Interleukin-12 Is Synthesized by Mesangial Cells and Stimulates Platelet-Activating Factor Synthesis, Cytoskeletal Reorganization, and Cell Shape Change

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Preliminary studies indicate the involvement of interleukin (IL)-12 in experimental renal pathology. In the present study, we evaluated whether cultured glomerular mesangial cells are able to produce IL-12 and whether IL-12 may regulate some of their functions, including the cytoskeletal reorganization, the change in cell shape, and the production of platelet-activating factor (PAF). The results obtained indicate that pro-inflammatory stimuli, such as tumor necrosis factor- α and bacterial polysaccharides, induce the expression of IL-12 mRNA and the synthesis of the protein by cultured mesangial cells. Moreover, cultured mesangial cells were shown to bind IL-12 and to express the human low-affinity IL-12 β 1-chain receptor. When challenged with IL-12, mesangial cells produced PAF in a dose- and time-dependent manner and superoxide anions. No production of tumor necrosis factor- α and IL-8 was observed. Moreover, we demonstrate that IL-12 induced a delayed and sustained shape change of mesangial cells that reached its maximum between 90 and 120 minutes of incubation. The changes in cell shape occurred concomitantly with cytoskeletal rearrangements and may be consistent with cell contraction. As IL-12-dependent shape change of mesangial cells was concomitant with the synthesis of PAF, which is known to promote mesangial cell contraction, we investigated the role of PAF using two chemically different PAF receptor antagonists. Both antagonists inhibited almost completely the cell shape change induced by IL-12, whereas they were ineffective on angiotensin-II-induced cell shape change. In conclusion, our results suggest that mesangial cells can either produce IL-12 or be stimulated by this cytokine to synthesize PAF and to undergo

shape changes compatible with cell contraction. (*Am J Pathol* 1999, 154:623–632)

Mesangial cells (MCs) are contractile cells that share features with smooth muscle cells and pericytes and that take part in the control of several glomerular functions, including the regulation of the glomerular hemodynamics and the processing of macromolecules and immunocomplexes.^{1,2} MCs are the target of vasoactive substances, such as angiotensin (AT)-II, vasopressin, nitric oxide, and endothelin.² Moreover, several lipid mediators, such as platelet-activating factor (PAF), eicosanoids, leukotrienes, and cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6, may affect MC functions by stimulating cell contraction, proliferation, or matrix production.³ Recently, it has been shown that lipid mediators may contribute to the biological activities exerted by certain cytokines. In particular, it has been shown that PAF, a phospholipid mediator of inflammation with a large spectrum of biological activity,^{4–6} directly stimulates MC contraction⁷ and that an endogenous production of PAF mediates the contraction induced by TNF- α ⁸ and endothelin-1.⁹ In several experimental models, PAF affects glomerular filtration and permeability and contributes to glomerular pathology.¹⁰ We have recently shown that the synthesis of PAF induced by IL-12 contributes to the activation of human neutrophils.¹¹ IL-12 is an heterodimeric cytokine, composed of a 40-kd and a 35-kd subunit, which displays a key role in the initiation of both innate and antigen-specific pro-inflammatory immunity.^{12–15} This cytokine is mostly produced by phagocytic cells and B lymphocytes in response to lipopolysaccharide (LPS) and other bacterial products.^{12,15} Recently, IL-12 has been also involved in the pathogenesis of au-

Supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC); by CNR, Target project Biotechnology; by Istituto Superiore di Sanità, target project Artificial Organs and Organ Transplantation; and MURST 40% to G. Camussi. B. Bussolati is a postdoctoral student in Nephrology, University of Parma, Italy.

Accepted for publication November 17, 1998.

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toimmune diseases.¹⁶ In particular, a prominent IL-12-dependent Th1 response has been demonstrated in some experimental glomerulonephritis.¹⁷ In MRL-Fas^{lpr} mice, which develop a lupus nephritis, an enhanced expression of IL-12 within the nephritic kidney has been shown.¹⁸ Cultured proximal tubular cells derived from the MRL-Fas^{lpr} mouse kidney were also capable of producing IL-12.¹⁸ However, the production of IL-12 from glomerular cells has not been investigated.

The aim of the present study was to evaluate whether MCs are capable of producing IL-12 and whether IL-12 may regulate some of the MC-related functions. In particular, we studied the ability of IL-12 to stimulate the production of PAF, superoxide anions (O₂⁻), and cytokines and to induce changes of the shape of MCs.

