Integrin-mediated stimulation of monocyte chemotactic protein-1 expression

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Abstract We investigated whether activation of integrin receptors could modulate the expression of monocyte chemotactic protein-1 (MCP-1) in human hepatic stellate cells (HSC), mesenchymal cells responsible for extracellular matrix synthesis within the liver. When compared to non-adherent cells, HSC plated on collagen types I or IV, or fibronectin, showed increased *MCP-1* gene expression and protein secretion in the conditioned medium. Increased MCP-1 secretion was also observed when cells were plated on dishes coated with a monoclonal antibody directed against the β1-integrin subunit, demonstrating that ligation of β1-integrins is sufficient to stimulate MCP-1 expression. Conversely, integrin-independent cell adhesion on poly-L-lysine did not modify MCP-1 secretion. Disruption of the actin cytoskeleton by cytochalasin D blocked the collagen-dependent increase in MCP-1 secretion. Chemotactic assay of HSC-conditioned medium showed that HSC plated on collagen secrete higher amounts of chemotactic factors for lymphomonocytes, and that MCP-1 accounts for the great majority of this effect. These findings indicate a novel mechanism of MCP-1 regulation possibly relevant in those conditions where HSC interact with an altered extracellular matrix.

Key words: Monocyte chemotactic protein-1; Integrin; Hepatic stellate cell; Collagen; Chemokine

1. Introduction

Infiltrating leukocytes are a salient feature of a variety of inflammatory conditions and often contribute to the pathogenesis of the underlying disease. Chemokines, or chemotactic cytokines, are a family of proteins which are potent chemotactic factors for different classes of leukocytes. Four subgroups are known, referred to as CXC, CX_3C, CC, or C chemokines, based on the position of conserved cysteine residues [1,2]. CC chemokines generally attract monocytes and lymphocytes but not neutrophils. Members of this group include monocyte chemotactic protein (MCP)-1, which is a major chemotactic factor for monocytes and T-lymphocytes [3–6]. MCP-1 is secreted by transformed and non-transformed cells [7–10] and its actions on target cells are mediated by the CCR2 chemokine receptor [11,12]. Expression of MCP-1 is up-regulated in different diseases, including atherosclerosis, pulmonary fibrosis, renal and liver diseases [13–16].

The liver response to injury is similar to the one observed in other tissues, leading to a reparative response and, when the injury is prolonged, to tissue fibrosis and scarring [17]. Accumulating evidence indicates that the hepatic stellate cells (HSC) play a pivotal role in the reparative response to liver injury, through proliferation and secretion of extracellular matrix (ECM) [17,18]. More recently, it has been reported that HSC may coordinate the recruitment of leukocytes through secretion of chemokines, including MCP-1 [19,20]. These events parallel those observed during the reparative response in other tissues, such as the kidney, lung, or blood vessels. Therefore, HSC may be regarded to as paradigmatic for other myofibroblast-like cells, such as kidney glomerular mesangial cells or vascular smooth muscle cells, involved in the repair process in other tissues.

The interaction of HSC with the ECM are mediated, at least in part, by integrins, a family of heterodimeric transmembrane adhesion receptors constituted of non-covalently associated α- and β-subunits [21]. We have recently characterized the main integrin receptors mediating HSC adhesion to collagens, fibronectin, and laminin [22]. In addition to their role as adhesion receptors, integrins function as signaling receptors, and have been shown to regulate cytoskeleton reorganization, protein kinase activation, and gene expression [23,24]. In the present study we provide evidence that interaction between β1-containing integrin receptors and ECM components stimulate MCP-1 expression in HSC. These data indicate a novel mechanism for MCP-1 secretion, potentially relevant for the pathophysiology of liver diseases and inflammatory states in general.

