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Chemokine and Chemokine Receptor Expression in a Novel Human Mesangial Cell Line

BERNHARD BANAS, BRUNO LUCKOW, MARCUS MÖLLER, CHRISTIANE KLIER, PETER J. NELSON, ERIK SCHADDE, MANFRED BRIGL, DANIEL HALEVY, HARRY HOLTHÖFER, BRIGITTE REINHART, and DETLEF SCHLÖNDORFF
Nephrological Center, Medical Policlinic, Ludwig-Maximilians University, Munich, Germany.

Abstract. Chemokines are thought to play a pivotal role in mediating the selective migration of leukocytes into sites of tissue injury. The local production of chemokines by mesangial cells (MC) has been linked to inflammatory processes within the glomerulus. To study the chemokine biology of human MC, an immortalized human MC line was generated and then chemokine and chemokine receptor expression was examined in response to various proinflammatory stimuli. The results show that human MC have a specific and limited repertoire of chemokine expression. The stimulus-specific regulation of the chemokines monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T cell expressed and secreted (RANTES), interleukin-8 (IL-8), and IP-10 was demonstrated using RNase protection assays. Transcripts for the chemokines MIP-1α, MIP-1β, I-309, or lymphotactin could not be detected. The expression of CC chemokine receptors was investigated by reverse transcription-PCR and RNase protection assays. MC stimulated with interferon-γ (IFN-γ) expressed mRNA for the chemokine receptor CCR1. The expression could be further increased by activating the cells with a combination of tumor necrosis factor-α (TNF-α), IL-1β, and IFN-γ. Under these conditions, no mRNA for CCR2, CCR3, CCR4, CCR5, or CCR8 was detected. A comparison of the immortalized human mesangial cells with primary cells showed identical expression patterns of chemokine receptors. To demonstrate functional activity of chemokine receptors expressed by human MC, chemotaxis assays were performed. MC stimulated with a combination of TNF-α, IL-1β, and IFN-γ, but not unstimulated MC, migrated toward a RANTES gradient. Eotaxin did not enhance the migratory activity of human MC. In summary, a novel human mesangial cell line was established and the pattern of chemokine expression was examined. For the first time, the inducible expression of functionally active CCR1 by human MC was shown.

Specific leukocyte infiltration is a characteristic feature of various renal disease processes. The influx of leukocytes from the glomerular capillaries into the mesangial space represents a multistep procedure involving rolling, and firm adhesion to the endothelial surface, followed by diapedesis and extravasation of the leukocyte. This overall process requires concerted interaction between the leukocyte and endothelium, involving a series of specific adhesion molecules, cytokines, mediators of inflammation, and chemotaxis (1,2). The selective attraction of different subsets of leukocytes to the site of tissue injury appears to be mediated to a significant extent by the expression of specific chemokines and chemokine receptors (3,4). In the kidney, the local generation of chemokines by mesangial cells (MC) appears to be important in the initiation and regulation of inflammatory processes within the glomerulus (5). Recently, chemokines and chemokine receptors have also been shown to play a role in noninflammatory processes, including normal cellular trafficking, hematopoiesis, angiogenesis, and organ development (6).

The human chemokines represent at least 40 different chemotactic cytokines that share conserved structural features, including four cysteine residues that help define the chemokine subfamilies. These include the C, CC, CXC, and CX_3C subfamilies (where X represents any intervening amino acid residue between the first two cysteines in the amino acid sequence) (7,8). Important members of the CC subfamily are the chemokines monocyte chemoattractant protein-1 (MCP-1) and regulated upon activation, normal T cell expressed and secreted (RANTES). Interleukin-8 (IL-8) represents a well-known chemokine of the CXC subfamily. Lymphotactin is thus far the only member of the C chemokine subfamily. It lacks the first and third cysteine in the 4-cysteine motif, but shows homology at its carboxy terminus with the CC chemokines (9). The CX_3C chemokine fractalkine represents a new subfamily of chemokines as a prototype of membrane-bound chemokines that induce adhesion of leukocytes (10).

The biologic effects of chemokines appear to be mediated through their interaction with a family of specific G protein-coupled seven-transmembrane receptors. More than a dozen human chemokine receptors have been characterized thus far, with many orphan receptors currently under investigation (4,11,12). Based on their specific chemokine ligands, the receptor proteins are named CC chemokine receptors (CCR),

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Correspondence to Dr. Bernhard Banas, Medical Policlinic, Pettenkoferstrasse 8a, 80336 Munich, Germany. Phone: +49 89 5160 3500; Fax: +49 89 5160 4439; E-mail: banas@medpoli.med.uni-muenchen.de

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CXC chemokine receptors (CXCR), C chemokine receptor (CR), and CX_3C chemokine receptor (CX_3CR).

The expression of chemokines by glomerular cells has been studied *in vivo* in several animal models of glomerular diseases. *In vitro* chemokine expression was shown for primary mouse, rat, and human MC, as well as rat and mouse MC lines (5). A recent report described binding of the mouse chemokine TCA3 to mouse MC, suggesting the expression of a functional and specific high-affinity receptor for TCA3 by these cells (13).

Because a human MC line showing typical properties of primary human MC was not available, we established and characterized a novel human mesangial cell line. This cell line was found to be a useful tool for studying human mesangial cell biology under reproducible conditions. We report here the time course and stimulus-dependent induction of chemokine expression by human MC. For the first time, the presence and regulation of the chemokine receptor CCR1 on human MC is described and its role in mesangial cell migration is characterized.