Materials and Methods

Materials

Polymyxin B, phospholipase A2, phospholipase A1, bovine serum albumin (BSA) fraction V (tested for not more than 1 ng of endotoxin per mg), Formyl-met-leu-phe (FMLP), sphingomyelin, and lyso-2-phosphatidylcholine (lyso-PC), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase from *Clostridium histolyticum* was from Boehringer (Mannheim, Germany); human factor VIII antiserum was from Nordic Immunology (Tilburg, The Netherlands); anti-smooth muscle cell myosin antibodies were from Immunotech (Marseille, France); and mouse monoclonal anti-cytokeratin antibodies, anti-collagen type IV antibodies, and anti-fibronectin were from Labometrics (Milano, Italy). IL-12 was a kind gift of G. Trinchieri, Genetics Institute (Cambridge, MA). The anti-IL-12 neutralizing monoclonal antibody (MAb) C.8.6 and the anti-IL-12 non-neutralizing MAb C.11.5¹⁵ were a kind gift from G. Trinchieri. Anti-IL-12 receptor 12Rβ.44 MAb¹⁹ was a kind gift from J. Ritz (Dana-Farber Cancer Institute, Boston, MA). All mouse anti-IL-12 MAbs and anti-IL-12 receptor MAbs were of the IgG1 isotype. The corresponding irrelevant isotypic control (mouse IgG1) was purchased from Cedarlane (Hornby, Ontario, Canada). Synthetic PAF (1-hexadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine) was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland); the stock solution in chloroform was stored at -20°C until use. The chloroform was evaporated, and saline containing 0.25% BSA fraction V, low endotoxin (Sigma), was added immediately before use. WEB 2170 was obtained from Boehringer and CV 3988 from Takeda Chemical Industries (Kyoto, Japan).

TNF-α and LPS from *Escherichia coli* (0111:B4) were purchased from Sigma. The stock solution of LPS was prepared by suspending 10 mg of LPS in 2 ml of 20 mmol/L EDTA and by sonicating until clarification (three to five times a 20-second burst at maximal intensity using a W375 sonicator with a number 419 microtip, Heat Systems-Ultrasonics (Farmingdale, NY). Aliquots of LPS stocks (200 μl) were stored at -20°C and when thawed

for use were sonicated for 15 seconds using a microsonicator (Microson, Heat Systems-Ultrasonics). LPS working dilutions were prepared in 10 mmol/L Hepes saline formulated using 1 mol/L Hepes stock (Gibco Laboratories, Grand Island, NY) and sterile, nonpyrogenic saline.

Culture of Human Mesangial Cells

Human glomeruli were isolated from surgical specimens of kidneys by the method described by Striker et al.²⁰ The separated cortex was sliced and forced through a graded series of stainless steel meshes, and isolated encapsulated glomeruli were recovered. MCs were obtained from collagenase-treated, isolated glomeruli to remove the epithelial cell component.²¹ Washed glomerular remnants were plated at a density of ~300 glomeruli/cm² in Dulbecco's modified Eagle's medium (DMEM) and 20% fetal calf serum tested for endotoxin levels less than 0.1 ng/ml (Sigma), 50 U/ml penicillin, and 50 μg/ml streptomycin; culture flasks were kept in a 95% air, 5% CO₂ environment at 37°C. After three weeks in primary culture, MCs were harvested with 0.05% trypsin, 0.02% ethylene-diamine-tetracetate (EDTA). Subcultures were grown in the same medium. The MCs used were characterized by the following criteria²⁰: 1) morphological appearance of stellate cells growing in interwoven bundles, 2) uniform fluorescence with FITC-phalloidin (F-PHD) specific for F-actin, 3) immunofluorescence staining for smooth muscle-type myosin, 4) immunofluorescence staining of extracellular matrix for type IV collagen and fibronectin using monospecific antisera, and 5) negative immunofluorescence staining for HLA-DR and leukocyte common antigen (CD-45) and human factor VIII antigens. In parallel experiments, cell viability was monitored by Trypan blue and ranged between 88% and 95%.

IL-12 Receptor Analysis

The presence of the IL-12 receptor on MCs was evaluated by cytofluorimetric analysis by assessing IL-12 binding to the putative receptor using the technique described in.²² MCs detached using 0.05 mol/L EDTA were first incubated with heat-inactivated human serum to block nonspecific sites. MCs (2 × 10⁶) in 100 μl of staining buffer (PBS containing 2% heat-inactivated human serum and 0.1% sodium azide) were sequentially incubated with IL-12 (10 ng/ml) for 1 hour, followed by incubation with the anti-IL-12 MAb C.11.5 or the neutralizing anti-IL-12 MAb C.8.6 or the irrelevant isotypic control for 30 minutes and finally with FITC-conjugated goat anti-mouse IgG for 20 minutes. All incubations were performed in staining buffer at 4°C, and cells were washed twice between incubations. Phytohemagglutinin-activated peripheral blood mononuclear cells, prepared as described previously,²² were used as positive control.

The stained cells were analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA).