2. Material and methods

2.1. Materials and matrix preparation

Types I and IV collagen were generously provided by Dr. D. Schuppan (Free University of Berlin, Germany). Fibronectin and polylisine hydrobromide (MW 70 000–150 000) were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-baboon MCP-1 antiserum was kindly provided by Dr. A.J. Valente (University of Texas Health Science Center at San Antonio, TX). This antiserum cross-reacts 100% with human MCP-1. Monoclonal antibodies raised against the human β1-integrin (AIIB2) were a kind gift of Dr. C.H. Damski (University of California, San Francisco, CA). The ECM proteins were dissolved in calcium- and magnesium-free PBS to the concentration of 20 μg/ml type I collagen, 10 μg/ml type IV collagen, 20 μg/ml fibronectin. For coating with AIIB2, the hybridoma supernatant was diluted 1:1 with 0.1 M carbonate buffer, pH 9.4. Coating of plastic dishes was carried out by overnight incubation at 4°C. The dishes were then incubated for 2 h at 37°C with 1% (w/v) BSA denatured at 70°C for 15 min, to block the non-specific binding sites on plastic, and washed with sterile PBS before cell plating. As control, dishes were directly blocked with denatured BSA. For poly-L-lysine coating, a 0.1 mg/ml solution was applied for 5 min, and the dishes were then washed once with sterile PBS.

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2.2. Cell culture

Human liver HSC were isolated from liver tissue unsuitable for transplantation by collagenase/pronase digestion and centrifugation on stractan gradients. Procedures used for cell isolation and characterization have been extensively described elsewhere [19,25]. Cells were cultured in Iscove's medium supplemented with 17% FCS.

2.3. Northern blot analysis

HSC were cultured on plastic dishes in complete medium until confluent. The cells were then washed twice with PBS and incubated in serum-free, insulin-free (SFIF) medium for 24 h. The medium was aspirated and the cells were trypsinized, centrifuged, and resuspended in fresh SFIF medium at a concentration of approximately 250 000 cells/ml. Eight milliliters of the cell suspension were then seeded onto 100-mm Petri dishes precoated as indicated above, and the cells were incubated for the indicated time points. The cells were then scraped in their culture medium, centrifuged, and total RNA was isolated as described by Chomczynski and Sacchi [26]. Northern blot analysis was carried out as previously described [27]. The filters were hybridized with a ^{32}P-labeled full-length baboon MCP-1 probe at 42°C, washed, and autoradiographed. After removal of the MCP-1 probe, the same filters were hybridized with a ^{32}P-labeled control probe encoding for the ribosomal protein 36B4 [28].

2.4. MCP-1 Western blot

HSC grown to confluence in complete medium were pre-incubated in SFIF medium for 24 h, trypsinized, and resuspended in SFIF medium at a concentration of approximately 100 000 cells/ml. Identical aliquots of cell suspension (750 μl) were then plated onto 12-well plastic dishes precoated with the appropriate ECM component as indicated above. After 24 h the conditioned medium was collected, briefly spun at 4°C to remove cells, and stored at −80°C until analyzed. Conditioned medium (50–100 μl) was dried and resuspended in 15 μl of Laemmli sample buffer [29]. Western blot analysis was carried out as described in detail elsewhere [30]. Briefly, samples were separated by 15% SDS-PAGE analysis and electroblotted on a PVDF membrane. The membrane was blocked with 3% BSA in PBS containing 0.1% Tween-20, then sequentially incubated with anti-baboon MCP-1 antibodies (1:500 in blocking buffer) and with horseradish peroxidase-conjugated anti-rabbit antibodies (1:5000). After extensive washing, the signal was detected by chemiluminescence (Amersham, Little Chalfont, UK) using the manufacturer's protocol.

2.5. MCP-1 ELISA

Cell culture supernatants were obtained and stored as indicated for Western blot analysis. MCP-1 concentration was determined using a commercial ELISA kit specific for human MCP-1 (R&D Systems, Minneapolis, MN). Samples were diluted 40-fold to obtain values fitting in the standard curve.

