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IL-12 Is a Heparin-Binding Cytokine

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Using an ELISA approach, we demonstrate that recombinant human IL-12 (rhIL-12) binds strongly to an immobilized heparin-BSA complex. This binding is completely displaceable with soluble heparin, IC50 ~ 0.1 µg/ml, corresponding to ~ 10 nM. By interpolation with our previous findings, this indicates an affinity for heparin greater than that of antithrombin III and comparable with that of FGF-2, two high-affinity heparin-binding proteins. Recombinant murine IL-12 also binds strongly to heparin. The binding of rhIL-12 to heparin shows specificity because chondroitin sulfates A and C fail to compete, whereas chondroitin B inhibits weakly. A highly sulfated heparan sulfate is a strong competitor, whereas other heparan sulfates show weak or no activity. Small heparin fragments inhibit binding, although activity decreases with size. An octasaccharide pool derived by cleavage of heparin with nitrous acid is a significantly stronger inhibitor than its heparinase I-derived counterpart, further indicating structural specificity in the interaction between rhIL-12 and heparin. The binding of recombinant p40 to heparin appears indistinguishable from that of the IL-12 heterodimer, implying that the heparin binding site is largely if not solely located in this subunit. These results show for the first time that IL-12 is a heparin-binding cytokine, a property common to the other Th1-response-inducing cytokines, IFN-γ and IL-2. Our findings strongly suggest that IL-12 will tend to be retained close to its sites of secretion in the tissues by binding to heparin-like glycosaminoglycans, thus favoring a paracrine role for IL-12. The Journal of Immunology, 1999, 162: 1064–1070.

Interleukin 12 is an immunostimulatory cytokine, molecular mass (Mn) 70 kDa, of unusual heterodimeric structure, being composed of p35 and p40 subunits (1, 2). It is secreted largely by macrophages and APCs and has important functions in the early stages of immune responses (reviewed in Ref. 3). IL-12 stimulates the production and secretion of several cytokines, in particular IFN-γ, by both resting and activated NK and T cells. Because IFN-γ in turn enhances IL-12 production, a positive feedback loop exists. IL-12 induces proliferation of NK and T cells, especially in the presence of costimulation through the B7-CD28 receptor-ligand pairing (4). It also enhances the cytotoxic activity within these cell populations (3). An important activity of IL-12, acting together with IFN-γ and IL-2, is to drive Th cell responses toward the Th1 rather than Th2 phenotype (3). Mice homozygous for a null mutation of the IL-12 p40 gene develop without hematological abnormalities, but show markedly reduced IFN-γ production on Ag challenge and substantially reduced delayed-type hypersensitivity responses (5). Studies of several animal models of infectious diseases have demonstrated a key role for the generation of resistance via Th1 responses to parasitic protozoa such as Leishmania major and Plasmodium cynomolgi (6–8). Furthermore, administration of rIL-12 has also been shown to generate potent antitumorigenic effects and is likely to take place at sites on the surface of the cytokine that are distinct from the binding sites for the IL-2R (rIL-2) binds to heparin with high affinity, Kd ~ 0.5 µM. This interaction is specific as chondroitin sulfates A, B, and C fail to compete, and among HS only a highly sulfated variant was found to be an effective competitor (13). The interaction of rIL-2 with heparin does not appear to either increase or decrease its biological activity in vitro, and is likely to take place at sites on the surface of the cytokine that are distinct from the binding sites for the IL-2R subunits (14). Overall, the binding of these cytokines to heparin is likely to retain them close to their sites of secretion, thus serving to maintain high local concentrations of these soluble mediators.

In the present study, we have employed our ELISA technique to demonstrate for the first time that rIL-12 is a cytokine with high affinity for heparin. We show that this interaction with glycosaminoglycan is selective as chondroitin sulfates A and C fail to compete.

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3Abbreviations used in this paper: HS, heparan sulfate; h, human; m, mouse; HSBI, HS from bovine intestinal mucosa; HSK, HS from bovine kidney.

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with the immobilized heparin complex. However, certain HS, particularly those with high sulfate densities, do interact with IL-12.

Materials and Methods

Reagents

Porcine intestinal mucosal heparin (sodium salt, grade I-A), chondroitin sulfate A (from bovine trachea), chondroitin sulfate B (dermatan sulfate, from bovine mucosa), chondroitin sulfate C (from shark cartilage), fuco-roid (from *Fucus vesiculosus*), and HS from bovine intestinal mucosa (HSBI) and from bovine kidney (HSK), were all purchased from Sigma-Aldrich (Poole, U.K.). Before use, heparin was exhaustively dialysed against initially 1 M NaCl and subsequently deionised water before freeze drying. Two HS isolated from porcine intestinal mucosa, HSA and HSE, were kindly provided by Dr. B. Mulloy (NIBSC, Hertfordshire, U.K.); HSBI and HSK have M, of 20 kDa and 8 kDa; sulfate to carboxylate ratios of 1 and 1.7; and, N-acetyl to carbohydrate ratios of 0.6 and 0.2, respectively (15). The clinical low m.w. heparins, Fragmin (KabiVitrum, Stockholm, Sweden), Fraxiparine (Sanofi Chimie, Notre Dame de Bondeville, France), and Tinzaparin (Logiparin, Novo Nordisk, Gentofte, Denmark) were obtained from their respective suppliers.