Immunoprecipitation and Western Blot Analysis Studies

MCs (20×10^6) were extracted with cold detergent-insoluble matrix buffer (50 mmol/L Pipes, pH 6.8, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 300 mmol/L sucrose, 5 mmol/L EGTA, 2 mmol/L sodium orthovanadate) plus 1% Triton X-100 and a mixture of protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml leupeptin, 0.15 U/ml aprotinin, 1 μ g/ml pepstatin A) for 20 minutes at 4°C and centrifuged at $15,000 \times g$ for 20 minutes. The clarified supernatant was precleaned for 1 hour with 50 μ l of Sepharose-protein A (3 mg/sample). The protein concentration of MC lysates was determined by the Bradford's technique, and the protein content of the samples was normalized to 250 mg/sample by appropriate dilution with the cold DIM buffer. The samples were then incubated with 2 μ g of 12R β .44 MAb or the isotopic control and adsorbed by anti-mouse IgG coupled to Sepharose-protein A. Bound proteins were washed several times in DIM buffer and eluted in boiling Laemmli buffer. Thirty microliters of eluted proteins were subjected to 8% SDS-polyacrylamide gel electrophoresis. Lymphocytes (20×10^6) were extracted by sonication. Proteins were then transferred electrophoretically to nitrocellulose; the filters were incubated with blocking solution (10% low-fat milk in 20 mmol/L Tris/HCl, pH 7.6, and 17 mmol/L NaCl) for 1 hour. The anti-IL-12 receptor 12R β .44 MAb (2 μ g) was then added at the same solution, and the incubation was carried out overnight at room temperature. For detection, the filters were washed four times (15 minutes each wash) with PBS, 0.5% Tween 20 and reacted for 1 hour at room temperature with horseradish-peroxidase-conjugated protein A. The enzyme was removed by washing as above. The filters were reacted for 1 minute with a chemiluminescence reagent (ECL) and exposed to an autoradiography film for 1 to 15 minutes. To reprobe, nitrocellulose filters were first stripped of antibody by 62 mmol/L Tris/HCl, pH 6.7, 2% SDS, 100 mmol/L β 2-mercaptoethanol.

IL-12 mRNA Expression

IL-12 p40-specific mRNA was detected in total RNA extracted from MCs by guanidinium thiocyanate phenol-chloroform and precipitated with isopropanol. One microgram of RNA was treated with 6 U of RNase-free DNase for 1 hour at 37°C and then for 5 minutes at 94°C; complementary DNA was obtained by using random hexamer primers (Perkin-Elmer Cetus, Norwalk, CT). Reverse transcription was carried out at 42°C for 60 minutes; in addition to 1 μ g of RNA, the reaction mixture (20 μ l) contained 10 mmol/L Tris/HCl, pH 8.3, 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 1.0 mmol/L dNTPs, 20 U of ribonuclease inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer Cetus). cDNA was then subjected to 35 cycles of amplification by the polymerase chain reaction (PCR) in an automated DNA thermal cycler (Perkin-Elmer Cetus) by using human IL-12 p40 mRNA-specific primer pairs (R&D Systems, Abingdon, UK). The

PCR reaction mixture (50 μ l) contained 10 mmol/L Tris/HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 20 pmol of (+) and (–) primers, and 2 U of thermostable DNA polymerase (Perkin-Elmer Cetus). Times and temperatures for denaturation, annealing, and extension were 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C, respectively. Amplification product (559 bp) was analyzed in 2% agarose gels containing 0.5 μ g/ml ethidium bromide.

Purification and Quantification of PAF

The production of PAF from MCs stimulated with IL-12 was studied. Cells were equilibrated for 15 minutes in Tris-buffered Tyrode's buffer containing 0.25% delipidized BSA (fraction V), as previously described,^{23,24} and incubated at 37°C for the indicated time with IL-12 at different concentrations. To assess the specificity of the reaction, IL-12 was preincubated for 10 minutes at 37°C with the neutralizing anti-IL12 MAb C.8.6 (10 μ g/ml). Selected experiments were conducted in the presence of 5 μ g/ml polymixin B for 30 minutes at 37°C to exclude LPS contamination. The supernatants and the cell pellets were extracted according to a modification of the Bligh and Dyer procedure,²⁵ with formic acid added to lower the pH of the aqueous phase to 3.0. Each individual experiment was performed in duplicate.

