Heparin Synergistically Enhances Interleukin-11 Signaling through Up-regulation of the MAPK Pathway*

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Using an animal model of heparin-induced osteoporosis we previously demonstrated that heparin causes bone loss, in part, by increasing osteoclast number and activity. Furthermore, we found that, although heparin alone has no effect, it is able to synergistically enhance Interleukin-11 (IL-11)-induced signal transducer and activator of transcription 3 (STAT3) activation and thus increase osteoclast formation in vitro. In the present study, we examine the effect of various serine kinase inhibitors on the ability of heparin to act synergistically with IL-11. Inhibition of the c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), or the phosphatidylinositol 3-kinase of the c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), or the phosphatidylinositol 3-kinase pathways had no effect on the ability of heparin to promote either IL-11-induced STAT3/DNA complex formation or osteoclast formation in vitro. In contrast, PD098059, a MAPK kinase inhibitor, completely abolished the synergy between heparin and IL-11. In an attempt to resolve the mechanism by which this was occurring, we examined the effect of heparin on STAT3 Ser-727 phosphorylation and extracellular signal-regulated kinases 1 and 2 (Erk1/2) activation, either in the presence or absence of IL-11. Heparin alone was found to have no effect on Ser-727 phosphorylation, nor did heparin alter the phosphorylation status of Ser-727 in the presence of IL-11. Heparin was, however, found to increase Erk1/2 activation in both a time- and dose-dependent manner. When taken together, these findings suggest that heparin enhances IL-11-induced STAT3 activation and thus osteoclast formation, by a mechanism that is independent of STAT3 Ser-727 phosphorylation but that involves up-regulation of the MAPK pathway.

Osteoporosis is a well recognized complication of long term heparin therapy (1–9). However, little is known about the mechanism by which heparin causes bone loss. To address this issue we developed several in vitro and in vivo models with which to study the effects of heparin on bone. By using these models, we showed that heparin causes both a time- and a dose-dependent decrease in cancellous bone and that this, in part, results from an increase in osteoclast number and activity (10–12). In addition, we found that heparin alone has no effect on osteoclast formation, but rather it acts synergistically with interleukin-11 (IL-11),3 a member of the IL-6 family of cytokines, to enhance both IL-11 signaling and in vitro osteoclast formation (13). When taken together, these findings suggest that IL-11 plays a critical role in the ability of heparin to induce osteoclast formation. This finding may account for the increase in bone resorption that we observe in our animal models of heparin-induced osteoporosis.

IL-11 belongs to a family of cytokines that includes IL-6, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and cardiotoxin-1 (14). All members of this family display multiple effects on both hematopoietic and non-hematopoietic cell populations, and as such, are known to stimulate osteoclast formation both in vitro and in vivo (15–18). Receptors for these cytokines are found on the osteoblast cell surface where they initiate signal transduction through a series of well defined steps. Thus, following binding to their respective α-chain receptors these cytokines form a tri-molecular complex with glycophorin (gp)130 and initiate signal transduction through activation of the Janus family of tyrosine kinases (Jak1, Jak2, and Tyk2) (19, 20). This activation results in the phosphorylation of gp130 at specific tyrosine residues located within its cytoplasmic domain and creates a docking site for members of the signal transducer and activator of transcription (STAT) family (20). Once STATs (STAT3 and STAT1) bind to gp130, they are also phosphorylated on specific tyrosine residues by Jak. Following phosphorylation, STATs form homo- or heterodimers and translocate into the nucleus where they act to promote transcription by binding to specific promoter elements within their various response genes (21). Although tyrosine phosphorylation of STATs (i.e. Tyr-705) is a necessary prerequisite for the dimerization and subsequent binding of STATs to DNA, STAT activity can also be influenced by serine phosphorylation (22–24). Thus, a number of studies have shown that serine phosphorylation, in particular phosphorylation...
tion of Ser-727, can enhance the ability of STATs to promote transcriptional activity (25, 26).