Materials and Methods

Immortalization of Human MC

Primary human MC were obtained from Clonetics Corp. (San Diego, CA). The cells had been prepared from a 16-wk-old female fetus within 36 h after spontaneous abortion and were cultured according to manufacturer's instructions. For immortalization, subconfluent primary human MC from passages 5 and 6 were stably cotransfected with the plasmids pUCInwt and pRc/CMV. The SV40 large T antigen expression plasmid pUCInwt (kindly provided by E. Fanning, Munich, Germany) contains SV40 sequences in pUC12 with a deletion in the late region extending from the *Bam*HI site (position 2533) to the *Pst*I site (position 1988). A 1-bp insertion at the SV40 origin causes a defect in replication. The plasmid pRc/CMV (Invitrogen, Carlsbad, CA) contains as a dominant selectable marker the neomycin gene under the control of the SV40 early promoter, allowing selection of stably transfected cells with the neomycin analogue Geneticin (G418). For transfection, 10 μg of pUCInwt digested with *Eco*RI and 2 μg of pRc/CMV linearized with *Xba*I were dissolved in 100 μl of TE buffer, combined with approximately 1×10^7 primary MC resuspended in 400 μl of phosphate-buffered saline and transferred to a GenePulser cuvette with a 4-mm electrode gap. Electroporation was performed at room temperature using a GenePulser apparatus (Bio-Rad Laboratories, Hercules, CA) set to 230 V and 960 μF. After a recovery period of 5 d, stably transfected cells were selected by adding G418 (Life Technologies, Eggenstein, Germany) at 400 μg/ml to the cell culture medium. Previous dose–response experiments had shown that this concentration was lethal for primary human MC. After 7 d of G418 selection, 52 foci were picked and further expanded. Four cell clones were grown for up to 9 mo (approximately 30 passages). Limited dilution cloning was performed to guarantee monoclonal cell lines. Clone 18 was chosen for additional studies and continuously cultured for more than 100 passages.

Cell Culture Conditions and Characterization of the Human MC Line

Immortalized human MC were grown in Dulbecco's modified Eagle's medium (Biochrom KG, Berlin, Germany) supplemented with 10% bovine serum (Serum Supreme, BioWhittaker, Walkersville, MD) and 1% penicillin-streptomycin (Biochrom KG, Berlin, Germany; 100 U/ml and 100 μg/ml) in an atmosphere of 95% air/5% CO_2 at 37°C. The cell line was characterized by indirect immunofluorescence stainings using antibodies against fibronectin (rat anti-fibronectin, 1:100; Serotec, Oxford, United Kingdom), smooth muscle actin (mouse anti-smooth muscle α actin, 1:50; Progen Biotechnik, Heidelberg, Germany), β1 integrin α1 and α5 chains (mouse anti-β1 integrin α1 and α5 chains, 1:100 each; Telios Pharmaceuticals, San Diego, CA), RCA I (*Ricinus communis*), lectin (mouse anti-*Ricinus communis*, 1:20; Vector Laboratories, Burlingame, CA), factor VIII (rat anti-factor VIII, 1:20; Dakopatts, Glostrup, Denmark), vimentin (mouse anti-vimentin, 1:50; Labsystems, Helsinki, Finland), and SV40 large T antigen (kindly provided by Ellen Fanning, Munich, Germany). Tetramethylrhodamine isothiocyanate- or FITC-labeled secondary antibodies were from DAKO Diagnostika (Hamburg, Germany). For staining, MC were cultured in 8-well plastic chamber slides (NUNC A/S, Roskilde, Denmark). Cells were fixed in methanol/acetone (1:1) for 2 min at room temperature and staining was done as described previously (14). Karyotype analysis on metaphase-arrested chromosome spreads was performed according to an established protocol (15).

Stimulation of Human MC and RNA Preparation

Before stimulation, human MC were cultured in serum-free medium for 24 to 48 h. After stimulation for 4 to 48 h with recombinant human tumor necrosis factor-α (TNF-α; 20 ng/ml), recombinant human IL-1β (2 ng/ml), recombinant human interferon-γ (IFN-γ; 250 U/ml), lipopolysaccharide (LPS; 100 μg/ml), phorbol myristate acetate (PMA; 100 ng/ml), or serum (10% Serum Supreme) cells were harvested, and total RNA was prepared using a standard method (16).

RNase Protection Assay and Reverse Transcription-PCR

Multi-probe template sets for human CC chemokines (hCK-5) and human CC chemokine receptors (hCR-5) for use in RNase protection assays were obtained from Pharmingen (San Diego, CA). Multi-probe RNase protection assays were performed according to the manufacturer's instructions, using 5 μg of total RNA from human MC to analyze the expression pattern of CC chemokines. To exclude incomplete digestion of the probes, tRNA controls were used (data not shown). Protected fragments were separated by denaturing polyacrylamide gel electrophoresis. Gels were dried down and exposed at −80°C on x-ray film using intensifying screens or exposed on Phosphor screens for use with a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Chemokine receptor mRNA expression was studied using reverse transcription (RT)-PCR and RNase protection assays. For RT-PCR, 2 μg of total MC RNA was dissolved in 9.5 μl of diethyl pyrocarbonate-treated water. After addition of 0.5 μl of oligo(dT) primer (1 μg/μl), the sample was heated for 10 min to 65°C, chilled on ice for 1 min, and briefly centrifuged. Subsequently, 10 μl of an RT premix was added. It consisted of 4 μl of Superscript 5× reaction buffer, 0.1 μl of bovine serum albumin (10 mg/ml), 3.5 μl of diethyl pyrocarbonate-treated water, 0.4 μl of 25 mM dNTP, 1 μl of 100 mM dithiothreitol, 0.5 μl (=20 units) of RNasin, and 0.5 μl (=100 units) of Superscript Moloney murine leukemia virus reverse transcriptase (Life Technologies). The composition of RT minus control samples was identical, except that there was no reverse transcriptase present. RT reactions were performed for 1 h at 42°C. Afterward, the nucleic acids were precipitated with ammonium acetate and ethanol in the presence of glycogen, washed with 80% ethanol, and dissolved in 20

