically cholesterol derived from plasma lipoproteins, stimulate the receptor expression in the circulating monocytes both in animal models and in humans [8–10]. Increased expression of CCR2 renders the monocyte hyper-responsive to chemotactic stimuli and we hypothesized that this may accelerate the rate by which monocytes are recruited to the atherosclerotic lesion. Interestingly, only the activity of the promoter P2 was enhanced in the monocytes from the hypercholesterolemic animals. A consensus sequence for transcription factor SREBP binding is present in the P2 promoter region in mouse as well as in the promoter of the human CCR2 gene. The exact mechanism by which cholesterol regulates CCR2 expression remains to be investigated.

In contrast to the stimulatory effect of cholesterol, activation of PPARγ very potently inhibits monocyte CCR2 expression both in the mouse as demonstrated here as well as in humans [12]. Rosiglitazone, a well-described ligand for PPARγ, is currently used for the treatment of type 2 diabetes, but it may also have additional athero-protective properties. Members of this class of drugs have been shown to inhibit cell proliferation in the vascular wall [20], decrease carotid artery intima-media thickness [21], and reduce atherosclerosis in animal models [22–24]. Previously, we have shown that treatment of human monocytes ex vivo with rosiglitazone inhibits CCR2 expression and consequently reduces the chemotactic activity in response to MCP-1 [10]. The in vivo data presented here demonstrate that rosiglitazone decreases CCR2 expression in circulating monocytes by repressing both P1 and P2 promoters.

In conclusion, we show here that the mouse CCR2 gene is under the control of two distinct promoters that give rise to two differential 5′ UTR splice variants encoding the same protein. Plasma lipoproteins and PPARγ ligands have the opposite effect on CCR2 expression. While an increase in CCR2 expression by cholesterol is considered to be pro-atherogenic, the decrease achieved with PPARγ may be athero-protective. We hypothesize that the beneficial effect of rosiglitazone is in part derived from its ability to inhibit monocyte trafficking in response to MCP-1, which ultimately may improve the prognosis of cardiovascular disease.

Acknowledgments

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