In the present study, we examine the mechanism by which heparin acts synergistically with IL-11 to induce both STAT3 activation and in vitro osteoclast formation. By using various serine kinase inhibitors we demonstrate that heparin enhances IL-11-induced STAT3 activation and thus, osteoclast formation, by a mechanism that is independent of STAT3 Ser-727 phosphorylation but involves up-regulation of the MAPK pathway. When taken together, these findings suggest a plausible mechanism by which heparin may cause bone loss when administered long-term.

**EXPERIMENTAL PROCEDURES**

**Materials**—Thirty-day-old Swiss Webster and 15- to 17-day-old pregnant female C57Bl/6 mice were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). Recombinant human IL-11 was purchased from R&D Systems (Minneapolis, MN), whereas phospho-STAT3 (Ser-727) antibodies were obtained from Cell Signaling Technology (Beverly, MA). All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Unfractionated heparin and the serine kinase inhibitors PD098059, wortmannin, SB202190, and SP600125 were all purchased from Sigma.

**Calvaria Cell Isolation**—Calvaria cells were isolated from parietal bones as described previously (27). Briefly, parietal bones were isolated from 3- to 5-day-old neonatal mice under sterile conditions and cut into 2- to 4-mm² pieces. The calvaria cells were then isolated from the pieces of parietal bone by sequential digestion with collagenase II at a concentration of 2.5 mg/ml. Isolated cells were washed by centrifugation, seeded at a concentration of ~30,000 cells/cm² in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum, and expanded for 7 days before use.

**Cell Lysis and Nuclear Extract Preparation**—Calvaria cells were plated in 15-cm diameter dishes at a concentration of 1 × 10⁶ cells/plate and then incubated for 48 h in α-MEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were then treated with various serine kinase inhibitors or the Jak2 inhibitor, AG490, before being cultured with either heparin (25 μg/ml), IL-11 (20 ng/ml), or a combination of both heparin and IL-11, for 24 h. Total RNA was isolated using an RNeasy mini kit (Qiagen), run on a 1% agarose/formaldehyde gel, and transferred onto a polyvinylidene difluoride membrane (Bio-Rad) prior to hybridization at 43 °C. An [α-32P]dCTP-labeled cDNA probe was created using a random primed labeling kit (Roche Diagnostics). A 734-bp PCR product corresponding to the partial coding region of gp130 was used as a probe. Membranes were exposed on Kodak X-Omat AP film at −80 °C for 3–7 days. Blots were stripped in a 0.1% SSC/0.5% SDS solution and rehybridized with a glycerol-aldehyde-3-phosphate dehydrogenase probe as a control for loading.

**Immunoblotting**—Murine calvaria were grown to 50–70% confluency in α-MEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin before being treated at 37 °C for increasing periods of time. Following incubation at 37 °C, cells were washed with phosphate-buffered saline and then lysed with 1% sodium deoxycholate, 1% Nonidet P-40, 0.5% sodium dodecyl sulfate, and 50 mM NaF in 50 mM Tris-HCl (pH 7.4) containing leupeptin (5 μg/ml), aprotenin (5 μg/ml), pepsatin A (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM), 0.1 mM Na3VO4, and EDTA (0.25 mM). The cell lysates were then run on 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked overnight with 5% casein at 4 °C. The membranes were then incubated with anti-p-Erk antibody (Santa Cruz Biotechnology) for 3 h before being incubated with horseradish peroxidase-conjugated secondary antibodies and visualized with an enhanced chemiluminescent system. Following visualization, the blots were stripped (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) and incubated with anti-Erk antibody (Santa Cruz Biotechnology). Protein was visualized by the same method as p-Erk.