2.6. Chemotactic assay

Human blood mononuclear cells were prepared from peripheral blood collected in ACD anticoagulant from healthy volunteers, using a metrizoate/polysaccaride solution (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) according to the manufacturer's protocol. The mononuclear cells were washed twice with Ca^{2+}- and Mg^{2+}-free HBSS, counted, and finally resuspendend in Iscove's medium containing 0.5% BSA at a concentration of 3×10^6 cells/ml. The chemotaxis assay was carried out using polyvinylpirrolidone-free polycarbonate 5 μm filters and modified Boyden chambers, as described by Valente et al. [3]. HSC-conditioned medium was serially diluted, pre-incubated with neutralizing anti-MCP-1 antibodies or non-immune rabbit serum for 1 h at 37°C, and placed below the filters. The cell preparation (200 μl) was placed above the filters and the chambers were incubated at 37°C for 90 min. After staining with Giemsa, cells migrating to the underside of the filters were quantitated as the mean number of cells in 10 high-power fields.

2.7. Reproducibility and data analysis

All the autoradiograms and autoluminograms shown are representative of at least three independent experiments with comparable results. Quantitative data were obtained by densitometry scan of the films. Statistical analysis was performed by Student's t test for paired data. $P<0.05$ was considered significant.

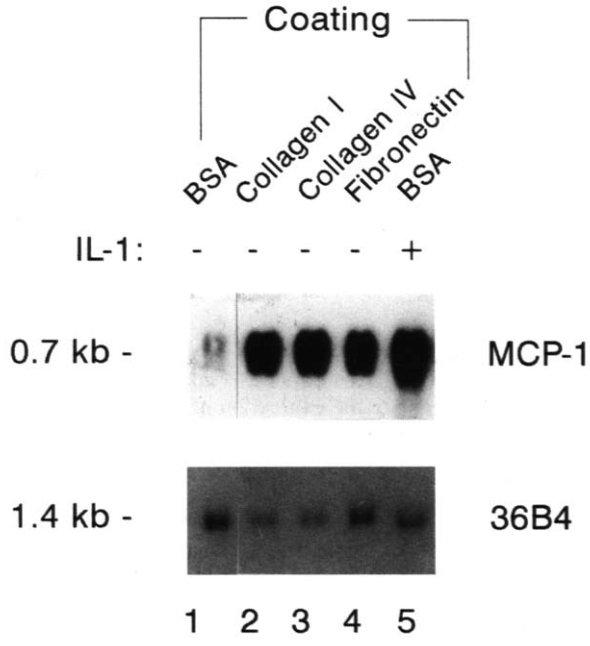

Fig. 1. Adhesion on ECM up-regulates *MCP-1* gene expression in HSC. Serum-starved human HSC were trypsinized and plated on dishes coated with BSA (lane 1, non-adhesion), on types I or IV collagen, or on fibronectin (lanes 2–4) for 4 h. Cells plated on BSA were also incubated in the presence of 25 U/ml IL-1α (lane 5). At the end of incubation, total RNA was prepared and Northern blot analysis was performed as described in Section 2.

3. Results and discussion

We first investigated if adhesion of HSC to different ECM proteins could affect *MCP-1* gene expression. Serum-starved cells were trypsinized and plated on dishes coated with BSA, types I or IV collagen, or fibronectin. Plating on BSA resulted in a complete lack of attachment, whereas all the cells adhered to collagens or fibronectin. MCP-1 mRNA was expressed at low levels in serum-starved, non-adherent HSC, whereas it was markedly up-regulated in cells adhering to collagen I, collagen IV, or fibronectin for 4 h (Fig. 1). The average increase in MCP-1 mRNA levels was 3.3, 3.8, and 2.6-fold, comparing types I and IV collagen and fibronectin, respectively, with BSA ($n=3$). Up-regulation of the message for MCP-1 was evident as early as 1 h after plating on type I collagen and peaked at 4 h (data not shown). To rule out the possibility that non-adherent HSC could be non-specifically altered in their ability to express MCP-1, cells plated on BSA were incubated with IL-1, a potent inducer of MCP-1 expression (Fig. 1, lane 5). Incubation with this cytokine resulted in marked up-regulation of MCP-1 mRNA abundance, indicating that non-adherent cells are capable of responding to cytokines in a similar fashion as adherent cells. To determine if plating on different ECM proteins results in differences in the amount of protein secreted in the culture medium, an identical number of HSC was plated on BSA, types I or IV collagen, or fibronectin, and kept in culture for 24 h. Aliquots of the conditioned medium were then assayed for MCP-1 using Western blot analysis. This technique is capable of detecting as little as 1 ng of recombinant human MCP-1 (Fig. 2A). MCP-1 in the conditioned media appeared as two bands of apparent MW around 10-14 kDa (Fig. 2B, Fig. 3). When

