Chemokine Gene Expression in Bone Marrow Stromal Cells: Downregulation With Sodium Salicylate

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Chemotactic cytokines, chemokines, have been shown to influence the proliferation of hematopoietic progenitor cells. Thus, regulation of chemokine production by bone marrow accessory cells is a critical aspect of stromal cell regulation of hematopoiesis. We have previously reported that monocyte chemotactic protein-1 (MCP-1 or MCP-1/JE) and interferon inducible protein 10 kDa (IP-10) are both induced in murine bone marrow stromal cells +/+ -1.LDA11 after stimulation with the inflammatory agents interleukin-1α (IL-1α), interferon-γ (IFN-γ), or lipopolysaccharide (LPS). In the present study, we have investigated the effect of sodium salicylate, an antiinflammatory agent, on the IL-1α-induced expression of MCP-1/JE and IP-10 genes in stromal cells. Sodium salicylate attenuates the levels of MCP-1/JE and IP-10 mRNA in a concentration- and time-dependent manner. The suppression of MCP-1/JE mRNA is reversible, whereas IP-10 mRNA expression is more or less irreversibly affected as its recovery from the effect of sodium salicylate is slow and partial. Sodium salicylate-mediated suppression of mRNA expression is attributable neither to de novo synthesis of intermediate(s) nor to the destabilization of mature mRNA transcripts. On the other hand, sodium salicylate downregulates the transcriptional activity of both genes. Furthermore, IL-1α induces activation of transcription factor nuclear factor (NF)-κB, and sodium salicylate suppresses it in a dose-dependent manner. We conclude that while posttranscriptional events remain unaffected, inhibition of NF-κB activation by sodium salicylate may account for the suppression of chemokine gene expression at the transcriptional level.

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THE COMPLEX hematopoietic pathways involved in the production of mature blood cells are regulated by the cooperative effects of numerous colony-stimulating factors (CSFs) and hematopoietic growth factors (HGFs) or cytokines that control the proliferation and differentiation of pluripotent stem cells and the progenitor cells.1-3 The various CSFs and cytokines that have been demonstrated to influence the hematopoietic progenitor cells include, but are not limited to, macrophage CSF (M-CSF), granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), interleukin-1 (IL)-1α, IL-3, IL-6, stem cell factor (SCF), and erythropoietin.4-9 Recent reports have suggested that the hematopoietic microenvironment or bone marrow stroma, which is comprised of a heterogenous mixture of various cell types (ie, fibroblasts, endothelial cells, macrophages, adipocytes, etc),10-12 plays a pivotal role in the regulation of hematopoiesis.13,14 Bone marrow stromal cells exert their influence on hematopoiesis by secreting CSFs and cytokines as well as extracellular matrix proteins, which assist in the stabilization and presentation of growth factors to the progenitor cells.15-18

Although the production of growth factors under basal conditions has been difficult to detect, stromal cells respond to a variety of inflammatory stimuli including tumor necrosis factor-α (TNF-α) and IL-1α, resulting in the production of CSFs and HGFs.17,19,20 In addition to producing the growth-promoting factors, stromal cells also emanate products that directly or indirectly inhibit proliferation of multilineage and committed progenitor cells.20 For example, TNF-α,21 transforming growth factor-β (TGF-β),22,23 and prostaglandins24 secreted by accessory cells have been shown to inhibit the proliferation of progenitor cells. More recently, many members of a superfamily of proinflammatory chemoattractant cytokines produced by accessory cells have been demonstrated to influence myelopoiesis.25 Recombinant macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and MIP-2 have been shown to have myelopoietic activity.26-28 Other chemotactic cytokines (eg, monocyte chemotactic and activating factor [MCAF], RANTES, Gro-protein, and platelet factor 4) have also been examined for effects on myelopoiesis.22 Very little is known about the synthesis and regulation of chemokine production by stromal cells. We have previously shown that the murine bone marrow-derived stromal cell line +/+ -1.LDA11 expresses chemotactic cytokines MCP-1/JE and IP-10 after stimulation with IL-1α or IFN-γ.25 Furthermore, both IL-1α and IFN-γ synergize with TGF-β1 or IL-4, causing upregulation of MCP-1/JE and IP-10 mRNA expression. Because IL-1α- and TNFα-mediated induction of protooncogenes and growth factor genes involves the products of arachidonic acid metabolism,36,40 and because antiinflammatory agents such as salicylic acid and sodium salicylate have been shown to influence gene transcription and activation of transcription factors,41-45 we examined the effect of sodium salicylate on the expression of MCP-1/JE and IP-10 chemokines in stromal cells. The results demonstrate that sodium salicylate inhibits the expression of both chemokines at the level of gene transcription, and the suppression of nuclear factor-κB (NF-κB) activation appears to be the part of the mechanism whereby sodium salicylate blocks the stromal cell expression of chemokine genes.