**Immunoprecipitation**—Murine calvaria were grown to 50–70% confluency in α-MEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin before being

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using polynucleotide T4 kinase. Nuclear extract (5 μg) were then incubated with 3 μg of poly(dl-dC) at 4 °C, for 15 min, before adding 100,000 cpm of radiolabeled SIE in 20 mM HEPES (pH 7.9), containing 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM Na3VO4, and 25% glycerol. After a 30-min incubation at room temperature, DNA-protein complexes were run on a 4% native polyacrylamide gel, before being exposed on X-Omat AP film (Eastman Kodak, Rochester, NY), and quantified using a PhosphorImager (Amersham Biosciences). Negative controls included excess unlabeled homologous SIE and a mutated SIE (5'-GTG CAT CCA CCG TAA ATC TTG TCT ACA-3'), which is unable to bind STAT3.
treated for 24 h with either PD098059 or SP600125. The cells were then treated with either heparin (25 μg/ml), IL-11 (20 ng/ml), or IL-11 plus heparin (20 ng/ml and 25 μg/ml, respectively). After a 20-min incubation, the cells were washed and then lysed with 1% deoxycholic acid, and Triton X-100 in 50 mM Tris-HCl (pH 7.2) containing EDTA (0.25 mM), leupeptin (5 μg/ml), aprotinin (5 μg/ml), pepstatin A (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and Na₃VO₄ (0.1 mM). The supernatants of the cell extracts were then incubated overnight at 4 °C with 2 μg of anti-STAT3 antibody (Santa Cruz Biotechnology) and then washed three times with lysis buffer, boiled, and then subjected to 7.5% SDS-PAGE before being transferred to a nitrocellulose membrane (Bio-Rad). The blots were then blocked overnight with 5% casein at 4 °C and then incubated with anti-STAT3 phospho-Ser-727 antibody (Cell Signaling) for 3 h before being incubated with a horseradish peroxidase-conjugated secondary antibody and visualized by an enhanced chemiluminescent system.

Real-time PCR—Calvaria were plated in 15-cm dishes at a concentration of 1 × 10⁶ cells/plate and then incubated for 48 h in α-MEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were then treated with PD098059 for 1 h, before being cultured with either heparin (25 μg/ml), IL-11 (20 ng/ml), or a combination of the two, for 6 days. Total RNA was then isolated using an RNeasy mini kit (Qiagen) and first strand cDNA synthesized from 4 μg of total RNA using random priming and Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR analysis of RANKL and 18 S rRNA was performed with an ABI Prism 7300 (Applied Biosystems, Foster City, CA) using specific primers for RANKL and 18 S rRNA. Denaturation took place at 95 °C for 15 s, and annealing and extension were at 60 °C for 1 min, for 40 cycles. Results were expressed as the -fold increase, over the respective 18 S rRNA controls.

siRNA Transfection—Calvaria cells were seeded into 24-well tissue culture dishes containing 13-mm Thermaxx coverslips at a cell density of 2 × 10⁵ cells/well. Twenty-four hours later, the cells were transfected with either 2 μg/ml PKD-MAPK1/Erk2-v1 siRNA expression plasmid (Upstate, Lake Placid, NY) or control plasmid, (pKD-NegCon-v1, Upstate) for 5 h using Lipofectamine reagent (Invitrogen). Following removal of the Lipofectamine reagent, the cells were allowed to incubate in normal growth media for 24 h before being used in an in vitro osteoclast formation assay as described below.

