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Effect of Antiinflammatory Agents on Synthesis of MCP-1/JE Transcripts by Human Blood Monocytes

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SUMMARY

The human monocyte chemoattractant protein (MCP)-1 encoded by the JE gene belongs to a family of low molecular weight secretory cytokines with monocyte-stimulating activity. JE transcripts are constitutively synthesized by normal and leukemic monocytes, as well as mesenchymal cells, including fibroblasts, vascular endothelial cells, and smooth muscle cells. Expression of MCP-1/JE is increased severalfold upon exposure of cells to recombinant human granulocyte-macrophage colony-stimulating factor but is down-regulated when cells are treated with lipopolysaccharide (LPS). Given the proinflammatory properties of MCP-1/JE, we have examined the modulatory effects of various antiinflammatory agents, including indomethacin, dexamethasone, cyclosporin A, and interleukin-4, on levels of MCP-1/JE transcripts either constitutively or inducibly expressed by human peripheral blood monocytes. Whereas indomethacin had no detectable effect on synthesis of MCP-1/JE transcripts and interleukin-4 treatment resulted in only a modest increase in steady state JE mRNA levels, exposure of monocytes to dexamethasone (DXS) led to a significant (2.5–10-fold) down-regulation of MCP-1/JE transcript levels. Studies examining the mechanism of down-regulation of JE mRNA by DXS indicated that DXS was acting transcriptionally and posttranscriptionally, by reducing the transcriptional rate of the MCP-1/JE gene and by destabilizing JE mRNA, a process requiring *de novo* RNA and protein synthesis. Although cyclosporin A by itself had no effect on synthesis of JE transcripts, it apparently relieved LPS-mediated down-regulation of JE transcript levels, by interfering with the destabilizing effect of LPS on JE mRNA. These results may provide new information regarding the action of antiinflammatory agents on synthesis of endogenous proinflammatory cytokines.

Recently, the murine JE gene has been cloned and expressed (1). When the human homologue of JE was molecularly identified (2), its encoded protein was shown to be identical to a low molecular weight cytokine called MCP-1 (3) [also referred to as macrophage chemoattractant and activating factor (4)]. This molecule belongs to a new family of cytokines, with monocyte-stimulating activities (see Ref. 5 for review), whose genes are clustered on human chromosome 17q11.2–12 (6). Recently, purified recombinant human MCP-1/JE has been produced in COS cells (7) and shown to induce chemotaxis, calcium flux, and the respiratory burst in human monocytes similarly to natural MCP-1 (8). MCP-1/JE is primarily produced by monocyte-containing tissues, suggesting that it acts in an autocrine fashion (9). Its expression is enhanced by platelet-derived growth factor, phorbol ester (phorbol 12-myristate 13-acetate) (1, 2, 10), IL-1, and tumor necrosis factor (11). Other cellular sources of MCP-1/JE include fibroblasts (1, 2, 10, 12), vascular endothelial cells (11–13), and smooth muscle cells (14).

Besides its proinflammatory properties, MCP-1/JE has been implicated in the tumoricidal capacity of monocytes *in vitro* (9) and *in vivo* (15) and in the tissue reaction to vascular injury and, thus, in early events during atherogenesis (11, 13, 14).

In the studies to be presented below, we have examined the effect of various antiinflammatory agents on expression levels of MCP-1/JE transcripts in human blood monocytes. Indomethacin, DXS, Cyc A, and rhIL-4 antagonize inflammatory responses by interfering with secretion of monocyte-activating molecules and, thus, were selected for our studies. Indomethacin antagonizes the proinflammatory action of eicosanoids, whereas DXS, Cyc A, and IL-4 are known to suppress secretion of various monocyte-activating cytokines, including IL-1, IL-6, and tumor necrosis factor (16–19). Studies on the effect of IL-4 on MCP-1/JE expression were also of interest, because IL-4 was shown to inhibit synthesis of IL-8 (20), whose effects in neutrophils are analogous to those described for recombinant human MCP-1/JE on monocytes.

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ABBREVIATIONS: MCP, monocyte chemoattractant protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; DXS, dexamethasone; Cyc A, cyclosporin A; act-D, actinomycin-D; LPS, lipopolysaccharide; IFN, interferon; GRE, glucocorticoid response element; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; MOPS, 3-[*N*-morpholino]propanesulfonic acid; CHX, cycloheximide; SCM, standard culture medium; UTR, untranslated region; rhGM-CSF, recombinant human granulocyte-macrophage colony-stimulating factor; rhIL-4, recombinant human interleukin 4.