PAF was quantified after extraction and purification by thin layer chromatography (silica gel plates 60 F254, Merck, Darmstadt, Germany) and high-pressure liquid chromatography (μ Porasil column, Millipore Chromatographic Division, Waters, Milford, MA) by aggregation of washed rabbit platelets, as previously reported.^{23,24} The biologically active material extracted from cells and supernatants in different experiments was characterized by comparison with synthetic PAF according to the following criteria^{23–27}: 1) induction of platelet aggregation by a pathway independent from both ADP and arachidonic acid/thromboxane-A₂-mediated pathways, 2) specificity of platelet aggregation as inferred from the inhibitory effect of 5 μ mol/L WEB 2170 or CV 3988, two different PAF receptor antagonists,^{28,29} 3) thin-layer chromatography and high-pressure liquid chromatography behavior and physico-chemical characteristics, such as inactivation by strong bases and by phospholipase A₂ treatment, but resistance to phospholipase A₁, acids, weak bases, and 5 minutes of heating in boiling water.^{26,27}

Cytokine Detection

The presence of IL-12 protein was measured in the supernatants from MCs unstimulated or stimulated with LPS (100 ng/ml) or TNF- α (10 ng/ml) with an ELISA kit that specifically detects only the heterodimeric form of the molecule (Genzyme, Cambridge, MA). The quantitative determination of TNF- α and IL-8 in the supernatant of IL-12-stimulated MCs was performed by ELISA using specific kits (Genzyme).

O_2^- Assay

Production of O_2^- was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome C.³⁰ MCs (2.5×10^6 cells) were incubated at 37°C in Tyrode's buffer (2.6 mmol/L KCl, 1 mmol/L $MgCl_2$, 137 mmol/L NaCl, 6 mmol/L $CaCl_2$, 0.1% glucose, 1 mmol/L Tris, pH 7.4) containing 80 μ mol/L cytochrome C with or without superoxide dismutase (50 U/ml) and appropriately stimulated. Basal O_2^- production was assessed in the absence of stimulating factors. Supernatants were removed at specified times and centrifuged, and the absorbance was measured in a spectrophotometer at 550 nm. The extinction coefficient of ferricytochrome C at 550 nm was taken as 2.1×10^4 (mol/L)⁻¹ cm⁻¹. Protein content of MCs was measured according to the Lowry technique. O_2^- production was expressed as nmol/L cytochrome C reduced/mg of protein.³⁰

Shape Change of MCs

MCs, seeded in small petri dishes (35-mm diameter) coated with dimethylpolysiloxane at subconfluent density, in DMEM with 0.25% BSA, were kept in an attached, hermetically sealed plexiglass Nikon NP-2 incubator at 37°C. Cells were stimulated with IL-12 (20 ng/ml), AT-II (10^{-7} mol/L), and PAF (10 nmol/L). To evaluate the role of PAF in IL-12-dependent shape change, cells were incubated for 10 minutes with WEB 2170 (3 μ mol/L) and CV 3988 (5 μ mol/L) before stimulation. Cell shape change was studied over 2-hour period under a Nikon Diaphot inverted microscope with a 20 $\times$ phase-contrast objective. Cell shape change was recorded using a JVC-1CCD video camera. Image analysis was performed with a MicroImage analysis system (Cast Imaging srl, Venice, Italy) and an IBM-compatible system equipped with a video card (Targa 2000, Truevision, Santa Clara, CA). Image analysis was performed by digital saving of image compared before stimulation and then at 5-minute intervals for 2 hours. The cell planar surface was calculated by the MicroImage software. The reduction of the planar cell surface >15% was used as a parameter of cell shape change compatible with a cell contraction. Both the number of contracted cells and the mean cell contraction were indicated. Between 10 and 25 cells were analyzed for each experimental condition and repeated at least four times. Values are given as mean $\pm$ SE.

Cytoskeleton Alterations

Actin microfilament alterations in IL-12-stimulated MCs was evaluated as binding of FITC-phalloidin. MCs seeded on glass coverslips coated with dimethylpolysiloxane were stimulated for 2 hours with IL-12 (20 ng/ml), with IL-12 plus the neutralizing anti-IL-12 C.8.6 MAb, with IL-12 plus the PAF-receptor antagonist WEB 2170 (3 μ mol/L), or with AT-II (10^{-7} mol/L) for 15 minutes. MCs were then fixed for 5 minutes at room temperature in 3% paraformaldehyde in PBS, pH 7.6, containing 2% sucrose. After rinsing in PBS, MCs were permeabilized by

Figure 1. Expression of mRNA specific for IL-12 p40 by MCs incubated with vehicle alone (DMEM plus 0.25% BSA, lane 2) or with 10 ng/ml TNF- α (lane 3) or 100 ng/ml LPS (lane 4) at 37°C for 18 hours. Lane 1 represents IL-12 p40 cDNA positive control, provided by R&D Systems. Comparable mRNA expression for β -actin in unstimulated (lane 2) and TNF- α -stimulated (lane 3) or LPS-stimulated (lane 4) MCs is indicated in the lower panel.

soaking the coverslips for 5 minutes in 20 nmol/L Hepes, pH 7.4, 300 nmol/L sucrose, 50 mmol/L $MgCl_2$, and 0.5% Triton X-100. To stain actin microfilaments, direct staining using F-PHD (30 minutes at 37°C at the concentration of 2 μ g/ml) was performed. F-PHD, which directly binds to F-actin, was used according to the method of Wulf et al.³¹