In Vitro Osteoclast Formation Assay—To obtain bone marrow cells for the assay, femurs from 30-day-old Swiss Webster mice were isolated, and the marrow was flushed out with phenol red-free MEM, using a syringe fitted with a 25-gauge needle (27). Once isolated, the cells were washed by centrifugation, and then co-cultured with murine calvaria cells (4 × 10⁵/cm²) at a concentration of 2.0 × 10⁶ cells/cm² in 24-well plates containing 13-mm Thermaxx coverslips. In some cases, the cells were treated for 24 h with either PD098059 or SP600125. The cells were then treated with either heparin (25 μg/ml), IL-11 (20 ng/ml), or IL-11 plus heparin (20 ng/ml and 25 μg/ml, respectively). After a 20-min incubation, the cells were washed and then lysed with 1% deoxycholic acid, and Triton X-100 in 50 mM Tris-HCl (pH 7.2) containing EDTA (0.25 mM), leupeptin (5 μg/ml), aprotinin (5 μg/ml), pepstatin A (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and Na₃VO₄ (0.1 mM). The supernatants of the cell extracts were then incubated overnight at 4 °C with 2 μg of anti-STAT3 antibody (Santa Cruz Biotechnology) and then incubated with protein A-Sepharose (Amersham Biosciences) for 1 h to precipitate the antigen-antibody complexes. Immunoprecipitates were then washed three times with lysis buffer, boiled, and then subjected to 7.5% SDS-PAGE before being transferred to a nitrocellulose membrane (Bio-Rad). The blots were then blocked overnight with 5% casein at 4 °C and then incubated with anti-STAT3 phospho-Ser-727 antibody (Cell Signaling) for 3 h before being incubated with a horseradish peroxidase-conjugated secondary antibody and visualized by an enhanced chemiluminescent system.
were also incubated with the serine kinase inhibitor PD098059 (25 μM) and either heparin (25 μg/ml), IL-11 (20 ng/ml), or a combination of heparin and IL-11. Nine days later, all adherent cells were fixed with a citrate/acetone solution and then stained for tartrate-resistant acid phosphatase (TRAP) activity (Sigma, Procedure No. 386). Following staining, the coverslips were air-dried and mounted onto slides, and the number of TRAP-positive multinucleated cells was determined microscopically at 200× magnification (27).

Statistical Analysis—All experiments were performed in triplicate. Analysis of variance was used to compare the results between the experimental and control groups. If a significant difference between experimental and control groups was detected, an unpaired Student’s t test was performed at each point. Significance levels were adjusted using a Bonferroni correction for multiple comparisons.

RESULTS

Effect of Heparin on IL-11-induced STAT3 Activation—We previously demonstrated that heparin enhances both IL-11-induced STAT3-DNA complex formation and transactivation without altering the tyrosine phosphorylation status of STAT3 (13). To further define the mechanism by which this occurs, we pretreated calvaria cells with various serine kinase inhibitors and then assayed, by use of an EMSA, the ability of heparin to promote STAT3-DNA complex formation in the presence or absence of IL-11. As shown in Fig. 1, nuclear extracts isolated from IL-11-treated cells were able to form STAT3-DNA complexes when incubated with radiolabeled SIE oligonucleotides. In contrast, no STAT3-DNA complexes were formed when the nuclear extracts were isolated from heparin-treated cells. Heparin was found, however, to enhance IL-11-induced STAT3-DNA complex formation (3.0 ± 0.2-fold; p < 0.01) when the nuclear extracts isolated from heparin- and IL-11-treated cells were compared with those that had been treated with IL-11 alone (Fig. 1). Pretreatment of the calvaria cells with the phosphatidylinositol 3-kinase inhibitor, wortmannin, or the p38 MAPK inhibitor, SB202190, had no effect on the ability of heparin to promote IL-11-induced STAT3-DNA complex formation. However, the MEK inhibitor, PD098059, was found to completely abolish the synergy between heparin and IL-11 (Fig. 1B). In addition, the JNK inhibitor, SP600125, while not affecting the ability of heparin to promote IL-11-induced STAT3-DNA complex formation, did partially reduce complex formation (46.6 ± 3.7%, p < 0.05) in the presence of IL-11 alone (Fig. 1A). These results suggest that the ability of heparin to synergistically enhance STAT3-DNA binding is dependent upon the ability of heparin to activate the MAPK pathway.