Materials and Methods

Reagents. *Escherichia coli*-derived LPS (10 ng/ml), indomethacin (10^{-6} to 10^{-8} M), DXS (10^{-5} to 10^{-8} M), act-D (5 μg/ml), and CHX (10 μg/ml) were purchased from Sigma Chemicals (Munich, Germany). Cyc A (Sandimmun) was obtained through Sandoz (Basle, Switzerland) and was used at a concentration of 1.0 μg/ml. Ketoconazole (100 μM) was obtained from Biomol (Philadelphia, PA). rhIL-4 was kindly provided by Dr. P. Trotta (Schering Plough Corp., Bloomfield, NJ) and was used at 10–100 ng/ml. rhGM-CSF and IL-3 were a gift of Dr. S. Gillis (Immunex, Seattle, WA) and were used at 25 ng/ml each.

cDNA probes. Plasmids and cDNA probes used were the 0.8-kilobase *Bam*HI-*Pst*I chicken α-actin fragment in pBR322 (21) and the 0.74-kilobase *Kpn*I fragment of pGEMhJE34 (2).

RNA preparation, Northern blotting, and nuclear run-on transcription assay. Cells were lysed with 4 M guanidinium isothiocyanate (Sigma) and extracted by the cesium chloride method (22). Samples were fractionated by electrophoresis through a 1% agarose gel in 0.02 M MOPS (Serva, Heidelberg, FRG), pH 7.0, 0.66 M formaldehyde, transferred to synthetic membranes (Schleicher and Schuell, Dassel, FRG), and hybridized to a minimum of 10^6 cpm/ml [α^{32}P] dCTP. cDNA probes (50 ng) were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Buchler, Braunschweig, FRG), using the hexanucleotide primer technique (23). The filters were washed to a final stringency of 0.1× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) at 65° and exposed to Kodak X-Omat films with intensifying screens for 1–3 days. For nuclear run-on transcription assays (24), cells (10^8) were lysed in RSB (10 mM Tris·HCl, 5 mM KCl, 3 mM $MgCl_2$) containing 0.5% Nonidet P-40 (Sigma) and were washed once in ice-cold phosphate-buffered saline. Nuclei were incubated at 26° in 15% glycerol, 70 mM KCl, 2.5 mM $MgCl_2$, 10 mM EDTA, 4 mM levels each of ATP, CTP, and GTP, 2 mM UTP, 0.5 mM dithiothreitol, 60 units/ml RNasin (Boehringer Mannheim, Mannheim, FRG), in the presence of 100 μCi of [^{32}P]UTP (3000 Ci/mmol; Amersham Buchler), for 30 min. The mixture was digested with DNase I and proteinase K, extracted with trichloroacetic acid and phenol/chloroform, and precipitated in 70% ethanol before hybridization of 5× 10^5 cpm/ml of hybridization buffer [50% formamide, 2× SSC, 1% SDS, 5× Denhardt's solution (1× Denhardt's is 0.02% Ficoll, 0.02% bovine serum albumin (fraction V; Sigma), 0.02% polyvinylpyrrolidone), 5 μg/ml tRNA]. Filters contained 10 μg each of linearized plasmids immobilized on nitrocellulose (Schleicher and Schuell), after blotting with a slot-blot apparatus (Schleicher and Schuell). After hybridization at 42° for 3 days, filters were rinsed in 2× SSC at 55°, 2× SSC containing 10 μg/ml RNase A at 37°, and finally 0.5× SSC at 55°, for 30 min each time, and were exposed to Kodak X-Omat film for 10 days.

Monocyte preparation and culture. Monocytes from various healthy donors were obtained by elutriation, as previously described (20), and were >95% pure by nonspecific esterase staining. Culture of monocytes (10^6ml) was done during the indicated time periods, in RPMI 1640 medium supplemented with 10% low-endotoxin fetal calf serum (Hazelton, Vienna, UT), 1% penicillin/streptomycin, and 2 mM L-glutamine (SCM), by using polypropylene flasks (Corning Glass Works, Corning, NY) to avoid activation due to adherence. In selected experiments, cells of the leukemic monoblast cell line U 937 (10^6/ml) (American Type Culture Collection, Bethesda, MD) were also investigated.

