Heparin Inhibits the Expression of Interleukin-11 and Granulocyte-Macrophage Colony-Stimulating Factor in Primate Bone Marrow Stromal Fibroblasts Through mRNA Destabilization

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Interactions between different cytokines, extracellular matrix components, and various cell types inside the bone marrow microenvironment are believed to play important roles in the regulation of hematopoiesis. We observed that both interleukin-1 (IL-1) and 12-O-tetradecanoylphorbol-13-acetate (TPA) can stimulate the expression of IL-11 and granulocyte-macrophage colony-stimulating factor (GM-CSF) genes in a primate bone marrow fibroblast cell line, PU-34. We also found that IL-1 or TPA-stimulated IL-11 and GM-CSF expression in PU-34 cells can be abolished by heparin, a class of molecules related to extracellular matrix components, glycosaminoglycans. Because the growth inhibitory signals provided by extracellular factors were less understood, the mechanisms of heparin inhibition of IL-11 and GM-CSF gene expression were further investigated. Our data demonstrate for the first time that heparin did not alter the transcription of endogenous IL-11 and GM-CSF genes or an exogenous IL-11 promoter construct containing an AP-1 sequence. Instead, heparin facilitated the degradation of the corresponding mRNAs. Through RNA gel shift assays, heparin-mediated mRNA destabilization was tentatively linked to its competition for mRNA binding proteins both in the cell-free system and in intact cells. Collectively, our findings suggest that varying degrees of heparin inhibition may provide a novel mechanism for the regulation of cytokine expression during the growth and differentiation of different lineages of hematopoietic cells.

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Fig 1. Heparin inhibits IL-11 and GM-CSF gene expression. (A) PU-34 cells were unstimulated (lane 1) or preincubated with different concentrations of heparin for 30 minutes and then stimulated with IL-
1α (25 U/mL; lanes 2 through 5) or TPA (25 ng/mL; lanes 6 through 9) in the presence of heparin (HEP). (B) Unstimulated cells were treated with different concentrations of heparin. After 6 hours of stimulation or treatment, RNA was isolated for Northern analysis. The same membrane was hybridized successively with IL-11, GM-CSF, and GAPDH cDNA probes. Two IL-11 transcripts were detected in PU-34 cells, as shown by arrows.

IL-11, antisense GM-CSF, and the plasmid DNA-containing human GAPDH cDNA insert were slot-blotted onto nitrocellulose membranes. Nuclei from approximately 3 x 10⁷ cells were collected by lysis with 0.5% NP-40 buffer (10 mmol/L Tris-HCl pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl₂, and 0.5% NP-40). The nuclear run-on assay was performed as previously described. The ³²P-labeled run-on transcripts were extracted with phenol/chloroform, precipitated with ethanol, washed, and counted. Equal counts of run-on products (3 x 10⁶ cpm per sample) were hybridized to each blot. After 24 hours of hybridization, the blots were washed until there was no background hybridization to the sense IL-11 negative control and were exposed to x-ray films. As a positive control, PU-34 cells were stimulated with TPA (25 ng/mL) for 1 hour, and transcriptional activation of JunB gene was measured by nuclear run-on assay.

Reporter gene expression. The human IL-11 genomic sequence has been determined previously. For reporter plasmid construction, a 160-bp 5' flanking sequence of the IL-11 gene (∼100 to +60 relative to the transcription initiation site) was prepared by polymerase chain reaction (PCR) and cloned into the BamHI/Sma I sites within the polylinker region of the firefly luciferase vector pXP2. Plasmid pXP2-100m was also constructed and had a point mutation from TGGAGTCA to GGAGTCA at the AP-1 site. For transient transfections, subconfluent PU-34 cells in 60-mm culture dishes were transfected with 4 µg of the reporter plasmid plus 1 µg of pCMV-β-galactosidase (Clontech, Palo Alto, CA) by the DOTAP method according to the manufacturer’s instructions. After 12 hours of incubation with DNA-DOTAP complex in medium containing 2% fetal calf serum (FCS), the cells were washed and incu- bated with medium containing 10% FCS for another 12 hours. In the presence of different concentrations of heparin, cells were stimulated with IL-1α or TPA and lysed after 12 hours for luciferase and β-galactosidase assays using standard procedures.