μl of TE buffer. One microliter of the cDNA was subsequently used as a template for PCR reactions with primer pairs specific for CCR1 (forward [for] 5′-AAGCCGGGATGGAAACTC-3′, reverse [rev] 5′-TTGGGTTGGCCTCCTATG-3′), CCR2 (for AACATGCTGTCCACATCTCG, rev CAACAATCAAACTGCTCCTCG), CCR3 (for GGAGAAGTGAAATGACAACCTC, rev AGGCAATTTTCTGCATCTACC), CCR4 (for AAATGAACCCCACGGATATAG, rev GACTCTGCATTTCACCATTTC), CCR5 (for GGAACAAGATGGATTATCAAGTGTC, rev CTGTGTATGAAAACTAAGCCATGTG), or CCR8 (for GCCTTGATGGATTATACACTTG, rev TCAATAAACACAATCACAGGC). PCR reactions were performed in 25 μl containing 2.5 μl of 10× PCR buffer with 15 mM $MgCl_2$, 4 μl of 1.25 mM dNTP, 2 μl (=20 pmol) of forward primer, 2 μl (=20 pmol) of reverse primer, 1 μl of cDNA, 13.3 μl of H_2O, and 0.2 μl (=1 unit) of AmpliTaq polymerase (Perkin Elmer, Foster City, CA). Amplifications were performed in a Robocycler (Stratagene, Heidelberg, Germany), using the following conditions: one cycle for 3 min at 94°C, 35 cycles for 1 min 15 s at 94°C, 1 min 15 s at 58°C, 2 min 30 s at 72°C, followed by one cycle for 10 min at 72°C. The PCR products varied in length from 1099 to 1278 bp and included the entire coding regions. The amplified products were resolved on agarose gels stained with ethidium bromide. Analysis of chemokine receptor expression in human MC by RNase protection was performed as described above using 25 μg of total RNA.

Chemotaxis Assays

For use in chemotaxis assays, human MC were stimulated with a combination of TNF-α, IL-1β, and IFN-γ for 24 to 36 h to induce chemokine receptor expression. After detaching with phosphate-buffered saline/10 mM ethylenedinitrilotetra-acetic acid, pH 8.0, cells were cultured on Transwell filter inserts (Costar Corp., Cambridge, MA) with a 12-μm pore size coated with fibronectin (Boehringer, Mannheim, Germany). Assay medium consisted of Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin (Sigma Chemie, Deisenhofen, Germany) and 10 mM Hepes (Life Technologies). Three hours before the assay, 1 to 3×10^4 cells (using a constant number of input cells across all conditions in one experiment) in a volume of 300 μl were plated onto each filter insert and incubated at 37°C. Recombinant human chemokines RANTES and eotaxin were obtained from Peprotech (Rocky Hill, NJ). Chemotactic factors (diluted in assay medium) were added to 12-well tissue culture plates in a final volume of 1200 μl. Transwells were inserted into each well, and the plate was then incubated for 2.5 to 4 h. Cells that had migrated to the bottom of the filter insert and/or the bottom chamber were collected by trypsinization and counted using flow cytometry. This counting method was found to be highly reproducible, and enabled gating on the human MC and the exclusion of debris.

Results

Generation and Characterization of the Human MC Line

To generate a human MC line as a novel model for human mesangial cell biology, primary human MC were immortalized by transfection with SV40 large T antigen and neomycin resistance genes. After selection for stable transfection, 52 clones were isolated. Four clones were cultured for up to 9 mo and 30 passages. To guarantee monoclonality of the subclones, limited dilution series were performed.

In phase contrast microscopy, these immortalized human MC showed the typical morphology of primary human MC. To rule out contamination with cells of non-human origin, karyotype analysis was performed and confirmed a human karyotype. Stable expression of SV40 large T antigen was demonstrated by immunofluorescence staining resulting in a strong nuclear signal (data not shown).

Since there is no specific mesangial cell marker available, the human MC line was characterized by indirect immunofluorescence using a panel of antibodies against cell surface and intracellular markers. The resulting staining patterns are listed in Table 1. The immortalized human MC showed a positive staining for fibronectin localized mainly in the cytoplasm. Also, staining patterns for smooth muscle actin, β1 integrin α1 and α5 chains, and RCA I (*Ricinus communis*) lectin were comparable to stainings we have previously described as typical for human MC in tissue sections *in vivo* and in primary MC *in vitro* (14). Immortalized human MC stained negative for vimentin, cytokeratin 18 and 19, and for factor VIII, respective markers for epithelial and endothelial cells (Table 1). This pattern of staining was maintained for over 40 passages.

On the basis of morphology and antigenic markers, one clone (clone 18) was selected for additional studies and continuously kept in culture for now more than 30 mo and 100

Table 1. Staining pattern of immortalized human MC[a]

Antigenic Marker	Staining Pattern of Immortalized Human MC	Typically Positive in Glomerular Cells[b]
Smooth muscle actin	Positive	Mesangial cells
Fibronectin	Positive (mainly cytoplasm)	Mesangial cells (cytoplasm), fibroblasts (nucleus)
β1 integrin α1 chain	Positive (strong)	Mesangial cells (strong), endothelial cells (weak), podocytes (weak)
β1 integrin α5 chain	Positive	Mesangial cells, endothelial cells
RCA I (*Ricinus communis*) lectin	Positive	Mesangial cells
Cytokeratin 18 and 19	Negative	Epithelial cells
Factor VIII	Negative	Endothelial cells

[a] MC, mesangial cells.
[b] According to reference 14.

passages. No changes in growth characteristics and morphology have been observed.