Effect of Various Serine Kinase Inhibitors on the Ability of Heparin to Enhance IL-11-induced gp130 Expression—Because the MEK inhibitor, PD098059, was able to abolish the ability of heparin to promote IL-11-induced STAT3-DNA binding, we next examined whether PD098059 would also prevent heparin from enhancing the transcription of gp130, a gene that contains a STAT3-binding element within its promoter (28). As seen in Fig. 2, gp130 expression was induced by IL-11, whereas heparin alone had no effect. However, when combined with heparin, the ability of IL-11 to induce gp130 expression was enhanced 2.0 ± 0.5-fold (p < 0.05) over that seen in its absence. Pretreatment of the calvaria cells with the MEK inhibitor, PD098059, while having no effect on IL-11-induced gp130 expression, completely abolished the ability of heparin to act synergistically with IL-11 (Fig. 2A). In contrast, the serine kinase inhibitors, wortmannin (a phosphatidylinositol 3-kinase inhibitor), and SB202190 (a p38 inhibitor), did not effect IL-11-induced gp130 expression, either in the presence or absence of heparin (Fig. 2B). However, similar to its effect on STAT3-DNA complex formation, the JNK inhibitor, SP600125, significantly decreased IL-11-induced gp130 expression (43.4 ± 6.3%, p < 0.05) without affecting the ability of heparin to act synergistically with IL-11.

Effect of Heparin and Serine Kinase Inhibitors on IL-11-induced STAT3 Serine Phosphorylation—Although tyrosine phosphorylation is a prerequisite of STAT3 activation, serine phosphorylation of STAT3, particularly at Ser-727, is known to enhance STAT3 transcriptional activity (25, 26). We therefore pretreated calvaria cells with either the MEK inhibitor, PD098059, or the JNK inhibitor, SP600125, before incubating the cells for 20 min with heparin, IL-11, or a combination of the two. The cells were then lysed, and the lysates were immunoprecipitated with an anti-STAT3 antibody before being immunoblotted for Ser-727 phosphorylation using an anti-STAT-phosphoserine 727 antibody. As seen in Fig. 3, incubation of murine calvaria cells with IL-11 resulted in a significant
Increase in serine 727 phosphorylation ($p < 0.01$). However, heparin alone had no effect on Ser-727 phosphorylation nor did it enhance Ser-727 phosphorylation in the presence of IL-11. In addition, pretreatment of the calvaria cells with either PD098059 (lanes 5 and 6) or IL-11 and heparin (lanes 4 and 6, respectively) did not affect IL-11-induced Ser-727 phosphorylation, either in the presence or absence of heparin. When taken together these results suggest that the ability of heparin to enhance STAT3 activation is independent of STAT3 Ser-727 phosphorylation.

**Effect of Heparin on Erk1 and Erk2 Activation**—Because the MEK inhibitor, PD098059, abolished the ability of heparin to promote both IL-11-induced STAT3-DNA complex formation and gp130 expression, we decided to determine directly if heparin was capable of activating Erk1 and Erk2. As shown in Fig. 4, heparin stimulated Erk1 and Erk2 activation ($2.8 \pm 0.5$-fold, $p < 0.05$) in both a dose (Fig. 4A) and time-dependent manner (Fig. 4B). Maximal stimulation of Erk was obtained when the calvaria cells were treated for 20 min with 25 $\mu$g/ml heparin and was sustained for at least 2 h before returning to control levels (data not shown). These findings demonstrate that heparin can directly up-regulate both Erk1 and Erk2 activation.

**Effect of Heparin and PD098059 on IL-11-induced RANKL Expression**—Because the expression of RANKL is a prerequisite of osteoclast formation/differentiation we next used real-time PCR to examine the ability of both heparin and PD098059 to affect IL-11-induced RANKL expression. As shown in Table 1, IL-11 induced RANKL expression by 39.1 $\pm 3.9$-fold ($p < 0.01$), whereas heparin alone had no effect. However, when heparin was combined with IL-11, the relative expression of RANKL was increased $2.7 \pm 0.7$-fold ($p < 0.05$) over that seen with IL-11 alone. In contrast, when the calvaria cells were first pre-treated with PD098059, heparin was unable to act synergistically with IL-11 and induce RANKL expression (Table 1). When taken together, these findings suggest that heparin increases IL-11-induced RANKL expression in an Erk-dependent manner.