MATERIALS AND METHODS

Stromal cell cultures. The murine bone marrow stromal cell line +/+ -1.LDA11 was a gift from Dr H.S. Boswell (Indiana University, Indianapolis) and was used in all experiments. The isolation, cloning, and characterization of this cell line have been previously described.21 Stromal cells were grown as monolayers in 75-cm² culture flasks in McCoy's 5A modified culture medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin (100 U/mg/ml), and amphotericin B.
mL), and streptomycin (50 μg/mL) purchased from GIBCO Laboratories (Grand Island, NY). Cells were maintained by passing 2 × 10^6 cells per flask (10 mL culture medium) prepared by trypsin-EDTA treatment of adherent cell monolayers. Cell growth was usually 100% confluent in 3 to 4 days. For experimental use, 2 × 10^6 cells in 10 mL culture medium were placed in each 100-mm tissue culture petri dish and incubated for 3 to 4 days at 37°C, 5% CO_2 and 95% relative humidity. Culture plates with confluent cell growth were washed once to remove nondherent cells before stimulation of cells as described in the individual experiments.

**Reagents.** Molecular biology-grade cesium chloride, anhydrous ethanol, sarcosyl, guanidine isothiocyanate, and formamidase were purchased from International Biotechnologies, Inc (New Haven, CT). Ultrapure agrose and sodium dodecyl sulfate (SDS) were obtained from GIBCO BRL (Gaithersburg, MD). TRIS for electrophoresis buffer was from BIO-RAD Laboratories (Richmond, CA). Dextran sulfate was from Pharmacia LKB (Uppsala, Sweden). Nytran membranes were obtained from Schleicher and Schuell (Keene, NH). The nick translation kit was purchased from Boehringer Mannheim (Mannheim, Germany). Both α-32P-deoxyctydylate triphosphate (dCTP) and γ-32P-deoxyadenosine triphosphate (dATP) were obtained from DuPont, New England Nuclear (NEN; Boston, MA). All other chemicals, including sodium salicylate, were purchased from Sigma Chemical Company (St Louis, MO).

**Cytokines.** IL-1α (1 × 10^6 U/mg) was a gift from Biological Response Modifier Repository, National Cancer Institute (NCI; Frederick, MD). Murine INF-γ (2.5 × 10^6 U/mg) was purchased from Enzyme Research Laboratories (Mannheim, Germany). Both [α-32P]-deoxycytidine triphosphate (32P-UTP; 200 μCi, 2,000 Ci/mmol UTP; DuPont, NEN), and 5 μL of RNAse (Promega) were added to each nuclear preparation. The reaction was performed at 30°C for 30 minutes, stopped by adding 15 μL RNAse-free DNase (5 μg/mL), and further incubated for 10 minutes at 30°C. Thirty-six microtainers of 10 × SET % SDS, 5 mmol/L EDTA, and 10 mmol/L Tris HCl pH 7.4, 10 μL of proteinase K (20 mg/mL), 15 μg of Escherichia coli tRNA were added, and the reaction was incubated at 37°C for 45 minutes. The reaction was extracted with an equal volume of phenol-chloroform-isomnyl alcohol (25:24:1) and RNA was precipitated in ethanol and 2.3 mol/L ammonium acetate for 20 minutes.