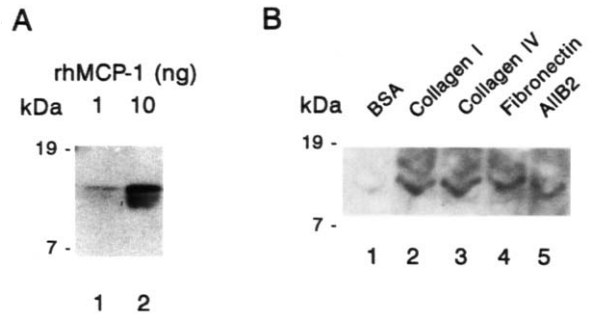

Fig. 2. Increased secretion of MCP-1 by HSC plated on ECM or on anti-β1-integrin antibodies. A: Different amounts of recombinant human MCP-1 were separated by 15% SDS-PAGE and analyzed by Western blotting with anti-MCP-1 antibodies as described in Section 2. B: Serum-starved human HSC were trypsinized and an identical number of cells was plated on dishes coated with BSA (lane 1, non-adhesion), on types I or IV collagen (lanes 2 and 3), on fibronectin (lane 4) or on dishes coated with the anti-β1-integrin antibody AIIB2 (lane 5) for 24 h. Aliquots of cell-free conditioned medium were analyzed by Western blot analysis for MCP-1. The migration of the molecular weight markers is shown on the left.

HSC were plated on collagens or fibronectin, a marked increase in the amount of MCP-1 secreted by HSC was observed in comparison to non-adherent cells (Fig. 2B, lanes 2–4). Average increase in MCP-1 secretion, as evaluated by densitometry scan of the autoluminograms, was approximately 3-fold for all ECM components tested ($n = 3$).

Types I and IV collagen and fibronectin interact with β1-containing integrin receptors on the cell surface [21]. Cultured HSC have been shown to express α1β1 and α2β1, which are ligands for types I and IV collagen, and αvβ1, which interacts with fibronectin [22,31]. To establish if the effect of ECM is mediated by interaction with these integrin receptors, we plated HSC on dishes coated with the monoclonal antibody AIIB2, raised against the human β1-subunit. Engagement of β1-containing integrins was associated with HSC adhesion, and resulted in up-regulation of MCP-1 secretion (3-fold over cells plated on BSA, $n = 3$) (Fig. 2B, lane 5). When non-immune mouse IgG were used, neither HSC adhesion nor increased secretion of MCP-1 were observed (data not shown). Thus, the effect of ECM proteins such as type I collagen on MCP-1 expression can be reproduced by interaction with β1-containing integrin receptors. In order to rule out that cell adhesion per se or the resulting shape change could be involved in the induction of MCP-1 expression, we analyzed MCP-1 levels in the conditioned medium of HSC plated on poly-L-lysine, which mediates integrin-independent cell adhesion. The number of adhering cells was similar comparing HSC plated on poly-L-lysine with those on type I collagen or on anti-β1-integrin antibodies, AIIB2. However, MCP-1 concentration in the conditioned medium was increased only when HSC were plated on collagen I or on AIIB2, whereas in cells plated on poly-L-lysine the levels of the chemokine were comparable to those of or non-adherent cells, i.e. plated on BSA (Fig. 3A,B). These data indicate that cell adhesion is not sufficient to stimulate *MCP-1* gene expression, and that interaction with an ECM protein, or engagement of an integrin receptor are required. Pretreatment of HSC with cytochalasin D, which disrupts the actin cytoskeleton, blocked the increase in MCP-1 secretion induced by collagen (Fig. 3C). Thus, an intact cytoskeleton is required to stimulate MCP-1 secretion in response to integrin activation.