**Effect of Heparin and PD098059 on IL-11-induced in Vitro Osteoclast Formation**—To determine if Erk1 and Erk2 activation by heparin was responsible for the ability of heparin to promote IL-11-induced osteoclast formation, we treated co-cultures of murine calvaria and bone marrow cells with IL-11 in

**TABLE 1**

| Conditions                        | Relative expression of RANKL mRNA |
|-----------------------------------|----------------------------------|
| Control                           | 1.0                              |
| Heparin                           | 1.0 $\pm 0.3$                    |
| IL-11                             | 39.1 $\pm 3.9^a$                 |
| IL-11 plus Heparin                 | 105.5 $\pm 26.0$                 |
| IL-11 plus PD098059               | 45.0 $\pm 13.3^a$                |
| IL-11 plus heparin plus PD098059  | 36.5 $\pm 7.5^a$                 |

$p < 0.01$ when compared with the expression of RANKL mRNA in control cells.

$p < 0.05$ when compared with the expression of RANKL mRNA in IL-11-treated cells.
the presence or absence of heparin and the MEK inhibitor, PD098059. Nine days later the cells were stained for TRAP activity, and the number of TRAP$^+$ multinucleated cells (MNCs) was determined. As shown in Fig. 5, the formation of TRAP$^+$ MNCs was stimulated when co-cultures of murine calvaria and bone marrow cells were treated with IL-11. Treatment with heparin alone had no effect. However, when co-cultures were treated with both heparin and IL-11, the ability of IL-11 to stimulate TRAP$^+$ MNC formation was enhanced $>2$-fold ($p < 0.01$). In contrast, heparin was unable to promote IL-11-induced TRAP$^+$ MNC formation when the co-cultures were treated in the presence of PD098059 (Fig. 5A) or when the murine calvaria cells were first transfected with Erk-specific siRNA (Fig. 5B). When taken together, these results suggest that the ability of heparin to synergistically enhance IL-11-induced osteoclast formation is dependent upon MEK activation.

**DISCUSSION**

Previously, we demonstrated that heparin acts synergistically with IL-11, a member of the IL-6 family of cytokines, to enhance both STAT3 activation and *in vitro* osteoclast formation (13). In the current study, we examine the mechanism by which this synergy occurs. Thus, by using various serine kinase inhibitors we demonstrate that the MEK inhibitor, PD098059, inhibits the ability of heparin to enhance both IL-11-induced STAT3 activation and *in vitro* osteoclast formation. In addition, we demonstrate that heparin can increase Erk1/2 activation but that its effect on IL-11-induced STAT3 activation is independent of STAT3 Ser-727 phosphorylation. When taken together, these findings suggest that heparin enhances IL-11-induced STAT3 activation, and thus osteoclast formation, by a mechanism that is independent of STAT3 Ser-727 phosphorylation, but which involves up-regulation of the MAPK pathway.

In a previous study, we demonstrated that heparin enhances both IL-11-induced STAT3-DNA complex formation and transactivation without altering either STAT3 tyrosine or serine 727 phosphorylation (13). In the current study, we confirm these findings and present evidence to support the hypothesis that heparin enhances IL-11-induced STAT3 activation by up-regulating the MAPK pathway. Thus, we demonstrate that heparin is able to increase Erk1 and Erk2 activation in both a time- and dose-dependent manner (Fig. 4). In addition, we show that the MEK inhibitor, PD098059, blocks the ability of heparin to enhance IL-11-induced STAT3 activation and as a consequence IL-11-induced gp130 expression (Figs. 1 and 2). Whether heparin is activating the MAPK pathway directly or
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Indirectly is unknown. However, heparin has been shown to increase the binding efficiency of a variety of cytokines and/or growth factors, some of which might activate the MAPK pathway upon binding to their receptor. For example, heparin has been shown to enhance basic fibroblast growth factor receptor binding and thereby suppress collagen synthesis in 21-day old fetal rat calvariae (29). In addition, heparin has been shown to interact with a variety of other growth factors, including insulin-like growth factor-binding proteins, heparin-binding EGF-like growth factors, and vascular endothelial growth factor (30–33).