Results

IL-12 Production

We studied the expression by cultured MCs of the IL-12 p40 subunit mRNA and the synthesis of the heterodimeric protein p70 in basal conditions and after stimulation with LPS and TNF- α . Figure 1 shows that the expression of IL-12 p40-specific mRNA in MCs was inducible on cell activation. MCs cultured for 18 hours in basal conditions did not express detectable IL-12 p40-specific mRNA by reverse transcription PCR. In contrast, after stimulation with LPS or TNF- α , MCs expressed the IL-12 mRNA. Moreover, MCs stimulated with LPS and TNF- α synthesized and released the IL-12 protein. The synthesis peaked at 24 hours to become undetectable after 48 hours (Figure 2A). MCs challenged with the vehicle alone as control did not synthesize detectable amounts of IL-12. Figure 2B shows the dose dependency of IL-12 synthesis induced by TNF- α . The inhibitory effect of cycloheximide, which prevents protein synthesis, suggests that the released IL-12 was newly synthesized (Figure 2B).

IL-12 Receptor Expression

IL-12 binding to the putative receptor was evaluated by incubating MCs with IL-12 followed by staining with the anti-IL-12 MAb C.11.5. As shown in Figure 3, MCs expressed significant levels of IL-12 binding. MCs unchallenged with IL-12 or incubated with the irrelevant isotypic control were not stained. IL-12 receptor detection specificity by this method was confirmed by the diminished staining observed when the C.11.5 MAb was replaced with the neutralizing anti-IL-12 C.8.6 MAb (Figure 3). Because the neutralizing anti-IL-12 C.8.6 MAb recognizes the IL-12 receptor-binding epitope, the reaction of

Figure 2. A.: Release of IL-12 in the supernatant of 1×10^6 MCs stimulated with LPS (100 ng/ml; striped bars) or TNF- α (10 ng/ml; solid bars) for different periods of time. Data are expressed as mean $\pm$ SD of three different experiments. B: IL-12 is synthesized by 1×10^6 MCs after stimulation with different doses of TNF- α (■) or of TNF- α in the presence of cycloheximide (○) as control. Data represent the mean $\pm$ SD of three individual experiments.

IL-12 with its receptor prevents binding of neutralizing anti-IL-12 MAb to cell-associated IL-12 as reported by Desai et al.²² As positive control, IL-12 binding to the putative receptor was detected on peripheral blood mononuclear cells activated for 72 hours with phytohemagglutinin (data not shown).

The expression of the IL-12 receptor β 1-chain by MCs was also evaluated by immunoprecipitation followed by Western blot analysis. The anti-IL-12 receptor 12R β .44 MAb was capable of recognizing a band of approximately 110 kd, which corresponds to the β 1 receptor chain,³² in MCs immunoprecipitated with the 12R β .44 MAb but not with the isotypic control (Figure 4). The expression of the β 1 receptor in unstimulated lymphocytes was used as control.

PAF Production by IL-12-Stimulated MCs

IL-12 induced a time-dependent (Figure 5A) and dose-dependent (Figure 5B) production of PAF from MCs. Time course studies showed a peak of PAF production 30 minutes after stimulation with IL-12 (Figure 5B). PAF de-

Figure 3. Detection of IL-12 receptor by flow cytometry on MCs. Binding of IL-12 to the putative receptor was evaluated by incubating MCs with IL-12 and then with the anti-IL-12 MAb C.11.5 (dark line) or with the neutralizing anti-IL-12 MAb C.8.6 (dashed line) or with an irrelevant isotypic control IgG (gray line), as described in Materials and Methods. Two experiments were carried out with similar results. In each individual experiment the Kolmogorov-Smirnov statistical analysis between C.11.5 MAb and the isotypic control or between C.5.11 and C.8.6 was significant ($P < 0.05$).

tested at that time was all cell associated, whereas 120 minutes after stimulation PAF was detected as released in the supernatant. Cell viability tested at the end of each experiment by Trypan blue dye exclusion test was $>90\%$. To evaluate the specificity of the effect induced by IL-12, the cytokine preparation was incubated for 30 minutes at 37°C with the neutralizing C.8.6 anti-IL12 MAb. Both cell-associated and cell-released PAF production was almost completely abrogated in the presence of the C.8.6 MAb, whereas the TNF- α -induced PAF synthesis was not (Figure 5C). The synthesis of PAF induced by IL-12 did not require protein synthesis, as treatment of MCs with cycloheximide before stimulation with IL-12 did not prevent the synthesis of PAF (Figure 5C).

Figure 4. MCs express the IL-12 receptor β 1 chain. MCs (2×10^7) were lysed and immunoprecipitated with the 12R β .44 MAb (lane 1) or with the irrelevant isotypic control IgG (lane 2). Unstimulated lymphocytes (2×10^7 ; lane 3) were used as positive control. The eluted proteins were subjected to 8% SDS-PAGE, and then the filter was immunoblotted with the anti-IL-12 receptor β 1 chain 12R β .44 MAb. The position of the IL-12 receptor β 1 chain is indicated by the arrow.