Expression of Chemokines by Human MC

In the newly established human MC line, the induction and regulation of chemokine expression was analyzed using commercial multi-probe RNase protection assays. This system contained probes specific for the chemokines MCP-1, RANTES, MIP-1α, MIP-1β (macrophage inflammatory protein 1 alpha/beta) I-309, IL-8, and IP-10 (interferon-gamma-inducible protein 10).

Chemokine expression was studied in proliferating MC as well as in growth-arrested MC starved for 24 to 48 h in serum-free medium. To simulate proinflammatory conditions, human MC were stimulated with LPS, PMA, TNF-α, IL-1β, or IFN-γ, either alone or in combination. To investigate time courses of chemokine expression, cells were harvested after stimulation for 4 to 48 h. The time course and pattern of chemokine expression differed for the various stimuli (Figures 1 and 2).

Stimulation of human MC with PMA, TNF-α, or IL-1β led to an induction of MCP-1 mRNA within 4 h. An upregulation of MCP-1 was also seen after stimulation with IFN-γ or LPS for 4 h but to a lower extent. Furthermore, fetal calf serum enhanced MCP-1 expression (Figure 1). In general, the increase of MCP-1 expression was followed by a rapid decline in mRNA levels for MCP-1, which at 12 h were close to basal. Stimulation of human MC with a combination of TNF-α, IL-1β, and IFN-γ resulted not only in a more than additive effect, but also in a prolonged expression with peak levels at 12 h (Figures 1 and 2).

A marked induction of IL-8 mRNA expression was found after stimulation with PMA or IL-1β for 4 h, whereas stimulation with TNF-α was less effective. LPS and IFN-γ did not induce IL-8 production. At 12 h, IL-8 mRNA had completely returned to basal levels. The combination of TNF-α, IL-1β, and IFN-γ did not further enhance IL-8 expression but resulted in a prolonged expression for more than 12 h (Figures 1 and 2).

Human MC expressed low basal levels of RANTES mRNA, and a weak induction could be detected after stimulation with IFN-γ for 24 or 48 h. Combined stimulation with TNF-α, IL-1β, and IFN-γ led to an earlier and enhanced expression of RANTES (Figures 1 and 2).

An expression of IP-10 was seen only after stimulation with a combination of TNF-α, IL-1β, and IFN-γ, resulting in a clear signal at 4 and 12 h (Figures 1 and 2).

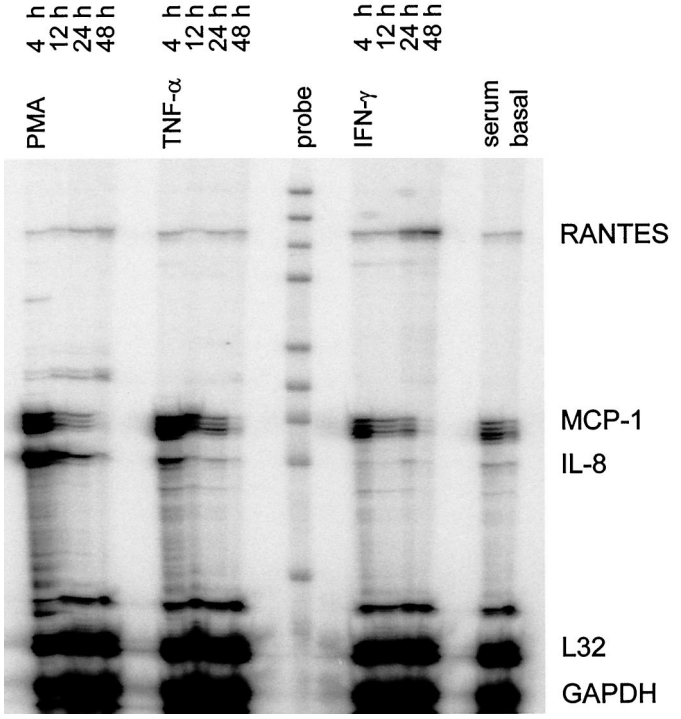

Figure 1. RNase protection assays for the detection of chemokine mRNA expression by human mesangial cells (MC) stimulated with phorbol myristate acetate (PMA), tumor necrosis factor-α (TNF-α), or interferon-γ (IFN-γ). Serum-deprived human MC were stimulated with PMA, TNF-α, or IFN-γ. Total RNA was extracted after 4 to 48 h. RNA prepared from human MC growing in medium containing 10% bovine serum (serum) or in a serum-free medium (basal) served as controls. Chemokine expression was analyzed by multi-probe RNase protection assays using ^{32}P-labeled templates specific for lymphotactin, regulated upon activation, normal T cell expressed and secreted (RANTES), IP-10 (interferon-gamma-inducible protein 10), MIP-1α, MIP-1β (macrophage inflammatory protein 1 alpha/beta), monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8), and I-309, as shown in the lane indicated as probe. Protected fragments were separated by polyacrylamide gel electrophoresis, and autoradiograms were analyzed using a PhosphorImager as described in Materials and Methods. Results are from one of four independent experiments that yielded comparable data. The protected fragments are indicated as RANTES, MCP-1, and IL-8, respectively. GAPDH and L32 gene products served as controls for equal amounts of RNA used.