STAT3-DNA binding and transactivation can be regulated in a number of ways. For example, to bind DNA, STAT3 must first dimerize through reciprocal phosphotyrosine-SH2 interactions, a process that requires phosphorylation of Tyr-705 (22–24). In addition, the transcriptional activity of STAT3 can be augmented by phosphorylation at Ser-727 (25, 26). How up-regulation of the MAPK pathway by heparin leads to enhanced IL-11-induced STAT3-DNA complex formation and transactivation is not known. Previous studies in our laboratory have shown that heparin does not affect either STAT3 Tyr-705 or Ser-727 phosphorylation, either in the absence or presence of IL-11. Furthermore, in the current study, we demonstrate that the MEK inhibitor PD98059, while capable of blocking the synergy between heparin and IL-11, has no effect on STAT3 Ser-727 phosphorylation. It is, however, possible that heparin is causing STAT3 phosphorylation at a serine residue other than Ser-727 and that this results in an enhancement of STAT3-DNA complex formation and transactivation. Indeed, there is evidence to suggest that the phosphorylation of STAT3 on serine residues other than Ser-727 might augment the transcriptional activity of STAT3 (34–36). Alternatively, it is possible that activation of the MAPK pathway by heparin can lead to an alteration in the ability of STAT3 to interact with any one of a variety of regulatory proteins. For example, it is possible that activation of the MAPK pathway could in some way lead to an attenuation of the ability of protein inhibitor of activated STAT3 (PIAS3) to bind and thus, inhibit STAT3 activity (37–40). In addition, it is also possible that activation of the MAPK pathway by heparin could either directly or indirectly affect STAT3 interactions with other co-activators of transcription such as P300 (41).

Our results demonstrate that the JNK inhibitor, SP600125, decreases both IL-11-induced STAT3-DNA complex formation and gp130 expression (Figs. 1 and 2). In contrast, the serine kinase inhibitors PD98059, wortmannin, and SB202190 were all found to have no effect on complex formation or gp130 expression in the presence of IL-11 alone. This suggests that maximum activation of STAT3 in the presence of IL-11 is in part dependent on JNK activation and that the MAPK, phos- phatidylinositol 3-kinase, or the p38 MAPK pathways have no effect on STAT3 activation. The mechanism by which JNK enhances STAT3 activation is unknown. Tenney et al. (42) recently reported that activation of the PI3-kinase pathway in 3T3-L1 adipocytes causes an increase in IL-11-induced STAT3 Ser-727 phosphorylation. However, we found that the ability of JNK to enhance STAT3 activation occurs independently of STAT3 Ser-727 phosphorylation (Fig. 3). Whether JNK is acting to phosphorylate other serine residues on STAT3 and/or other transcription factors, which could act cooperatively with STAT3, is unknown.

Previously, we demonstrated that the ability of IL-11 to induce osteoclast formation was enhanced in the presence of heparin even though heparin alone had no effect (13). In addition, we reported that heparin was able to enhance IL-11-induced osteoclast formation, because it acted synergistically with IL-11 to induce the expression of receptor activator of nuclear factor-κB ligand (RANKL) on the osteoblast cell surface. RANKL expression, in the presence of macrophage-colony-stimulating factor, has been shown to be a prerequisite of osteoclast formation both in *vitro* and *in vivo* (43–48). In the current study, we confirm the ability of heparin to synergistically enhance both IL-11-induced RANKL expression (Table 1) and IL-11-induced osteoclast formation (Fig. 5). In addition, we demonstrate that heparin not only enhances IL-11-induced RANKL expression but also IL-11-induced osteoclast formation through up-regulation of the MAPK pathway. Whether heparin can also act synergistically with other cytokines to induce osteoclast formation is unknown. However, we have found that heparin does not enhance either leukemia inhibitory factor, oncostatin M or IL-6 plus sIL-6R-induced osteoclast formation in co-cultures of murine calvaria and bone marrow cells.4

In summary, we have demonstrated that heparin enhances IL-11-induced STAT3 activation by a mechanism that is independent of STAT3 Ser-727 phosphorylation but involves up-regulation of the MAPK pathway. This provides a plausible mechanism by which heparin may synergize with IL-11 to increase osteoclast formation and helps to explain why heparin causes bone loss when administered long term.

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