**Plasmids.** Plasmids containing MCP-1/JE and P-10 cDNA sequences were provided by Dr T.A. Hamilton, Cleveland Clinic (Cleveland, OH). The α-tubulin cDNA probe was obtained from the American Type Culture Collection (Rockville, MD). All cDNA probes (1 μg of plasmid DNA) were labeled with 32P-dCTP by the method of nick translation using a nick translation kit for radiolabeling as specified by the manufacturers (Boehringer Mannheim). The resultant specific activity was approximately 10^6 cpm/μg, which was used at 8 × 10^6 to 10 × 10^6 cpm per blot.

**Oligonucleotide probes.** The synthetic double-stranded oligonucleotide containing the consensus sequence for the CTAE binding site, GGGGAC'ITTCC, was purchased from Promega (Madison, WI).

**Total cellular RNA preparation and Northern analysis.** For analysis of the chemokine mRNA expression by Northern hybridization, total cellular RNA was extracted by the guanidine isothiocyanate-cesium chloride method.45 The level of specific mRNA was analyzed by Northern hybridization as described elsewhere.45 Briefly, equal amounts of RNA (15 to 20 μg) were denatured and subjected to electrophoresis in a 1% agarose formaldehyde gel. The RNA was then blotted by capillary transfer to Nytran membranes. The blots were prehybridized for 20 hours at 42°C in 50% formamide, 1% SDS, 5x saline sodium citrate (SSC), 1× Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin [BSA], 0.02% polyvinylpyrrolidone), 0.25 mg/mL denatured salmon sperm DNA, and 50 mmol/L sodium phosphate buffer, pH 6.5. Hybridization was performed at 42°C for 12 to 16 hours with 10^6 cpm of denatured probe. The filters were washed for 30 minutes in a solution of 2% SDS, 0.2× SSC at room temperature, followed by a wash at 55°C in 0.1% SDS, 0.2× SSC. The blots were then exposed using XAR-5 x-ray film with DuPont (Wilmington, DE) Cronex Lightening Plus intensifying screens at −70°C. In some experiments, blots were stripped and rehybridized with 32P-labeled α-tubulin cDNA. The expression of α-tubulin was used as an internal control for the quantity of total mRNA. In addition, the RNA load per lane was assessed by ethidium bromide staining of the original agarose gel after capillary transfer. Autoradiograms were quantified by a scanning densitometer using GS365W software from Hoefer Scientific Instruments (San Francisco, CA), and the strength of the signal is presented in arbitrary units.

**Nuclear run-on transcription assay.** The nuclear run-on assay was performed using a slight modification of a previously described method.46 Briefly, nuclei were prepared from stromal cells (5 × 10^6) that were untreated or treated with IL-1α (10^5 U/mL) alone or IL-1α plus sodium salicylate (40 mmol/L) for 3 hours. Cells were harvested by trypsin-EDTA treatment, washed, and lysed by incubating on ice for 5 minutes in lysis buffer (10 mmol/L Tris HCl pH 7.4, 2 mmol/L MgCl₂, 10 mmol/L NaCl, 0.5% NP-40). Nuclei were resuspended in 200 μL of freezing buffer (5 mmol/L MgCl₂, 50 mmol/L Tris HCl pH 8.3, 0.1 mmol/L EDTA, 40% glycerol). For elongation of transcripts, 60 μL of 5 × run-off buffer (25 mmol/L Tris HCl pH 8.0, 12.5 mmol/L MgCl₂, 750 mmol/L KCl, and 1.25 mmol/L ATP, CTP, and GTP), 20 μL of 32P-UTP (32P-UTP; 200 μCi, 3,000 Ci/mm mol UTP; DuPont, NEN), and 5 μL of RNAse (Promega) were added to each nuclear preparation. The reaction was performed at 30°C for 30 minutes, stopped by adding 15 μL RNAse-free DNase (5 μg/mL), and further incubated for 10 minutes at 30°C. Thirty-six microtainers of 10 × SET % SDS, 5 mmol/L EDTA, and 10 mmol/L Tris HCl pH 7.4, 10 μL of proteinase K (20 mg/mL), 15 μg of Escherichia coli tRNA were added, and the reaction was incubated at 37°C for 45 minutes. The reaction was extracted with an equal volume of phenol-chloroform-isomnyl alcohol (25:24:1) and RNA was precipitated in ethanol and 2.3 mol/L ammonium acetate for 20 minutes.