RNA gel shift assay. To generate IL-11 or GM-CSF transcripts, full-length or 3' untranslated region (UTR) IL-11 cDNA and full-length GM-CSF cDNA were subcloned into pGEM-7Z vector (Promega, Madison, WI) within the polylinker region, and the plasmids were linearized with BamHI or EcoRI for in vitro transcription. Cold or ³²P-labeled IL-11 and GM-CSF transcripts were obtained using SP6 or T7 RNA polymerase according to standard procedures. Cytoplasmic extracts of PU-34 cells were prepared as previously described. Ten micrograms of cytoplasmic extract and 3 x 10⁶ cpm of ³²P-labeled transcripts were mixed in a total volume of 20 µL containing 20 mmol/L HEPES pH 7.9, 10% glycerol, 50 mmol/L KCl, and 0.5 mmol/L EDTA. For competition, cold RNA transcripts or heparin were mixed with cytoplasmic extracts before the
addition of 3P-labeled RNA probes. After 30 minutes of incubation at room temperature, the unbound RNA probes were digested with 10 U RNase T1 for another 10 minutes, and the reaction mixture was analyzed through electrophoresis on a 5% polyacrylamide gel in 0.5x TBE buffer. The gel was dried and exposed to x-ray film.

RESULTS AND DISCUSSION

Heparin inhibits IL-11 and GM-CSF gene expression at the posttranscriptional level. We report here that treatment of bone marrow stromal fibroblast cells with heparin altered the cellular response to cytokine or mitogen stimulation. In the absence of heparin, as shown in Fig 1A, IL-1α and TPA both drastically increased the steady state levels of IL-11 and GM-CSF mRNAs, although IL-1α-induced elevation of GM-CSF mRNA was less prominent as compared with TPA induction. The induction of IL-11 and GM-CSF can be inhibited by the addition of heparin to the culture medium in a dose-dependent manner. At a concentration as low as 10 μg/mL, heparin completely inhibited IL-1α induction of IL-11 and GM-CSF mRNAs, whereas the complete inhibition of TPA induction of GM-CSF mRNA required a heparin dose higher than 250 μg/mL. Heparin also inhibited basal IL-11 mRNA levels in unstimulated cells (Fig 1B). The inhibition of IL-11 and GM-CSF expression mediated by heparin is relatively selective, as the GAPDH mRNA level was obviously not affected by heparin under our experimental conditions.

To determine at what stage of gene expression heparin exerts its inhibition on PU-34 cells, nuclear run-on assays were performed to measure transcription rates of IL-11 and GM-CSF genes after different treatments. As shown in Fig 2A, the transcription of IL-11, GM-CSF, and GAPDH genes in PU-34 cells was not significantly affected by IL-1α and TPA stimulations in the presence or absence of heparin. These results suggested that increases of IL-11 and GM-CSF mRNA levels after IL-1α or TPA stimulation is not due to changes in transcription of the IL-11 and GM-CSF genes in PU-34 cells, and heparin inhibition of IL-11 and GM-CSF mRNAs is likely to be a posttranscriptional event. Under the same experimental conditions, TPA activated transcription of the JunB gene (Fig 2B).

Our previous promoter studies have shown that IL-11 gene transcription is controlled by a JunD/AP-1 complex in PU-34 cells. To demonstrate that JunD/AP-1 complex-mediated IL-11 gene transcription in PU-34 cells is, indeed, not affected by heparin, IL-11 promoter constructs were generated and transfected into PU-34 cells, and the transfected cells were incubated under different concentrations of heparin. As shown in Fig 3, in accordance with the nuclear run-on assays, transient expression of the reporter gene driven by the IL-11 promoter was not affected appreciably by IL-1α or TPA. Although transcription from the wild-type IL-11 promoter construct (pXP2-100) conferred a strong luciferase activity, a point mutation from TGAGTCA to GGAGTCA at the AP-1 site (pXP2-100m) resulted in almost a 10-fold loss in the luciferase activity, demonstrating that IL-11 promoter
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activity is, indeed, controlled by the AP-1 recognition sequence. However, under concentrations shown to abolish IL-1α - and TPA-induced expression of the endogenous IL-11 gene (Fig 1), heparin did not show any significant effect on the expression of exogenous AP-1-containing luciferase reporter constructs (Fig 3). Our finding is different from that of a previous report in which Fos- and Jun/AP-1-mediated reporter gene transcription can be trans-repressed by heparin in primary vascular smooth muscle cells, as well as in cultured HeLa cells. The inability of heparin to inhibit transcription of the IL-11 gene in cultured bone marrow stromal fibroblasts probably reflected the difference among various cell types or reporter gene constructs used in these studies.