Expression of Chemokine Receptors by Human MC

In a recent report, the presence of chemokine-binding receptors on the surface of mouse MC was suggested (13). Therefore, a systematic search was done to investigate a potential expression of chemokine receptors by human MC. RNA prepared from unstimulated or stimulated human MC were screened for the presence of CC chemokine receptor transcripts by RT-PCR using primer pairs specific for CCR1, CCR2, CCR3, CCR4, CCR5, or CCR8. Proliferating or serum-deprived human MC did not express mRNA for any of these CCR. A clear and reproducible signal for CCR1 was seen in human MC stimulated for 24 h with IFN-γ alone or in combination with TNF-α and IL-1β (Figure 3A). The identity of the CCR1 fragment amplified by PCR was confirmed by restriction analysis (Figure 3A). Despite performing PCR reactions with high sensitivity (35 cycles), no CCR1-specific products could be amplified from cDNA prepared from human MC stimulated with PMA, LPS, TNF-α, or IL-1β. No other CC chemokine receptors were reproducibly expressed by the MC line under any of the above-described conditions.

A comparison of the chemokine receptor expression pattern of immortalized MC with primary MC was done to rule out induction of CCR1 expression by the immortalization. Com-

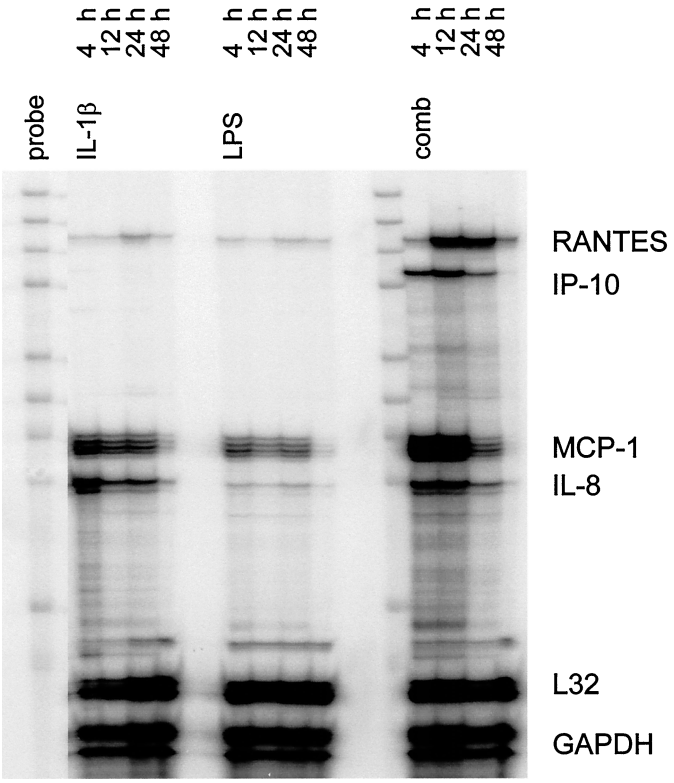

Figure 2. RNase protection assays for the detection of chemokine mRNA expression by human MC stimulated with IL-1β, lipopolysaccharide (LPS), or a combination of tumor necrosis factor-α (TNF-α), IL-1β, and interferon-γ (IFN-γ). RNA prepared from human MC stimulated with IL-1β, LPS, or a combination of TNF-α, IL-1β, and IFN-γ (comb) for 4 to 48 h was analyzed by multi-probe RNase protection assays for the presence of chemokine transcripts as described in the legend of Figure 1. Protected fragments are indicated as RANTES, IP-10, MCP-1, and IL-8, respectively. The figure is representative for one of four experiments, which resulted in comparable data.

parable to the transfected MC line, primary human MC expressed no chemokine receptor mRNA under basal conditions, but had detectable mRNA levels for CCR1 upon stimulation with IFN-γ alone or in combination with TNF-α and IL-1β for 24 h (Figure 3B). Furthermore, in primary human MC, PMA, LPS, TNF-α, or IL-1β failed to induce CCR1 (data not shown), and no other CC chemokine receptor was reproducibly detected by RT-PCR.

To confirm this expression of CCR1 by human MC, RNase protection assays were performed using probes specific for human CCR1, CCR2, CCR3, CCR4, CCR5, and CCR8. Analyzing 20 to 30 μg of total RNA prepared from the human MC line, CCR1 expression could be detected after stimulation with IFN-γ for 24 h. CCR1 was not induced after stimulation with PMA, LPS, TNF-α, or IL-1β. CCR1 expression was found to be further enhanced after stimulating the cells with IFN-γ for 48 h or using a combination of TNF-α, IL-1β, and IFN-γ (Figure 4). Under none of the above conditions could other CC chemokine receptors reproducibly be detected.

In summary, an inducible expression of CCR1 could be demonstrated for both immortalized and primary human MC. Two different methods, RT-PCR and RNase protection assay, revealed identical results that further confirm this novel and unexpected finding.

Selective Chemotactic Response of Stimulated Human MC to Human RANTES and Eotaxin

To examine the functionality of the chemokine receptor in human MC, chemotaxis assays were performed. After induction of CCR1 by stimulating the cells with the combination of TNF-α, IL-1β, and IFN-γ, the migratory activity of human MC was analyzed in a sensitive transwell migration assay. Unstimulated cells served as control. The chemotactic response to different concentrations of the CCR1-ligand RANTES was assessed. Only human MC stimulated with TNF-α, IL-1β, and IFN-γ and expressing CCR1 migrated through the 12-μm pores of the filter membranes toward a RANTES gradient. The migration indices ranged from 1.5 to 2.2, with maximum response at 100 ng/ml RANTES (Figure 5). Unstimulated cells showed no enhanced migratory activity upon RANTES stimulation. Eotaxin, a ligand only for CCR3, failed to attract unstimulated or stimulated human MC (Figure 5).