**Preparation of nuclear extracts.** Nuclear proteins were isolated by the method of Dignam et al.47 with slight modifications. In brief, stromal cell monolayers were first treated with sodium salicylate for 1 hour, and then IL-1α (1,000 U/mL) was applied for 30 minutes. Cells were cold-harvested by trypsin-EDTA treatment, washed with ice-cold phosphate-buffered saline (PBS), resuspended in hypotonic buffer (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L diethiothreitol [DTT], 0.5 mmol/L phenylmethylsulfonyl fluoride [PMSF], and 0.6% NP-40), allowed to swell on ice for 15 minutes, and vortexed gently for lysis. Nuclei were pelleted by centrifugation at 12,000g for 1 minute, resuspended in buffer (20 mmol/L HEPES pH 7.9, 25% glycerol, 0.4 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF), and shaken for 30 minutes at 4°C. The nuclear extract was centrifuged for 10 minutes at 12,000g, the supernatant was collected, and protein contents were measured using a protein assay kit from Sigma Diagnostics (St Louis, MO).

**Electrophoretic mobility shift assay (EMSA).** The DNA binding reaction was performed using 10 μg of protein in 20 μL total reaction mixture containing 20 mmol/L HEPES pH 7.9, 100 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 50 μg/mL poly dIdc, 8% glycerol, and 5 × 10^6 cpm (32P)-endlabeled oligonucleotide. After incubation for 20 minutes at room temperature, samples were loaded onto a 6% polyacrylamide gel in low ionic strength buffer (22.3 mmol/L Tris, 22.2 mmol/L borate, 0.5 mmol/L EDTA) (0.25 × TBE).
The gels were run for 2 hours at 150 V/cm and then vacuum-dried before being subjected to autoradiography for analysis.

RESULTS

Sodium salicylate inhibits MCP-1/JE and IP-10 mRNA expression in stromal cells. Because chemokines have been shown to modulate transcription of genes, we asked how sodium salicylate might affect the induction of MCP-1/JE and IP-10 genes in stromal cells. Unstimulated +/+−1.LDA11 stromal cells express only minimally detectable levels of MCP-1/JE or IP-10 mRNA; however, the message for these chemokines can be readily induced after stimulating cells with a variety of inflammatory agents, including LPS, IL-1α, and IFN-γ. Monolayers of stromal cells were stimulated with LPS, IL-1α, or IFN-γ in the absence or presence of varying concentrations of sodium salicylate for 4 hours. Total cellular RNA was isolated and analyzed for MCP-1/JE and IP-10-specific mRNA levels by Northern blot analysis using radiolabeled gene-specific cDNA probes. Blots were exposed by autoradiography for 16 hours. Similar results were obtained in three different experiments.

These results indicate that sodium salicylate inhibits the induction of chemokine mRNA in bone marrow stromal cells.

As maximum suppression of mRNA was observed at relatively high concentrations of sodium salicylate, we wished to determine the effect of similar concentrations of sodium salicylate on the viability of cells. For this purpose, monolayers of cells were exposed to different concentrations of sodium salicylate for 4 hours and were harvested by trypsin-EDTA treatment. Cells were washed, and viability was determined by dye exclusion method using trypan blue. No significant change in the viability of cells was found after 4 hours of treatment with various concentrations of sodium salicylate (96%, 96%, 97%, 95%, and 94% viability at 0, 5, 10, 20, and 40 mmol/L sodium salicylate concentrations, respectively). Thus, suppression of MCP-1/JE and IP-10 mRNA at 40 mmol/L concentration of sodium salicylate is not due to the loss of cell viability.

Characterization of sodium salicylate-mediated suppression of chemokine mRNA. To further characterize sodium salicylate-mediated suppression of chemokine mRNA induction, we asked whether the decrease in mRNA levels in cells treated with sodium salicylate might be attributed to an actual inhibition of mRNA induction or to a shift in the time course of mRNA production in the presence of sodium salicylate. For this purpose, we analyzed the levels of mRNA expressed at various periods of time in the presence of inducer alone (IL-1α) or inducer plus sodium salicylate. Treatment of cells with IL-1α caused a rapid increase in the level of MCP-1/JE mRNA, reaching a peak level at 6 hours (Fig 2A and B). Induction of IP-10 mRNA was somewhat slower compared with MCP-1/JE mRNA induction; however, it also peaked at 6 hours (Fig 2B). In both cases, although the levels of mRNA were highly reduced at all time points in the presence of sodium salicylate, the kinetics of MCP-1/JE and IP-10 mRNA expression were comparable in the absence or presence of the drug.