Immunoprecipitation of MCP-1/JE protein. Supernatants of monocyte cultures exposed to GM-CSF in the presence or absence of DXS, Cyc A, IL-4, or indomethacin (in concentrations as indicated above) were concentrated using a YM-5 membrane (Amicon, Danvers, MA), and material soluble in 45% $(NH_4)_2SO_4$ was dialyzed extensively against 20 mM Tris·HCl (pH 8.0). Thereafter, this material was mixed with an equal volume of cold RIPA buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride), and rabbit anti-JE antiserum (2) (Advanced Magnetics, Boston MA) was added. Incubation continued on ice for 1 hr, after which 50 μl of a 50% slurry of *Staphylococcus aureus* protein A-agarose beads (Bio-Rad Laboratories, Munich, Germany) were added. Tubes were gently rocked at 4° for an additional 1 hr. Beads were washed twice with cold RIPA buffer and once with cold 1 M NaCl, 50 mM Tris·HCl (pH 7.5), and were suspended in Laemmli sample buffer. The slurry was boiled for 1 min, the beads were centrifuged, and the supernatant was analyzed by SDS-polyacrylamide gel electrophoresis.

Results

Constitutive and inducible expression of MCP-1/JE transcripts. MCP-1/JE mRNA was detectable, by Northern blot analysis, in 15 μg of RNA from monocytes isolated by elutriation. Culture of these cells in SCM for up to 6 hr under nonadherent conditions did not alter MCP-1/JE transcript levels. Similarly, MCP-1/JE transcripts were also detectable in the same amount of total RNA isolated from U 937 monoblasts, and the intensity of hybridization signals was 2-fold greater than that of signals obtained from hybridization of monocyte RNA with MCP-1/JE-specific cDNA, as shown by laser densitometric scanning of autoradiographs after normalization of MCP-1/JE transcript levels for α-actin mRNA.

Exposure of monocytes and U 937 cells to rhGM-CSF for 6 hr resulted in 8.3- and 11.0-fold, respectively, increases in MCP-1/JE transcript levels, whereas LPS treatment led to a 80% decrease, compared with monocytes cultured in SCM only. Interestingly, recombinant human IL-3 also augmented MCP-1/JE transcripts in monocytes 4.3-fold but was completely inactive on U 937 cells (Fig. 1). This might be due to the low IL-3 receptor number found on U937 cells (25).

Effect of antiinflammatory agents on MCP-1/JE transcript levels. As shown in Fig. 2A, treatment of monocytes for 6 hr with indomethacin at various dose levels or with Cyc A did not alter MCP-1/JE mRNA accumulation. However, when Cyc A was combined with either LPS alone or LPS plus rhGM-CSF, LPS-mediated suppression of MCP-1/JE transcripts was abolished (Fig. 2B). Exposure of monocytes to various concentrations of rhIL-4 increased synthesis of MCP-1/JE transcripts by maximally 1.8-fold, when 25 ng/ml was used. Higher concentrations of IL-4 did not further increase MCP-1/JE transcript levels (Fig. 3).

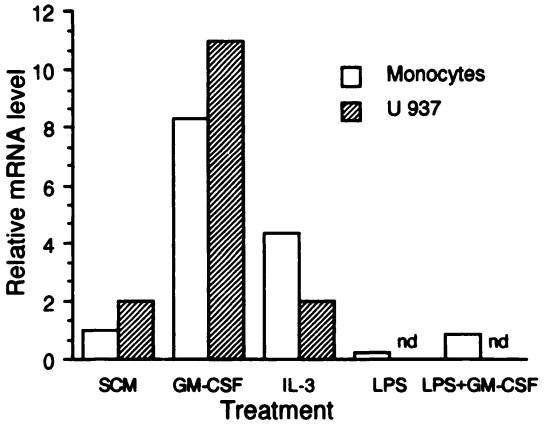

Fig. 1. Effect of GM-CSF, IL-3 (25 ng/ml each), and LPS (10 ng/ml) on synthesis of MCP-1/JE transcripts in monocytes and U 937 monoblasts. Cells were cultured for a total of 6 hr before extraction of RNA for Northern hybridization. Shown is a densitometric scanning analysis. The values obtained from multiple exposures were normalized to the levels of α-actin mRNA. The intensity of MCP-1/JE mRNA signals in cells stimulated with SCM only was arbitrarily set to 1. Experiments were repeated twice, and representative data are shown.