Figure 5. PAF production by MCs stimulated with IL-12. **A:** Time course of PAF synthesis from 1×10^6 MCs unstimulated (□) or stimulated with 20 ng/ml IL-12. PAF was detected as cell-associated (●) and as released in the supernatant (○). Data represent the mean $\pm$ SE of six individual experiments. **B:** Dose-dependent PAF production from 1×10^6 MCs stimulated with IL-12 for 30 minutes. Data are the mean $\pm$ SE of three individual experiments. **C:** Cell-associated (striped bars) and released (solid bars) PAF after stimulation with 20 ng/ml IL-12 or with IL-12 preincubated (30 minutes at 37°C) with the neutralizing anti-IL-12 C.8.6 MAb (10 μ g/ml; C.8.6) or with cycloheximide (10 μ g/ml) or after stimulation with TNF- α (10 ng/ml) or TNF- α preincubated with the C.8.6 MAb used as control. Data are mean $\pm$ SE of four individual experiments. ANOVA with Dunnett's multicomparison test was performed among MCs stimulated with IL-12 *versus* MCs stimulated with IL-12 plus the anti-IL-12 C.8.6 MAb or MCs stimulated with IL-12 plus cycloheximide, or between MCs stimulated with TNF- α and TNF- α plus the anti-IL-12 C.8.6 MAb. * $P < 0.05$.

Cytokine and O_2^- Production by IL-12-Stimulated MsC

We evaluated the production of TNF- α and IL-8 by MCs stimulated with various doses of IL-12 (5 to 20 ng/ml) for 8, 12, 24, and 48 hours. IL-12 failed to stimulate the production of TNF- α or IL-8 by MCs at all concentrations and times tested (data not shown).

IL-12 induced O_2^- production by MCs, evaluated as reduction of cytochrome C. The production of O_2^- peaked 1 minute after the addition of IL-12 and was

Figure 6. Time course of the production of O_2^- from MCs (2.5×10^6 cells) unstimulated (○) or stimulated with 20 ng/ml IL-12 (■) or with 20 ng/ml IL-12 preincubated (30 minutes at 37°C) with the neutralizing anti-IL-12 C.8.6 MAb (10 μ g/ml; ▲). Values are given as mean $\pm$ SE of five individual experiments.

abrogated by preincubation of IL-12 with 10 μ g/ml C.8.6 neutralizing anti-IL12 MAb (Figure 6).

Shape Change of MCs

Shape change of MCs, compatible with a cell contraction, was evaluated as changes in planar surface area in response to different stimuli. As shown in Table 1, IL-12 induced a reduction of the cell planar surface of $>15\%$ in 84% of MCs. Figure 7 is representative of MC shape change observed after stimulation with IL-12. Preincubation with neutralizing C.8.6 anti-IL-12 MAb prevented the IL-12-induced reduction of the cell planar surface (Table 1). A similar reduction of the cell planar surface was obtained with AT-II and synthetic PAF. However, the kinetics of cell shape change were different depending on

Table 1. Planar Surface Reduction of MCs Induced by IL-12, AT-II, and PAF

	% retracted cells	Mean area reduction
Alone	5	2.7 ± 1.3
IL-12	84	37.5 ± 1.3
IL-12 + C.8.6	9	5.5 ± 2.1
IL-12 + WEB 2170	25	9.1 ± 2
IL-12 + CV 3988	10	4.3 ± 0.8
AT-II	86	24.5 ± 3.1
AT-II + WEB 2170	83	25.2 ± 4.1
WEB 2170	3	1.1 ± 1.6
CV 3988	4	1.4 ± 1.5
PAF	73	37.9 ± 5.4

MCs seeded in small Petri dishes coated with dimethylpolysiloxane at subconfluent density and kept at 37°C were stimulated, and the cell planar surface was analyzed before stimulation and then every 5 minutes for a 2-hour period. Cell shape change induced by IL-12 (20 ng/ml); AT-II (10^{-7} mol/L), and PAF (10 nmol/L) was studied. In selected experiments, cells were preincubated with the anti-IL-12 neutralizing C.8.6 MAb (10 minutes at 37°C) or with the PAF receptor antagonists WEB 2170 (3 μ mol/L) or CV 3988 (5 μ mol/L) (30 minutes at 37°C) and then challenged with IL-12 or the vehicle alone. Image analysis was performed as described in Material and Methods. The percentage of cells showing a reduction $>15\%$ of the planar area and the mean area reduction were evaluated. Between 10 and 25 cells were analyzed for each experimental condition, and each experiment was repeated at least four times. Values are given as mean $\pm$ SD.