Because time dependency may provide important clues regarding the mechanism of sodium salicylate-mediated suppression of MCP-1/JE and IP-10 mRNA, experiments were next performed to determine the effect of time-delayed addition of sodium salicylate on the expression of the two mRNAs. Sodium salicylate was added to the cultures simultaneously with IL-1α, or its addition was delayed 0.5, 1, or 2 hours after stimulation with IL-1α. The suppression of MCP-1/JE mRNA expression was greatest when sodium salicylate was added concurrently with IL-1α (Fig 3A and B).
 Delaying the addition of sodium salicylate more than 1 hour had no effect on the levels of MCP-1/JE mRNA. IP-10 mRNA was also maximally decreased if cells were treated with IL-1α and sodium salicylate simultaneously. Although the level of suppression decreased as the addition of sodium salicylate was delayed, suppression was still evident even when sodium salicylate was added as late as 2 hours after IL-1α. Thus, unlike the progressive decrease in the suppression of MCP-1/JE mRNA seen by delaying sodium salicylate treatment, suppression of IP-10 mRNA by sodium salicylate persisted at least up to 2 hours after stimulation with IL-1α.

To determine whether stromal cells are transiently or permanently affected by sodium salicylate treatment, we explored the effect of pretreatment of cells with sodium salicylate, followed by its removal before stimulating cells with IL-1α. Stromal cells were pretreated with 40 mmol/L sodium salicylate for 2 hours, washed, and then stimulated with IL-1α at 0.5, 1, 2, 4, or 24 hours after the removal of sodium salicylate. It is apparent from Fig 4 that preincubation with sodium salicylate for 2 hours followed by its removal before application of IL-1α resulted in only slight decrease in MCP-1/JE mRNA levels, regardless of whether cells were stimulated with IL-1α as early as 0.5 hours or as late as 24 hours after removal of sodium salicylate (left panel). In contrast, preincubation with sodium salicylate followed by its removal markedly affected the levels of IP-10 mRNA. IP-10 mRNA levels remained below the levels of mRNA induced in the continuous presence of sodium salicylate (IL-1α + Na-Sal) up to 4 hours after removal of sodium salicylate (right panel). Although mRNA production appears to have recovered somewhat at 24 hours later, it was still markedly reduced compared with the mRNA levels induced by IL-1α alone. These data suggest that, at least in the case of the IP-10 gene, pretreatment with sodium salicylate irreversibly alters the response of stromal cells to IL-1α with respect to the transcription of this gene.

Suppression of mRNA expression by sodium salicylate is not dependent on de novo protein synthesis. If sodium salicylate-mediated suppression of MCP-1/JE and IP-10 mRNA is caused by de novo protein synthesis, then the suppressive effect might be blocked in cells cotreated with a protein synthesis inhibitor such as cycloheximide (CHX). To test this possibility, stromal cultures were stimulated with IL-1α (Fig 5, lane 2), IL-1α plus sodium salicylate (Fig 5, lane 3), CHX (Fig 5, lane 4), IL-1α plus CHX (Fig 5, lane 5), or IL-1α plus CHX plus sodium salicylate (Fig 5, lane 6). As seen in the preceding experiments, sodium salicylate inhibited the levels of IL-1α–induced MCP-1/JE and IP-10 mRNA. Treatment with CHX alone also induced both mRNAs, which was further increased in cells treated with IL-1α plus CHX. However, inclusion of sodium salicylate in cultures treated with a combination of IL-1α plus CHX...
resulted in reversal of both MCP-1/JE and IP-10 mRNA levels to the levels seen in cells treated with IL-1α plus sodium salicylate (lane 3). Sodium salicylate also blocked the expression of mRNAs induced with CHX alone (data not shown). Thus, the inability of CHX to reverse sodium salicylate-mediated suppression of MCP-1/JE and IP-10 mRNA suggests that synthesis of new protein(s) is not required for suppression of these genes by sodium salicylate.