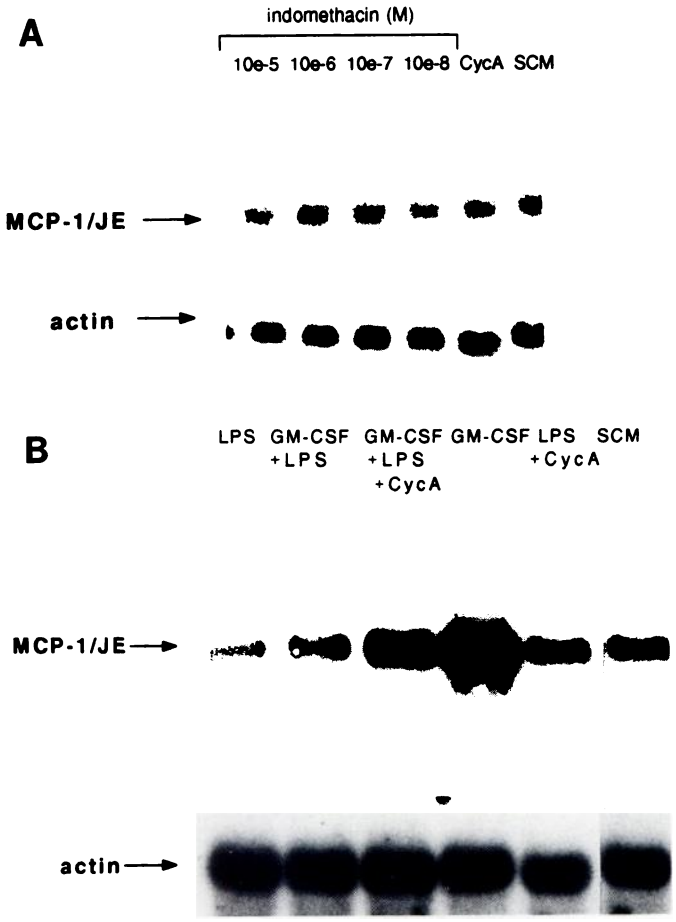

Fig. 2. A, Effect of indomethacin, Cyc A (1.0 μg/ml), and medium only (*SCM*) on MCP-1/JE transcript levels in human monocytes. Cells were exposed to the various agents for 6 hr before RNA extraction. Each *lane* contains 15 μg of RNA. Rehybridization of filters with an α-actin cDNA confirms comparable RNA loading in each lane. B, Effect of LPS (10 ng/ml), Cyc A (1.0 μg/ml), GM-CSF (25 ng/ml), and combinations thereof on MCP-1/JE transcript levels in human monocytes. The treatment period was 6 hr. Each *lane* contains 15 μg of cytoplasmic RNA. Probing of α-actin transcript levels confirms comparable RNA loading in each lane.

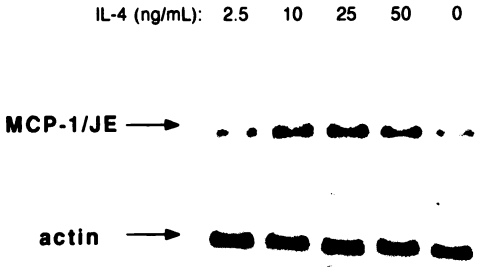

Fig. 3. Effect of IL-4 on MCP-1/JE mRNA synthesis in human monocytes. The treatment period was 6 hr. For details, see the legend to Fig. 2.

In contrast to the effects observed with indomethacin, Cyc A, and IL-4, culture of monocytes (Fig. 4) and U 937 cells (data not shown) with DXS was associated with down-regulation of MCP-1/JE transcripts in the presence or absence of rhGM-CSF. A DXS concentration of 10^{-8} M was sufficient to suppress MCP-1/JE transcripts by >60%, compared with cultures performed under identical conditions without DXS, irrespective of the presence of rhGM-CSF.

Effect of DXS on the transcriptional activity of the MCP-1/JE gene. To study the molecular level at which the inhibitory action of DXS on MCP-1/JE mRNA levels took place, in a first set of experiments transcriptional nuclear run-on assays were performed. To this end, only GM-CSF-stimulated cultures were investigated. As shown in Fig. 5, addition of DXS to GM-CSF-stimulated monocyte cultures led to an approximately 70% reduction of the transcriptional rate of the MCP-1/JE gene at 3 hr, whereas transcription of the α-actin gene was unaffected by DXS at that time. These results suggest that down-regulation of MCP-1/JE transcripts in monocytes is, at least in part, regulated transcriptionally.