Figure 7. Micrographs representative of MCs, seeded in Petri dishes coated with dimethylpolysiloxane at subconfluent density, in DMEM with BSA 0.25% stimulated with IL-12 (20 ng/ml). Cell shape change was studied over a 2-hour period under a Nikon Diaphot inverted microscope with a 20 $\times$ phase-contrast objective using a JVC-1CCD video camera. **A:** Morphological aspect of MCs before stimulation with IL-12. **B:** Morphological changes of MCs, consistent with cell contraction, observed 120 minutes after stimulation with IL-12. Magnification, $\times 200$.

the stimuli (Figure 8). Whereas the reduction of the cell planar surface induced by IL-12 was delayed and sustained, reaching its maximum between 90 and 120 minutes of incubation, reduction of the cell planar surface induced by AT-II was rapid and transient. The cell shape change induced by PAF was also rapid, but sustained up to 60 minutes. As the kinetics of cell shape change induced by IL-12 was concomitant with that of PAF production (Figure 5), we evaluated whether the production of PAF could mediate the shape change of MCs induced by IL-12 using WEB 2170 and CV 3988, two structurally different PAF-receptor antagonists.^{28,29} As shown in Table 1, the receptor antagonists significantly reduced cell shape change induced by IL-12, as well as that induced

by synthetic PAF. In contrast, as previously reported,³³ AT-II-induced contraction was not prevented by the addition of the PAF-receptor antagonists. The changes in cell shape of MCs were reversed by replacement of the stimuli with fresh medium. No significant cell shape change was observed in MCs stimulated with the vehicle alone.

Cytoskeleton Alterations

Morphological alteration of IL-12-stimulated MCs were associated with cytoskeleton changes. Resting MCs showed an elaborate array of microfilament bundles of the stress fiber type after staining with F-PHD, which binds specifically to F-actin (Figure 9A). Within 1 to 2 hours after addition of IL-12, MCs lost their regular array of microfilament bundles, and F-actin appeared to be predominantly associated with the cell periphery; most stress fibers disappeared, and ruffles were often seen (Figure 9B). Several MCs showed leading edges and a prominent tail compatible with cell movement (Figure 9C). These changes of cytoskeleton were similar to those induced by PAF⁸ and were inhibited by WEB 2170 (Figure 9D).

Figure 8. Kinetics of change of MC shape after stimulation with 20 ng/ml IL-12, 10^{-7} mol/L AT-II, or 10 nmol/L PAF evaluated using a video-recording system, as described in Materials and Methods. Cell retraction is expressed as the reduction of the planar surface evaluated before stimulation. Between 10 and 25 cells were analyzed for each experimental condition and repeated at least four times. Values are given as mean $\pm$ SD.

Discussion

The results of the present study demonstrate that MCs synthesize IL-12 after stimulation with LPS and TNF- α and that IL-12 interacts with MCs and induces cell shape change and cytoskeletal reorganization by a mechanism

Figure 9. Micrographs representative of F-actin staining of human MCs. **A:** Control MCs show an elaborate array of microfilament bundles of the stress fibers. **B** and **C:** Effect of treatment with 20 ng/ml IL-12 for 2 hours. Stress fibers tend to disappear, and F-actin appeared to be predominantly associated with the cell periphery (**B**) or fuse in axial microfilaments bundles in cells showing a leading edge and a prominent tail compatible with cell movement (**C**). **D** shows the inhibitory effect of WEB 2170 on IL-12-induced changes of MC cytoskeleton. Magnification, $\times 400$.

involving the synthesis of PAF. IL-12 is involved in both the innate resistance mediated by phagocytic and NK cells and the adaptive immune response mediated by T and B cells.¹² IL-12 regulates the cytotoxic activity, the proliferation, and the cytokine production of NK and T cells.^{13–15} IL-12 also regulates the inflammatory cell recruitment in tissues, acting as a chemotactic agent.^{34,35} In adaptive immunity, IL-12 induces a Th1-type immune response that is particularly beneficial in infectious processes involving intracellular pathogens and parasites.¹⁶ Furthermore, IL-12-dependent Th1 responses have been demonstrated to cause or exacerbate autoimmune diseases.¹⁶ IL-12 has been implicated in several experimental and human autoimmune diseases, including autoimmune encephalitis in mice,³⁶ diabetes in NOD mice,³⁷ and Wegener granulomatosis in humans.³⁸ Moreover, a prominent IL-12-dependent Th1 response has been implicated in some experimental glomerulonephritis.¹⁷ In particular, the host's propensity to develop a Th1-type response has been correlated with the susceptibility and the severity of a crescentic glomerulonephritis in mice and rat.^{39,40} The pathogenetic role of the Th1 response has been also demonstrated in a lupus-like glomerulonephritis in mice.⁴¹ In this experimental model, the inhibition of the Th1 response was capable of ameliorating the disease and of down-regulating the appearance of Th1-