In order to validate the results of Western blot analysis and to obtain quantitative data, we measured the concentration of MCP-1 in HSC-conditioned medium using a specific ELISA. These experiments confirmed the increase in MCP-1 secretion when HSC plated on type I collagen or on the anti-β1-integrin antibodies, AIIB2, were compared to non-adherent cells (Fig. 4A). Previous studies have indicated that adhesion of monocytes to the ECM induces the expression of immediate early genes, including those encoding for several cytokines [32,33]. However, in that system, adhesion was not sufficient for protein translation and secretion without exposure to a second activation signal, such as that provided by LPS [23,32]. In this report, we provide evidence that interaction of HSC with the ECM is an effective stimulus for MCP-1 expression and secretion in the absence of any additional factors, and that this effect is mimicked by ligation of β1-integrins. To our knowledge, this is the first report of integrin-dependent stimulation of MCP-1 secretion in any cell type, and of a direct relationship between integrins and chemokines in ECM-producing cells.

Different chemotactic factors for monocytes have been individuated, and several of these molecules can be produced by cultured cells, including HSC. We therefore investigated whether interaction of HSC with ECM was associated with an actual increase in the chemotactic activity for mononuclear cells, and the contribution of MCP-1 to this biologic effect.

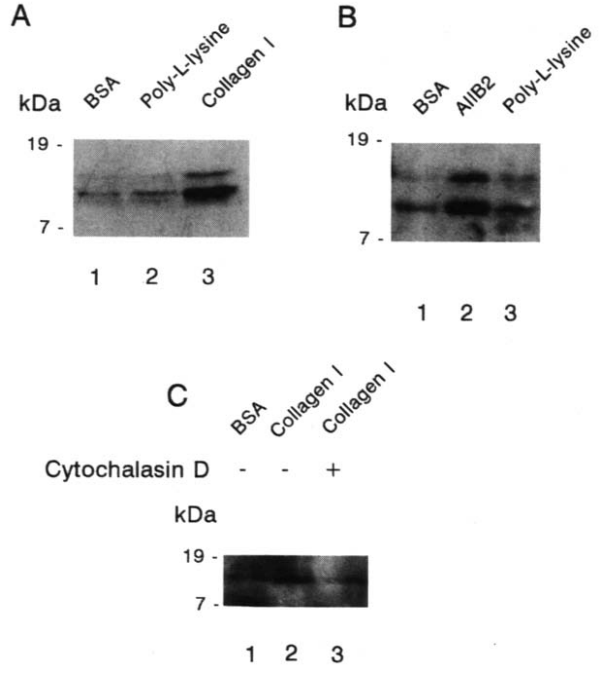

Fig. 3. Integrin-independent cell adhesion does not increase MCP-1 secretion. A: Serum-starved human HSC were trypsinized and an identical number of cells was plated on dishes coated with BSA, on poly-L-lysine, or on type I collagen for 24 h. MCP-1 Western blot analysis of conditioned medium was carried out as described in Fig. 2. B: Serum-starved human HSC were trypsinized and an identical number of cells was plated on dishes coated with BSA, on dishes coated with the anti-β1-integrin antibody AIIB2, or on poly-L-lysine for 24 h. C: Serum-starved HSC were incubated for 30 min with 5 μg/ml cytochalasin D or its vehicle (DMSO, final concentration 0.1%) for 30 min before plating on BSA or collagen I, as indicated, for 24 h. MCP-1 Western blot analysis of conditioned medium was carried out as described in Fig. 2. The migration of the molecular weight markers is shown on the left.