Discussion

There is increasing support for the hypothesis that during inflammatory glomerular processes, the production of chemotactic mediators by both infiltrating and resident cells, and especially MC, plays an important role in the regulation of the leukocyte influx (5). To investigate the expression and regulation of chemokines and their receptors by human MC, a novel cell line was established. Human MC were immortalized by transfection with expression plasmids of SV40 large T antigen and a neomycin resistance gene. This strategy allowed for the selection of clones that stably overexpress the viral antigen. The resultant novel human MC line proved a useful model for the study of human MC biology. In contrast to primary MC, the human MC line could be easily grown using standard cell culture conditions. Dedifferentiaton of the MC line was not seen during a 30-mo cultivation period. Because a typical mesangial cell marker is lacking, the cell line had to be characterized using a variety of antigenic markers we have previously described to be helpful for the detection of MC *in vivo* and *in vitro* (14). The cells stained positive for smooth muscle actin, fibronectin, β1 intergrin α1 chains, β1 integrin α5 chains, and *Ricinus communis* lectin I, markers typically found to stain positive on mesangial cells. Since these markers do not exclusively stain MC (*e.g.*, anti-β1 integrin antibodies also stain endothelial cells), contamination by endothelial or epithelial cells was ruled out by negative staining for factor VIII and cytokeratin 18 and 19, respectively. Contamination with cells of non-human origin was excluded by karyotype analysis, which confirmed a human karyotype.

After establishing the human MC line, the expression of chemokines was examined in this model system. The expression of chemokines by MC has been previously described by us and several other groups (17–19). MCP-1, a chemotactic agent for monocytes and T cells, is rapidly upregulated in mouse, rat,

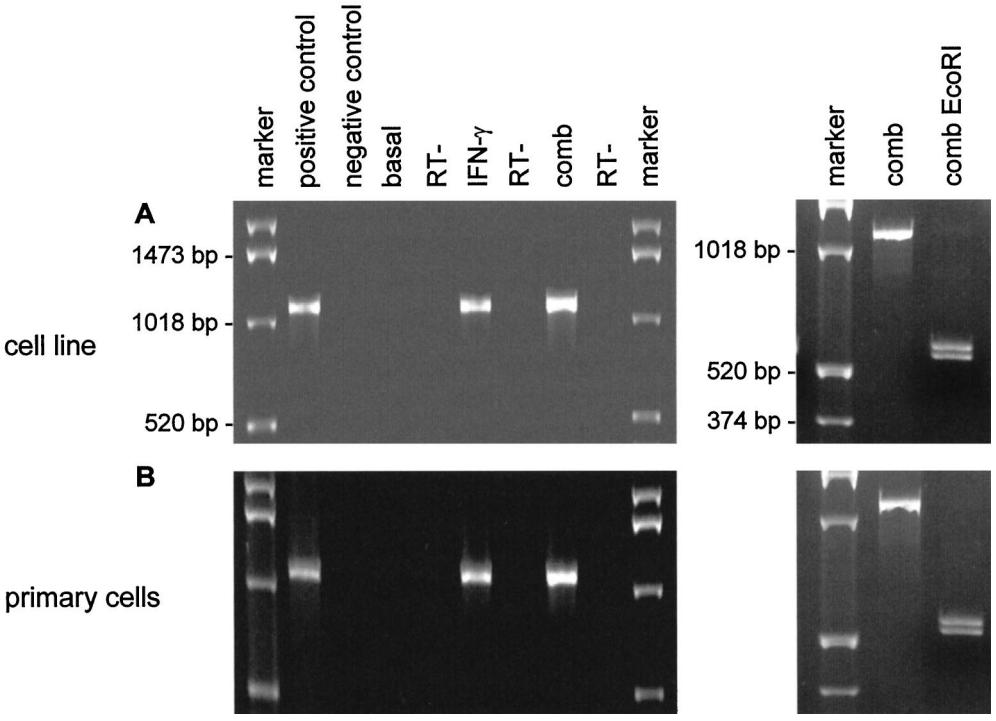

Figure 3. Expression of CC chemokine receptor 1 by the human MC line and primary human MC as detected by reverse transcription (RT)-PCR. Immortalized human MC (Panel A) and primary human MC (Panel B) were cultured under basal conditions or stimulated with IFN-γ or a combination of TNF-α, IL-1β, and IFN-γ (comb), respectively, for 24 h before RNA extraction. RT-PCR was performed using primers specific for human chemokine receptor CCR1 as described in detail in Materials and Methods. CCR1-specific PCR products had a length of 1106 bp and were visualized by ethidium bromide staining after separation on a 1.2% agarose gel (left side). To confirm the identity of CCR1, PCR products were digested with *Eco*RI (comb *Eco*RI). The two resulting fragments of 565 and 541 bp were separated on a 2% agarose gel and are shown on the right side. Results representative for a series of four independent experiments are shown. Genomic DNA or water served as positive and negative controls. Contamination of RNA with genomic DNA was controlled in reactions without reverse transcriptase (RT−).

and human MC after activation of the cells by a variety of stimuli (20–22). RANTES, a chemoattractant of T cells, monocytes, eosinophils, and basophils, is expressed within 2 h by mouse MC (23,24) and was also found to be expressed by primary human MC (25). IL-8, a potent neutrophil attractant, is expressed by rat and human MC (26,27). The expression of IP-10 mRNA has been described for mouse and human MC (28,29).