mediated nephritogenic IgG subclasses of antibodies. Recently, it has been demonstrated that in MRL-Fas^{lpr} mice, which spontaneously develop a lupus nephritis, IL-12 is up-regulated in the nephritic kidney.¹⁸ Moreover, cultured proximal tubular cells derived from MRL-Fas^{lpr} mice produce IL-12. This observation is of interest as only a few nonhematopoietic cells, such as keratinocytes,⁴² have been previously shown to produce IL-12. In the present study, we could show that, *in vitro*, human MCs are capable of producing IL-12 in response to pro-inflammatory stimuli such as TNF- α and LPS. Additional studies are needed to confirm whether MCs synthesize IL-12 *in vivo* in experimental or human glomerular injury. Although MCs produce only low quantities of IL-12 compared with LPS-activated monocytes,¹⁵ a local production of IL-12 by MCs could contribute to glomerular injury either by promoting recruitment of inflammatory cells or by stimulating the development of a Th1 immune response. Recently, the presence of Th1 cells and cytotoxic lymphocytes has been shown in glomeruli of rats developing Heymann nephritis and has been correlated with the onset of proteinuria.⁴³

MCs were also shown to be directly stimulated by IL-12. The functional high-affinity IL-12 receptor is composed of two β -type cytokine receptor subunits, each independently exhibiting a low-affinity binding for IL-12.⁴⁴

Whereas the $\beta 1$ subunit is expressed in basal conditions on lymphocytes,⁴⁵ the distribution of the $\beta 2$ subunit is more restricted, and its expression appears to be related to lymphocyte activation or differentiation.^{46,47} Herein, we demonstrate that cultured MCs bind IL-12 and express the human low-affinity IL-12 $\beta 1$ chain receptor under basal conditions. A similar expression of the low-affinity IL-12 receptor $\beta 1$ chain has been observed in unstimulated T lymphocytes,⁴⁵ in which IL-12, even in the absence of the $\beta 2$ subunit, induces an efficient production of interferon- γ ¹² and in neutrophils, in which IL-12 stimulates the synthesis of PAF.¹¹ We observed that IL-12 induces a dose-dependent synthesis of PAF also by MCs. IL-12-induced synthesis of PAF by MCs started rapidly and was detected both as associated to the cellular fraction and as released in the supernatant. On phagocytic cells, IL-12 induces production of interferon- γ and of other cytokines, such as granulocyte/macrophage colony-stimulating factor, IL-8, and TNF- α .^{12,48,49} As MCs may synthesize PAF with a cytokine-dependent mechanism, we evaluated whether IL-12 could induce cytokine synthesis by MCs. We failed to detect synthesis of TNF- α or IL-8 by IL-12-stimulated MCs. Moreover, treatment of MCs with cycloheximide before stimulation with IL-12 did not prevent the synthesis of PAF, suggesting a direct stimulatory action of IL-12 on the synthesis of this mediator rather than a cytokine-dependent synthesis. Previous studies have shown that PAF exerts several effects on renal function.¹⁰ Beside a vascular effect, PAF reduces the glomerular filtration by inducing the contraction of mesangial cells.⁷ The contraction of mesangium is in fact correlated with a decrease of the filtration area and may therefore affect the coefficient of filtration.¹⁻³ Herein, we demonstrate that IL-12 can also induce shape change of MCs compatible with cell contraction. The cell shape change induced by IL-12 was delayed and sustained, reaching its maximum between 90 and 120 minutes of incubation. The changes in cell shape occurred concomitantly with cytoskeletal rearrangements; in particular, the fluorescence of F-actin decreased and its distribution appeared more associated with the cell periphery. These alterations were similar to those induced by PAF and by TNF- α , which has been shown to act via the synthesis of PAF.⁸ Moreover, the kinetic of IL-12-dependent MC shape change was concomitant with that of PAF production. A role for PAF in IL-12-dependent cell shape change was investigated using two different PAF receptor antagonists. Both antagonists almost completely inhibited the cell shape change induced by IL-12 although they were ineffective on AT-II-induced cell shape change, as previously reported by Neuwirth et al.³³

In conclusion, these results suggest that cultured MCs produce IL-12, possess the IL-12 low-affinity $\beta 1$ receptor, and can be directly stimulated by this cytokine to produce PAF and to change their shape with a cell retraction. IL-12 produced by MCs could, therefore, have an autocrine effect by regulating MC functions and a paracrine effect by activating inflammatory cells and stimulating a Th1 response within the glomeruli.

Acknowledgments

We thank Dr. L. Pegoraro and Dr. F. Malavasi for the discussion and helpful suggestions, Dr. G. Trinchieri and Genetics Institute (Cambridge, MA) for providing IL-12 and anti-IL-12 MAb (C.8.6 and C.11.5), and Dr. J. Ritz (Dana-Farber Cancer Institute, Boston) for providing the anti-IL-12 receptor 12R β .44 MAb.

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