Lack of involvement of arachidonic acid metabolites in the effect of DXS on MCP-1/JE expression. DXS is a potent inhibitor of arachidonic acid formation by inhibiting phospholipase A_2 (26). Moreover, arachidonic acid metabolites, particularly leukotriene B_4, have been shown to be involved in the phorbol ester- and dimethylsulfoxide-mediated transcriptional activation of cytokine genes (27, 28). Therefore, it was of interest to determine whether DXS-mediated inhibition of MCP-1/JE transcriptional activation stimulated by GM-CSF could be due to inhibition of phospholipase A_2 activity and, thereby, due to decreases in the major arachidonic acid metabolites, i.e., prostaglandins and leukotrienes. Inhibition of prostaglandine synthesis by indomethacin had no effect on GM-CSF-induced MCP-1/JE expression (Fig. 2A). Similarly, preincubation of monocytes (30 min) with ketoconazole, an inhibitor of 5-lipoxygenase (29), before exposure to GM-CSF also failed to affect GM-CSF-induced MCP-1/JE expression (data not shown), suggesting that neither arachidonic acid metabolite plays a role in GM-CSF-mediated induction of MCP-1/JE. Indeed, it has recently been shown that GM-CSF is not capable of promoting arachidonic acid liberation, 5-lipoxygenase activation, or leukotriene synthesis (30). Also, the finding that GM-CSF down-regulates cell surface binding of leukotriene (31) is of note in this regard.

Effect of DXS on the stability of MCP-1/JE mRNA. Next, the possibility was investigated that DXS was also acting posttranscriptionally, influencing the balance between factors stabilizing or destabilizing mRNA. As shown in Fig. 6, steady state levels of MCP-1/JE mRNA induced by GM-CSF stimulation were investigated in the presence of the transcription inhibitor act-D or the translation inhibitor CHX. Culture of monocytes with DXS increased the turnover of MCP-1/JE mRNA. Addition of both act-D and CHX, however, increased MCP-1/JE mRNA stability. Addition of act-D and CHX to DXS-treated cultures abolished the inhibitory effect of DXS on MCP-1/JE mRNA levels. These results suggest that destabilization of MCP-1/JE mRNA by DXS required both *de novo* mRNA and protein synthesis.

Mechanism of the effect of Cyc A on LPS-mediated inhibition of MCP-1/JE mRNA accumulation. Because Cyc A was shown to reduce the MCP-1/JE mRNA decay-accelerating effects of LPS, we next examined at which stage Cyc A was acting in this regard. Transcriptional run-on analysis indicated that LPS had no detectable effect on the transcriptional activity of the MCP-1/JE gene stimulated by GM-CSF. Similarly, Cyc A and a combination of LPS and Cyc A did not alter MCP-1/JE gene transcription (data not shown). However, studies investigating the effect of LPS on steady state mRNA levels induced by GM-CSF revealed that LPS treatment of monocytes was associated with increased MCP-1/JE mRNA turnover that could be prevented when Cyc A was present in