The half-lives of IL-11 and GM-CSF mRNA were measured at 0, 3, and 6 hours post-actinomycin D treatment. In unstimulated cells, the half-life of IL-11 mRNA was measured to be less than 1 hour (Fig 4A, lanes 1 through 3). The stability of IL-11 and GM-CSF mRNAs in PU-34 cells was greatly increased after IL-1α and TPA stimulation. As shown in Fig 4B, IL-1α treatment (lanes 10 through 12) or TPA treatment (lanes 13 through 15) of PU-34 cells maintained high levels of IL-11 and GM-CSF mRNAs up to 6 hours after gene transcription was stopped by addition of actinomycin D to the stimulated cells. However, in the presence of heparin the half-life of IL-11 mRNA in IL-1α-treated cells (Fig 4A, lanes 4 through 6) or TPA-treated cells (Fig 4A, lanes 7 through 9) was greatly decreased as compared with that under stimulation in the absence of heparin. Because of the very low level of GM-CSF mRNA in unstimulated PU-34 cells, it was difficult to estimate its stability. In other cell systems, the half-life of GM-CSF mRNA was reported to be less than 30 minutes. The half-life changes of IL-11 mRNA were summarized in Fig 4C after densitometric scanning of the mRNA signals.

In summary, these results suggest that both IL-11 and GM-CSF genes are constitutively transcribed in bone marrow stromal fibroblast cells. The steady-state levels of these mRNAs are relatively low because of their short half-lives. IL-1α or TPA stimulation of these cells greatly increases the stability of both IL-11 and GM-CSF mRNAs, elevating the expression of IL-11 and GM-CSF genes. Heparin abolishes the induction of IL-11 and GM-CSF gene expression through blockage of mRNA stabilization.

Heparin inhibits IL-11 and GM-CSF gene expression, possibly through competition for mRNA-binding proteins. The expression of many cytokine and oncogene genes has long been recognized to be regulated by differential mRNA stability. Although intensive studies have been performed, the mechanisms underlying transient stabilization of cytokine and oncogene mRNAs remain elusive. It was proposed that cytokine mRNA degradation and stabilization is, in part, mediated by the AUUUA motif, which has recently been redefined as the UUAUUUAUU motif. These AU-rich sequences are also present in the 3' UTRs of IL-11 and GM-CSF mRNAs. Proteins that interact with labile mRNAs, such as AUUUA-binding factors or factors binding to other sequence within the 3' UTR, have been suspected to be involved in mRNA stabilization. As heparin has been reported previously to be capable of binding to a variety of proteins, such as certain plasma proteins and fibroblast growth factors, we speculated that heparin may also interact with RNA-binding proteins present in the cytoplasm of PU-34 cells.

To test the notion that heparin may destabilize IL-11 and GM-CSF mRNAs through competing with these mRNA transcripts for RNA binding proteins, RNA gel shift assays were performed using in vitro-labeled RNA transcripts as the probes. As shown in Fig 5A, the interaction of PU-34 cytosolic proteins with the full-length IL-11 transcript generated two RNA-protein complexes (indicated by arrows) that are resistant to RNase T1 digestion. The intensity of these two bands was not affected by IL-1α or TPA stimulation (lanes 2 through 4). The specificity of these two bands was supported by competition with excess amounts of unlabeled IL-11 transcript (lanes 5 through 7). When 32P-labeled antisense IL-11 transcript was used as the probe, no RNA-protein complex was observed in the gel shift assay (data not shown). Addition of increasing amounts of heparin resulted in decreasing intensity of the RNA-protein bands (lanes 8 through 10). Furthermore, the formation of RNA-protein complexes and heparin competition were not affected by unlabeled proteins such as BSA (lanes 11 through 14). The RNA sequences interacting with the RNA-binding proteins appeared to be within the 3' UTR of IL-11 mRNA, because RNA probe containing the 3' UTR of the IL-11 transcript generated a similar gel shift pattern as the full-length IL-11 probe (lanes 15 through 17).