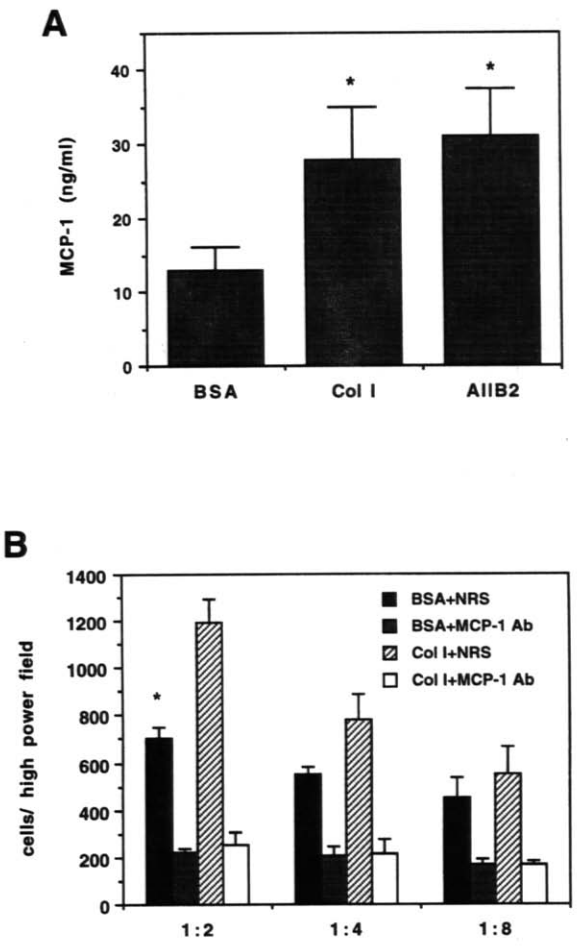

Fig. 4. A: Analysis of MCP-1 secretion using ELISA. HSC-conditioned media were obtained from cells plated for 24 h on dishes coated with BSA, type I collagen, or with the anti-β1-integrin antibody AIIB2, as described in legend to Fig. 2B. The medium was diluted and assayed using a commercial ELISA kit. Data are mean ± SEM from 5 independent experiments. $^*P < 0.05$ vs. BSA. B: Integrin-mediated increase of mononuclear cell chemotactic activity secreted by HSC. HSC conditioned medium was obtained from cells plated for 24 h on dishes coated with BSA or type I collagen. The media were incubated with non-immune rabbit serum (black columns or hatched columns for BSA and collagen, respectively) or with neutralizing anti-MCP-1 serum (gray columns or white columns for BSA and collagen, respectively). The chemotactic assay was carried out as described in Section 2, using different dilutions of the conditioned medium. Data are mean ± SEM of three independent experiments. The number of cells migrating with non-conditioned medium was subtracted from each sample. $^*P < 0.05$ vs. type I collagen.

Plating on type I collagen significantly increased the chemotactic activity of HSC conditioned medium in comparison to cells plated on BSA (Fig. 4B) or poly-L-lysine (data not shown). However, when the conditioned medium was pre-incubated with neutralizing anti-MCP-1 antibodies, the chemotactic activity was identical, regardless of where the cells were plated (Fig. 4B). These data indicate that MCP-1 accounts for the great majority of the effect of ECM, while the contribution of other chemotactic factors is negligible.

Upon injury, the liver undergoes a tightly regulated sequence of events aimed at the restoration of the integrity of the damaged tissue, similar to what is observed during the 'wound healing' process in other tissues, such as the kidney or the skin. These events include an inflammatory response, in which leukocytes are recruited, and a proliferative phase, in which myofibroblasts, endothelial cells and other cell types migrate into the wound, begin to proliferate, and secrete ECM. The HSC, as well as myofibroblasts in other organs, participate in both phases of the reaction, through proliferation and secretion of ECM components, and via secretion of chemotactic factors. Data from the present study identify a novel level of interaction in this process. In the normal liver, HSC are located in a space almost devoid of ECM, except for occasional collagen fibers and a thin, amorphous matrix [34]. Upon liver damage, expression of collagen types I, III, IV, and VI, fibronectin and other ECM components is dramatically increased, and the ECM surrounding HSC becomes thick and fibrillar [34–37]. According to the data from this study, ECM production in the reparative phase could participate in the maintenance of the inflammatory infiltrate by inducing secretion of MCP-1 by HSC. This interaction is likely to be relevant also in other tissues where this sequence of reparative events takes place, such as during atherosclerosis, or during glomerular injury in the kidney.

In summary, this study shows that interaction between integrin receptors and the ECM represents an effective stimulus for *MCP-1* gene expression and secretion in HSC. These findings indicate a novel mechanism of regulation for MCP-1, possibly relevant in the hepatic wound healing response and in other conditions where ECM alterations occur.

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