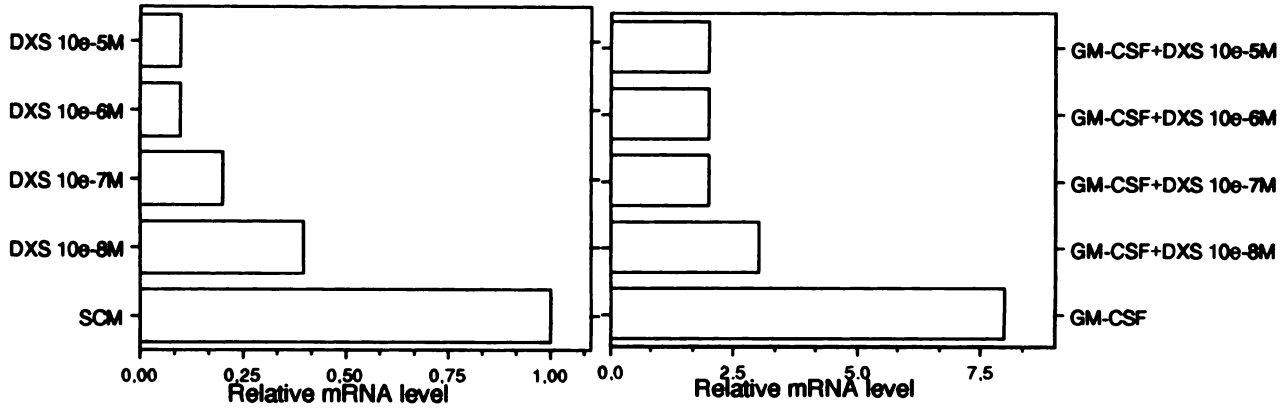

Fig. 4. Effect of DXS (10^{-5} to 10^{-8} M) on constitutive (*left*) and GM-CSF-induced (*right*) synthesis of MCP-1/JE mRNA in monocytes. Shown is a densitometric scanning analysis of autoradiographs. The intensity of MCP-1/JE mRNA signals in cells stimulated with SCM (6 hr) was arbitrarily set to 1. For details, see the legend to Fig. 1. Experiments were repeated twice, and representative data are shown.

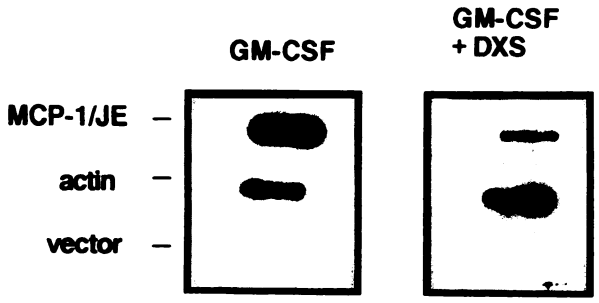

Fig. 5. Run-on assays with nuclei of GM-CSF-stimulated monocytes. Cultures were treated for 3 hr with 10^{-6} M DXS or left untreated (GM-CSF only). RNA from a plasmid containing α-actin cDNA and the pGEM-7Zf(+) plasmid (vector) were used as positive and negative controls, respectively.

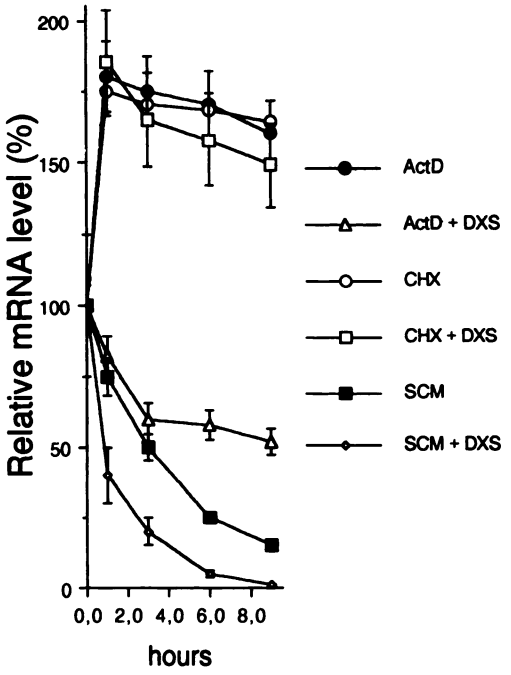

Fig. 6. Effect of DXS (10^{-6} M) on steady state levels of MCP-1/JE mRNA. Monocytes were first exposed to rhGM-CSF (25 ng/ml). After a 6-hr incubation period, act-D (5 μg/ml), CHX (10 μg/ml), or SCM was added, with or without DXS. Northern blot analysis was performed using 25 μg of RNA extracted at the indicated time points. The quantity of RNA was determined by densitometric scanning of autoradiographs. The relative quantity of MCP-1/JE mRNA was calculated on the assumption that the amount of RNA before drug treatment was 100%. Experiments were repeated four times.

cultures (data not shown). These results suggest that Cyc A may interfere with the destabilizing action of LPS on MCP-1/JE mRNA.

Effect of IL-4, Cyc A, and indomethacin on GM-CSF-induced MCP-1/JE protein secretion. To examine whether IL-4, Cyc A, and indomethacin inhibit GM-CSF-induced MCP-1/JE expression at the protein level, immunoprecipitation analyses, using an anti-JE antiserum (2), were performed with concentrated supernatants of monocytes exposed to GM-CSF for up to 48 hr, with or without IL-4, Cyc A, or indomethacin. The amount of MCP-1/JE protein detectable by this method was, however, not significantly affected by these antiinflammatory agents (data not shown).