**Sodium salicylate does not affect the stability of mRNA.**

The inhibition of IL-1α–induced MCP-1/JE and IP-10 mRNA by sodium salicylate may involve posttranscriptional regulation of mRNA; i.e., the stability of primary transcripts and/or mature cytoplasmic mRNA. Whether this drug affects the stability of mRNA was determined under two different treatment conditions. We first stimulated stromal cells with IL-1α for 4 hours, and then we treated with actinomycin D alone to prevent further transcription or with actinomycin D and sodium salicylate together. At different time points, specific mRNA levels were assessed, and the fraction of mRNA remaining was plotted versus post-actinomycin D treatment time to compare the rate of degradation of mRNA. If sodium salicylate decreases the stability of mRNA, then the rate of decay of mRNA will be faster in the presence of sodium salicylate. The results show that levels of MCP-1/JE and IP-10 mRNA, after a slight increase initially, subsequently decreased slowly over 8 hours at a comparable rate in the absence as well as presence of sodium salicylate (Fig 6A and B). In a second approach, we compared the rate of decay of mRNA produced in the absence or presence of sodium salicylate. Stromal cells were treated with IL-1α with or without sodium salicylate at a reduced concentration (20 mmol/L for 4 hours and then treated with actinomycin D, and mRNA levels were compared at various time points as described (Fig 6C and D). Again, the degradation kinetics of MCP-1/JE mRNA induced in the absence or presence of sodium salicylate were comparable (Fig 6C). The rate of
decay of IP-10 mRNA induced in the presence of sodium salicylate was somewhat slower compared with mRNA produced in its absence (Fig 6D). These results indicate that, by and large, sodium salicylate does not affect the stability of IL-1α--induced MCP-1/JE or IP-10 mRNA.

Sodium salicylate inhibits transcription of MCP-1/JE and IP-10 genes. The rapid induction of MCP-1/JE and IP-10 by IL-1α suggested that IL-1α might induce the expression of these mRNA by the transcriptional activation of the genes in question. To investigate specifically whether attenuation of mRNA production by sodium salicylate might involve inhibition of transcription of these genes, nuclei were isolated from untreated cells or cells that were treated with IL-1α alone or a combination of IL-1α and sodium salicylate, and newly transcribed mRNA was analyzed by nuclear run-on. A low level of constitutive transcription of MCP-1/JE and IP-10 was present in untreated (control) cells (Fig 7). The transcription of both genes was increased after stimulation with IL-1α, without a significant change in the transcription of the α-tubulin gene. In contrast, the addition of sodium salicylate to cultures dramatically reduced the basal and induced transcriptional activity of MCP-1/JE and IP-10 genes.

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Fig 5. Effect of CHX treatment on sodium salicylate-mediated inhibition of chemokine mRNA expression. Cells were preincubated with CHX (10 µg/mL) for 30 minutes and then treated with IL-1α (1,000 U/mL) or IL-1α + Na-Sal (40 mmol/L), as indicated, for 4 hours. Total RNA was prepared, and mRNA levels were analyzed by Northern hybridization as described. Similar results were obtained in two separate experiments.

Fig 6. Effect of sodium salicylate on the stability of MCP-1/JE (A and C) and IP-10 (B and D) mRNA. In one set of experiments (A and B), cells were first treated with IL-1α (1,000 U/mL) for 4 hours, after which they were then treated with actinomycin D (10 µg/mL; ) or actinomycin D + Na-Sal (40 mmol/L; ) for an additional 8 hours. In the second set (C and D), cells were treated with IL-1α (1,000 U/mL) in the absence ( ) or presence of 20 mmol/L sodium salicylate ( ) for 4 hours, and then with actinomycin D (10 µg/mL) for another 8 hours. Individual cultures were harvested at the indicated times, and total cellular RNA was subjected to Northern blot analysis. Specific mRNA levels were quantitated by scanning densitometry. Similar results were obtained in two separate experiments.
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Fig 7. Effect of sodium salicylate on transcription rates of MCP-1/JE and IP-10. Nuclear run-on transcription assays were performed on nuclei isolated from untreated cells (control) or cells that were treated with IL-1α (10^6 U/mL) alone or a combination of IL-1α and sodium salicylate (40 mmol/L) for 3 hours, as described in Materials and Methods. Equalized cpm of run-off products were hybridized to the indicated slot-blotted cDNA probes (7 pg each). Autoradiographs were exposed for 5 days.