To demonstrate that heparin also interacts with RNA-binding proteins in intact cells, cytoplasmic extracts prepared from untreated and heparin-treated PU-34 cells were used for the RNA gel shift assay. When intact cells were pre-
Fig 4. Heparin blocks stabilization of IL-11 and GM-CSF mRNAs. (A) PU-34 cells were unstimulated or preincubated with heparin (Hep; 250 μg/mL) for 30 minutes and then stimulated with IL-1α (25 U/mL) or TPA (25 ng/mL) for 6 hours in the presence of heparin. Cells were then treated with actinomycin D (Act-D; 5 μg/mL). Total RNA from unstimulated cells (lanes 1 through 3), from IL-1α-stimulated cells (lanes 4 through 6), and from TPA-stimulated cells (lanes 7 through 9) were analyzed at 0, 3, and 6 hours post-actinomycin D addition. (B) As a comparison, PU-34 cells were stimulated with IL-1α (25 U/mL; lanes 10 through 12) or TPA (25 ng/mL; lanes 13 through 15) for 6 hours in the absence of heparin, and then treated with actinomycin D (Act-D; 5 μg/mL). The cells were lysed at 0, 3, and 6 hours post-actinomycin D addition for the measurement of mRNA stability. The same membrane was probed with IL-11, GM-CSF, and GAPDH probes. (C) Effects of IL-1α, TPA, and heparin on IL-11 mRNA half-lives were summarized after densitometric scanning and normalization according to GAPDH signals.

treated with heparin, the intensity of RNA-protein bands in the RNA gel shift assay was greatly reduced as compared with those from untreated cells (Fig 5B, lanes 18 through 23). When a GM-CSF transcript was used as the RNA probe, as shown in Fig 5C (lanes 24 through 31), at least two specific RNA-protein complexes were observed by the gel shift assay. The mobility of these bands apparently differed from those using IL-11 RNA as the probe, indicating that proteins binding to IL-11 and GM-CSF probes are probably of different molecular weights. Although heparin attenuates specific and nonspecific RNA-protein interactions, the affinity of heparin toward different RNA-binding proteins apparently is not the same (as shown in Fig 5C).

Our results suggest that heparin inhibition of IL-11 and GM-CSF mRNAs in PU-34 cells may be linked to its competition for mRNA-binding proteins. However, we cannot rule out the possibility that heparin may interfere with certain protein kinase cascade(s) leading to mRNA stabilization.
Protein bindings to IL-11 or GM-CSF transcripts appear not to be affected by IL-1α or TPA stimulation, as shown by the RNA gel shift experiments, raising the possibility that these RNA-binding proteins can bind to these transcripts before cellular stimulation. If these proteins are, indeed, involved in the upregulation of IL-11 and GM-CSF gene expression, they may participate in mRNA stabilization after cellular stimulation through posttranslational mechanisms such as protein phosphorylation. This is consistent with our previous observation that genistein-sensitive protein tyrosine kinase(s) is involved in IL-1-induced IL-11 mRNA stabilization in PU-34 cells. If heparin destabilizes IL-11 and GM-CSF mRNAs through competition for the RNA-binding proteins as was suggested here, we would expect that heparin should decrease the basal and inducible levels of IL-11 and GM-CSF mRNAs. In our study, the IL-11 mRNA level in unstimulated cells is, indeed, decreased by treatment with heparin, although less drastically as compared with the inhibition of IL-1α- and TPA–induced IL-11 expression.

Heparin is well known for its inhibitory effect on the proliferation of certain cells, and the antiproliferative activity has been linked to the blockage of the G1 phase of the cell cycle, the inhibition of a heparin-sensitive, mitogen-activated protein kinase pathway, and the control of attenuated levels of c-fos and c-myc mRNAs. As most early response gene products are encoded by short-lived mRNAs,
our study implies that the antiproliferative activity of heparin may, in part, be mediated by facilitated degradation of these labile mRNAs, thus blocking the signal transduction pathways leading to cell proliferation. In addition, previous studies have indicated that the metabolism of heparin inside bone marrow stroma may change depending on culture conditions that favor myeloid or lymphoid proliferation and differentiation, raising the possibility that the expression of other cytokine genes in bone marrow stromal fibroblasts may also be modulated by heparin.

Collectively, these studies suggest that heparin may affect hematopoiesis in at least two aspects: outside the bone marrow stromal fibroblasts, heparin participates in the compartmentalization of cytokines and growth factors on their target cells; inside the bone marrow stromal fibroblasts, heparin exerts its control over the expression of cytokine genes at the posttranscriptional level. The interplay of extracellular and intracellular signaling events may ultimately establish a delicate balance within the bone marrow microenvironment in which the production of matrix molecules and cytokines and their actions on hematopoietic cells are tightly regulated.

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