Discussion

In this study, we have investigated the action of the antiinflammatory agents indomethacin, Cyc A, IL-4, and DXS on synthesis by human monocytes of transcripts coding for the proinflammatory MCP-1/JE protein. Only DXS treatment of monocytes was associated with decreases in MCP-1/JE transcript levels that were either constitutively expressed or induced by GM-CSF. The inhibitory action of DXS on MCP-1/JE mRNA levels was partly due to a reduction of the transcriptional rate of the MCP-1/JE gene, as has been previously reported by Kawahara and Deuel (32) and Mukaida *et al.* (33), investigating the effect of DXS on transcription of the JE/MCAF gene by murine 3T3 fibroblasts and fibrosarcoma cells, respectively, and partly due to an increase of MCP-1/JE mRNA turnover. The increased turnover of this mRNA did not occur in the presence of inhibitors of transcription or translation and, thus, required *de novo* synthesis of mRNA and protein. The fact, however, that the effect of act-D plus DXS was not the same as that of act-D alone, whereas the effect of act-D plus DXS was almost the same as that of CHX alone (Fig. 6), may indicate that the dependency of the DXS effect on new RNA synthesis was not complete. Nevertheless, these findings are in contrast to previous observations made by others, analyzing the effect of DXS on the regulation of IFN-β_1 gene expression and demonstrating that the increased turnover of

IFN-β_1 mRNA mediated by DXS was due to activation of preexisting ribonucleases targeting the AU-rich sequences in the 3′ UTR of the IFN-β_1 gene and, thus, occured in the absence of synthesis of mRNA and protein (34). It is presently unknown whether AU elements, which are also present in the 3′ UTR of the MCP-1/JE gene (2), contribute to the destabilizing activity of DXS on MCP-1/JE mRNA. Current experiments are, therefore, in progress that are aimed at using expression vectors for MCP-1/JE containing various deletions in the 3′ UTR (particularly deletion of AU sequences) and investigating the effects of DXS on the stability of the altered MCP-1/JE mRNA mutant, upon transfection into appropriate cells. Recently, an RNA-binding protein was shown to target AU sequences specifically, leading to degradation of the respective RNA (35). The involvement of DXS in induction of such an RNA-binding protein needs to be elucidated.

It is also of note that inhibition of MCP-1/JE transcript synthesis was, within the family of glucocorticoids, not restricted to DXS, because other related hormones, including prednisone, prednisolone, and hydrocortisone, also decreased MCP-1/JE transcription and increased turnover of its mRNA (data not shown).

Glucocorticoids exert their effect by binding to intracellular receptors, which directly modulate gene transcription by targeting specific DNA binding sites called positive GRE (36). Binding of positive GRE may represent a potent signal for transcription, inasmuch as induction of mRNA-degrading proteins, as shown here, may be due to transcriptional activation. However, binding of GRE may also lead to repression of expression of genes (negative GRE), as has been shown for the prolactin, gonadotropin, and collagenase genes (37–39). Recent studies demonstrating that a negative GRE represses the activity of c-*jun*, thereby displacing a transcriptional activator (40), may be of note in this regard and may point to a possible analogous mechanism of down-regulation of MCP-1/JE transcription by DXS.

Other antiinflammatory agents investigated, including indomethacin, IL-4, and Cyc A, failed to decrease MCP-1/JE transcript and protein levels. In contrast, IL-4 treatment of monocytes led to a small increase of MCP-1/JE mRNA levels. Although IL-4 has been demonstrated to induce MCP-1/JE synthesis in human endothelial cells (41), this observation was surprising to us, because previous studies have shown that, in monocytes, this cytokine exerts inhibitory effects upon mRNA synthesis of various inflammatory mediators, including the neutrophil chemoattractant protein IL-8, which belongs to the same family of genes as MCP-1/JE (42). Similarly, Cyc A, also a potent inhibitor of synthesis of inflammatory cytokines (43, 44), enhanced synthesis of MCP-1/JE transcripts on the transcriptional level but required the presence of endotoxin for this effect.

Given previous findings demonstrating that DXS also decreases mRNA stability of other molecules involved in inflammation, such as IL-1 and IL-8 (18, 45), the major conclusion of our study is that glucocorticoids may exert their antiinflammatory effects by inhibiting the synthesis of proinflammatory cytokines.

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