Using the human MC line, a systematic investigation of chemokine expression by human MC was possible and revealed reproducible results. Multi-probe RNase protection assays were performed because this method allowed the simultaneous analysis of eight chemokines for each stimulatory condition and time point. Thus, a comprehensive chemokine expression profile for human MC could be described. Comparable to the data in the literature under proinflammatory conditions, the human MC line upregulated mRNA encoding for the chemokines MCP-1, RANTES, IL-8, and IP-10, whereas no expression of the chemokines MIP-1α, MIP-β, I-309, and lymphotactin was seen. The various stimuli tested produced a different pattern, and time course, of chemokine response. The human MC line strongly induced MCP-1 and IL-8 mRNA within 4 h, and by 12 h had already downregulated expression to basal level. A combination stimulation with TNF-α, IL-1β, and IFN-γ led to a more than additive and prolonged expression of these chemokines. These results may be of particular interest because during an inflammatory process *in vivo*, a variety of proinflammatory mediators is released. In contrast to previously published data (25) in the human MC line, RANTES was induced only after stimulation with IFN-γ for at least 24 h, but the combined stimulation with TNF-α, IL-1β, and IFN-γ led to an earlier and enhanced expression of RANTES. A weak IP-10 expression by human MC was seen only after stimulating the cells with a combination of TNF-α, IL-1β, and IFN-γ, again indicating the difference in stimuli and time courses for the different chemokines.

Induction of specific chemokines may contribute to the various inflammatory cell infiltrates seen in different forms of glomerular injury. This is suggested by studies of animal models of inflammatory kidney diseases that describe glomerular expression of MCP-1, RANTES, IL-8, and IP-10 by local and infiltrating cells (30–35). In a recent article, a mouse model of crescentic glomerulonephritis was investigated. Blocking the function of MCP-1 and RANTES resulted in a significant decrease of glomerular leukocyte influx, crescent formation, and interstitial fibrosis (36). Chemokines may also play an important role in human glomerular disease. Using immunohistology in kidney biopsies of patients with prolifer-

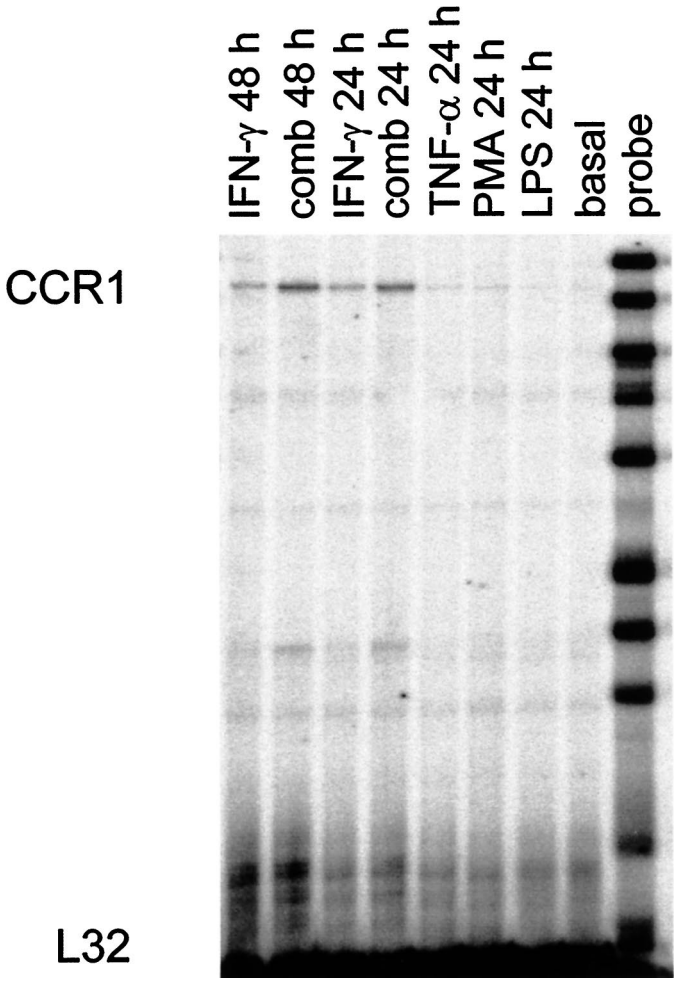

Figure 4. Detection of CC chemokine receptor 1 mRNA expression in stimulated human MC by RNase protection assay. For the analysis of CCR1 expression of human MC by RNase protection assays, cells were stimulated with IFN-γ alone or with a combination of TNF-α, IL-1β, and IFN-γ (comb) for 24 or 48 h before RNA extraction. RNA prepared from unstimulated cells and after stimulation with TNF-α, PMA, or LPS alone for 24 h served as controls. Multi-probe RNase protection assays were performed using 25 μg of RNA as described in Materials and Methods. The probes are shown in the last lane, the protected fragments are indicated as CCR1 using L32 gene products as housekeeping controls. Results are from one of three independent experiments, which gave reproducible data.