without altering the transcription of the α-tubulin gene. These data suggest that inhibition of MCP-1/JE and IP-10 mRNA by sodium salicylate is mediated at the level of transcription of these genes.

Sodium salicylate inhibits NF-κB activation. Because sodium salicylate has been shown to affect activation of transcription factors, including the activation of the nuclear factor that binds to the kB nucleotide consensus sequence (NF-kB), which is present in the promoter region of many cytokine genes and participates in the transcription of these genes, we determined the effect sodium salicylate has on the activation of NF-kB. Nuclear protein extract prepared from stromal cells that were treated or not with sodium salicylate before stimulation with IL-1α was analyzed for NF-kB using [32P]dATP-radiolabeled oligonucleotide probe recognizing the kB site-specific sequence by EMSA (Fig 8). IL-1α-stimulated stromal cells expressed increased levels of NF-kB binding to the kB probe (lane 2) compared with unstimulated cells (lane 1). Sodium salicylate inhibited the activation of NF-kB in a dose-dependent manner (lanes 3 through 6). Maximum suppression of NF-kB by sodium salicylate was observed at 40 mmol/L (greater than 80% suppression), and suppression was less effective at lower concentrations. By itself, sodium salicylate did not alter the gel shift profile of unstimulated cells (lane 7). In contrast to the effect on NF-kB expression, the level of AP1 transcription factor was not affected by sodium salicylate (data not shown).

DISCUSSION

A functionally intact bone marrow microenvironment provided by stromal cells is obligatory for the proliferation and differentiation of hematopoietic progenitor cells. Virtually all growth-promoting effects of stromal cells are mediated through the production of various CSFs and cytokines with HGF activity. Included among the various growth factors produced by stromal cells are low-molecular-weight chemotactic cytokines that have been recently shown to influence myelopoiesis. We have previously reported that...
the murine bone marrow stromal cell line +/+-LDA11 expresses mRNA for chemokines MCP-1/JE and IP-10 when stimulated with proinflammatory cytokines. As sodium salicylate is an antiinflammatory agent and has been shown to affect the transcription of genes, we have examined the regulation of chemokine gene expression in stromal cells by this agent. The results demonstrate that sodium salicylate inhibits the production of mRNA for MCP-1/JE and IP-10 chemokines induced with IL-1β, IFN-γ, or LPS. The suppressive effect of sodium salicylate was concentration-dependent, and complete suppression of inducible mRNA occurred at a rather high concentration (40 mmol/L). Although suppression of mRNA is observed at relatively high concentrations of sodium salicylate, higher doses of this drug are also required for an antiinflammatory effect in vivo. Such high concentration might seem to affect the viability of cells, which may account for the decrease in the levels of mRNA. This presumption is not supported by the experimental result, because comparable concentrations of sodium salicylate showed no effect on the viability of cells. This is further supported by the finding that, at least in the case of MCP-1/JE, cells pretreated with sodium salicylate followed by its removal remained fully competent in their expression of mRNA.