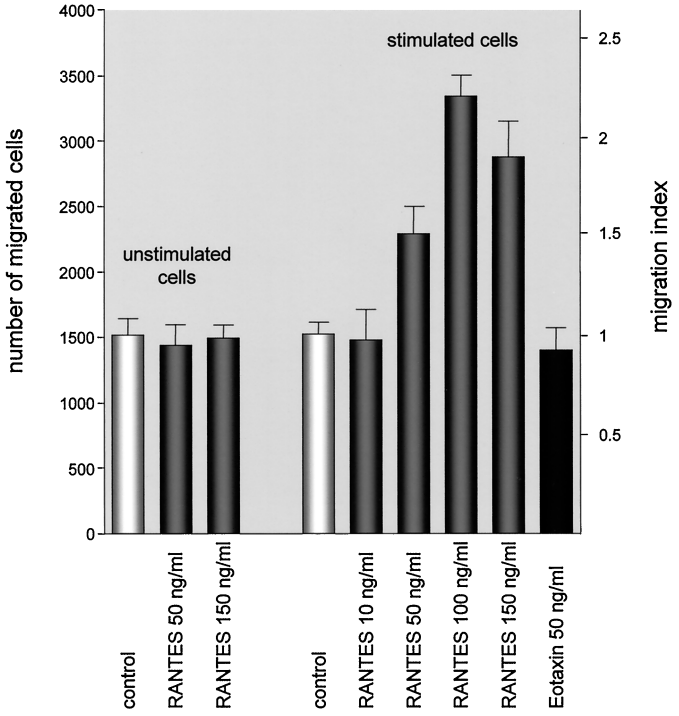

Figure 5. Chemotactic responses of human mesangial cells to RANTES and eotaxin. Unstimulated human MC (expressing no chemokine receptors, left side) and human MC stimulated with a combination of TNF-α, IL-1β, and IFN-γ (expressing CCR1, right side) were assessed in Transwell migration assays for chemotaxis toward different concentrations of the CCR1-ligand RANTES and the CCR3-ligand eotaxin. A total of 2×10^4 cells was placed on the top well of the filter insert. The cells that had migrated into the bottom chamber containing buffer $\pm$ chemokines were counted by flow cytometry as indicated on the left. The migration indices are shown on the right. The data are mean $\pm$ SD of triplicates performed for each experimental condition. The figure is representative for five experiments resulting in comparable data.

ative glomerulonephritis, Wegener's disease, and lupus nephritis, a positive staining with mesangial distribution was seen for MCP-1 (37). In other publications, a high urinary MCP-1 excretion was strongly correlated with disease activity of human lupus nephritis (38,39) and IgA nephropathy (40). A significant upregulation of glomerular MCP-1 expression was observed in kidney biopsy specimens from patients with cryoglobulinemic glomerulonephritis (41).

Our observation of inducible CCR1 expression in human MC is of interest, as most chemokine receptors are known to be expressed by leukocytes only, and their function is considered to be restricted to the recruitment of leukocytes to sites of tissue injury. However, over the past few years chemokine receptor function has been recognized as playing a role in hematopoiesis and angiogenesis (42). For example, mice lacking CXCR4 die *in utero* and have major defects in vascular development, hematopoiesis, and cardiogenesis. Furthermore, expression of these chemokine receptors also occurs on neuronal tissue and developing vascular endothelial cells, supporting a role for CXCR4 in organ development (6).

Two reports by Dorf *et al.* suggested the presence of chemokine receptors on the surface of mouse MC (13,43). In the first article, binding of the mouse CC chemokine thymus-derived chemotactic agent 3 (TCA3) to mouse MC was seen using Scatchard plot analysis. The binding of the chemokine led to adhesion to fibronectin, chemotaxis, and proliferation of the mouse MC (13). Subsequently, thymus-derived chemotactic agent 4 (TCA4) was described as a new mouse CC chemokine acting as a chemoattractant with activity on mature T cells and mouse MC (43). However, the corresponding chemokine receptors are still not identified.

Thus far no reports on the expression of known CC chemokine receptors by mouse or human MC have been published. It

therefore was of interest to show that stimulation of human MC with IFN-γ alone or in combination with TNF-α and IL-1β leads to an expression of CC chemokine receptor 1 mRNA. This finding could be confirmed by two independent methods, *i.e.*, RT-PCR and RNase protection assay. In addition, the expression pattern for CCR1 was identical for primary and immortalized human MC. Under basal conditions, no CCR1 mRNA was detectable and other stimuli when tested alone failed to induce CCR1. Interestingly, the combination of TNF-α and IL-1β with IFN-γ caused a stronger CCR1 expression than IFN-γ alone. No transcripts of the chemokine receptors CCR3, CCR4, CCR5, or CCR8 could be detected in human MC under any of the experimental conditions. A faint band for CCR2 was occasionally but not reproducibly noted.

To test the functionality of the CCR1 on MC, chemotaxis assays were used. Using this sytem, human MC migrated toward a RANTES gradient. Because the cells expressed none of the other known RANTES receptors (CCR3–5), the effect of RANTES on human MC appears to be mediated by CCR1. This is further supported by the congruent observation that only cytokine-stimulated MC expressed CCR1 and migrated in response to RANTES. To exclude unspecific effects of chemokines on human MC, the potential migration of MC toward eotaxin was assessed. As expected, eotaxin, which binds only to CCR3 but not to CCR1, induced no chemotactic response.

The finding that MC cannot only express chemokines but also at least one type of chemokine receptor, *i.e.*, CCR1, is novel. CCR1 binds MIP-1α, RANTES, and MCP-3, and has been detected previously on neutrophils, monocytes, lymphocytes, and eosinophils (44). Interestingly, in a preliminary report knockout mice for CCR1 were noted to have worse renal disease in response to nephrotoxic serum than wild-type mice (45). This was interpreted as indicating a potential anti-inflammatory effect of CCR1. In this context, it is tempting to speculate that the expression of CCR1 by human MC could be involved in modulating the local response to immune injury in the glomerulus, a hypothesis that needs further testing. Furthermore, CCR1 on MC could be involved in MC migration. For example, during experimental mesangiolytic glomerulonephritis, MC repopulate the mesangial stalk by migrating in from the glomerular vascular pole (46). The responsible mediator(s) and their respective receptors have not been identified to date. CCR1 could be a potential candidate especially as RANTES is upregulated in mesangiolytic glomerulonephritis (23) and caused mesangial cell migration in our studies. These intriguing speculations deserve further study especially as effective chemokine and chemokine receptor antagonists are becoming available.

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