Sodium salicylate could block chemokine gene expression by transcriptional and/or posttranscriptional mechanisms. The reduced accumulation of both mRNAs was found to be due to an actual inhibition of mRNA induction rather than a shift in the time course of mRNA production in the presence of sodium salicylate. Both mRNAs, whether induced in the presence of the inhibitor or not, followed similar kinetics of mRNA production with the difference that at all time points, the level of mRNA produced in the presence of sodium salicylate was dramatically suppressed. The results of time-delayed addition of sodium salicylate suggest that in the case of MCP-1/JE some very early event(s) initiated after stimulation with IL-1β are blocked by sodium salicylate. In contrast, attenuation of IP-10 mRNA by sodium salicylate even if added to the cultures as late as 2 hours after stimulation with IL-1β indicates that both early and late events involved in the expression of the IP-10 gene are susceptible to sodium salicylate. The difference in the susceptibility of these two genes to the suppressive effects of sodium salicylate is also apparent in experiments in which cells were first pretreated with sodium salicylate before stimulation with IL-1β. Whereas the expression of MCP-1/JE mRNA was readily restored after removal of sodium salicylate, that of IP-10 mRNA remained blocked even after 24 hours. These data suggest that IL-1β-induced intracellular pathways leading to the expression of the MCP-1/JE gene are transiently and reversibly affected, whereas those involved in the expression of the IP-10 gene are more or less permanently blocked by sodium salicylate. Although the molecular signaling events involved in the expression of these genes and their differential display of sensitivity to sodium salicylate remain to be elucidated, de novo synthesis of an intermediate protein is not required for sodium salicylate-mediated suppression of the two mRNAs. This conclusion is based on the finding that CHX, an inhibitor of protein synthesis, did not reverse the suppression of mRNAs by sodium salicylate. In fact, CHX enhanced the IL-1β-induced levels of mRNA of both genes, suggesting that mRNA induced by IL-1β alone might be suboptimal because of synthesis of an inhibitory protein. Collectively, these results suggest that de novo synthesis of an intermediary protein may be part of the mechanism by which IL-1β induces MCP-1/JE or IP-10 expression; however, it is not a requisite for the suppression of these mRNAs by sodium salicylate. In addition, we tested the possibility that sodium salicylate might posttranscriptionally affect mRNA by reducing its stability. The stability of both mRNAs was not significantly altered whether mRNA was generated in the absence or presence of sodium salicylate (Fig 6). This conclusion is based on the finding that decay rates of mRNA were similar in cells treated with sodium salicylate or not. Thus, the decrease in the levels of mRNA by sodium salicylate is not due to destabilization of mRNA transcripts.

The lack of significant effect of sodium salicylate on the time course of mRNA expression and its degradation kinetics suggests that inhibition of MCP-1/JE and IP-10 mRNA accumulation by sodium salicylate might result from interference with transcriptional activity of these genes. Indeed, the results of nuclear run-on analyses (Fig 7) clearly show that sodium salicylate inhibits both inducible and basal transcription rates of chemokine genes. The magnitude of suppression of transcriptional activity by sodium salicylate is consistent with the extent of reduction in mRNA levels seen in all experiments presented in this report. Thus, the primary mechanism by which sodium salicylate abrogates mRNA induction appears to be at the level of gene transcription.

The transcription of several cytokine genes (IL-1, IL-6, IL-8, IFN-β, TNF-α, etc) and adhesion molecules (endothelial leukocyte adhesion molecule-1 [ELAM-1], intercellular adhesion molecule [ICAM]-1, vascular cell adhesion molecule [VCAM]-1, etc) involves activation of NF-κB, an inducible transcription factor of the rel family. Further, transcriptional activation of the MIP-2, MCP-1/JE, and IP-10 genes has also been shown to involve NF-κB binding motifs present in the promoter regions of these genes. Our results demonstrate that IL-1β activates NF-κB in stromal cells, and sodium salicylate inhibits it in a dose-dependent manner. The participation of NF-κB in transcription of numerous cytokines/chemokines genes and the inhibition of NF-κB and chemokine mRNA expression by sodium salicylate allow us to speculate that sodium salicylate blocks MCP-1/JE and IP-10 expression, at least in part, by inhibiting activation of NF-κB transcription factor. These findings are consistent with previous reports in which salicylates have been shown to influence the activation of heat shock and NF-κB transcription factors.

Although the precise mechanism of sodium salicylate effect remains to be determined, our findings suggest that inhibition of chemokine gene expression by sodium salicylate might involve an effect on early signaling events essential to the activation sequence. As many proinflammatory cytokines, including IL-1β, induce the release of products of arachidonic acid metabolism that have been demonstrated to play an important role in induction of protooncogenes and
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growth factor genes, the inhibition of these mediators with sodium salicylate may abort signal transduction for gene expression by suppressing NF-κB activation.

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