Human serum albumin (~99% purity) and p-nitrophenol phosphate (pNPP) tablets were purchased from Sigma-Aldrich. Nunc Maxisorp ELISA plates were obtained from Life Technologies (Paisley, Scotland).

**Abs and recombinant cytokines.**

rhIL-12 and and recombinant murine IL-12 (rmIL-12), as well as recombinant p40, were routinely purchased from R&D Systems (Abingdon, U.K.), although rhIL-12 was also obtained from PharMingen (San Diego, CA). Goat polyclonal Ab specific for either human or murine IL-12 were also obtained from R&D Systems. Murine anti-human IL-12 mAbs recognizing the human IL-12 p40 subunit were kindly donated by R. Carter (Imperial Cancer Research Fund, Leeds, U.K.) (16). Alkaline phosphate-coupled rabbit anti-goat and sheep anti-mouse IgG secondary Abs were purchased from Sigma-Aldrich.

**Heparin-binding ELISA**

A heparin-BSA complex in which heparin chains are covalently coupled via their reducing ends to the protein using sodium cyanoborohydride was synthesized as described previously (13), except that the coupling reaction mixture contained 680 mg of heparin together with 34 mg BSA. Mock-treated BSA was prepared by exposing the same concentration of BSA to sodium cyanoborohydride in the absence of heparin. In some experiments (data not shown), heparin-BSA complex (purchased from Sigma-Aldrich) was used in place of the above complex.

Heparin-binding ELISA were performed essentially as described previously (13) but with slight modification. Briefly, ELISA plate wells were coated with 100 μl 50 mM Tris-HCl buffer, pH 7.4, containing 12.7 mM EDTA and either 0.08 μg complex (measured by BSA content) or the same amount of mock-treated BSA. After washing three times with PBS containing 0.05% (v/v) Tween 20 and blocking with 2% (w/v) dried skim milk powder (Marvel, Premier Beverages, Adbaston, Staffordshire, U.K.), wells were incubated for 2 h with rhIL-12 diluted in PBS. Wells were then washed three times with PBS-Tween and anti-IL-12 polyclonal Ab or mAb was added at a dilution of 1/1000 in blocking buffer. Following three further washes in PBS-Tween, the corresponding alkaline phosphatase-coupled secondary Ab was added at a dilution of 1/1000 in blocking buffer for 30 min incubation. After five washes in PBS-Tween, 100 μl 0.2 M Tris buffer, pH 10, containing 1 mg/ml p-nitrophenol phosphate was added, and absorbances were read at 405 nm after 30- to 90-min incubation at room temperature. In some experiments, a competitive variant of the ELISA was used in which cytokine diluted in PBS was preincubated with soluble glycosaminoglycan for 5 min before the addition of 100 μl aliquots of this mixture to coated and blocked wells.

**Heparin-derived oligosaccharides**

Porcine intestinal mucosal heparin, 10 mg/ml, was dissolved in 250 mM sodium acetate buffer, pH 7.5, containing 2.5 mM calcium acetate. Heparinase I from *Flavobacterium heparinum* (Grampian Enzymes, Aberdeen, Scotland) was added at 0.07% (v/v), and the mixture was incubated at 30°C for 10 h. After boiling, the mixture was freeze dried and reconstituted in 2% (w/v) ammonium bicarbonate buffer, pH 7.6, before gel filtration on a 5 × 100-cm column of Biogel P6 equilibrated in the same buffer. The eluate was analyzed for the presence of glycosaminoglycan by a microtiter plate dimethylene blue binding assay, and the size and homogeneity of the oligosaccharide fractions was verified by PAGE, both methods being fully described elsewhere (17). Oligosaccharides were also obtained by cleavage of heparin with nitrous acid (18) as fully described elsewhere, with similar fractionation of the resulting oligosaccharide size pools.

**Results**

To investigate whether IL-12 might bind to heparin, we incubated rhIL-12 with an immobilized complex of heparin covalently coupled to BSA. As may be seen in Fig. 1A, dose-dependent binding to the heparin complex was observed. This binding is detected with goat polyclonal anti-IL-12, and absorbances were read after 30 min incubation with substrate. The curves shown are typical of six independent experiments. A, Binding of rhIL-12 was detected with goat polyclonal anti-IL-12, and absorbances were read after 30 min incubation with substrate. The curves are typical of two independent experiments, and absorbances were read after 1 h. C, Binding of rmIL-12 was detected with monoclonal Ab. The curves are typical of two independent experiments and were obtained after 30 min incubation. Throughout blank absorbance values, typically 0.1–0.15, obtained with wells containing only substrate have been subtracted from the values shown. Error bars indicate ± SEM of the quadruplicates, where this is larger than the symbol size.
dose-response curves were obtained with the alternative anti-human IL-12 mAbs, 1.3A3 and 2.1B3 (data not shown). These findings establish that the observed heparin binding is not an artifact of the Abs employed in the detection of bound IL-12. In some experiments, absorbance readings taken at early times of substrate incubation show that the binding curves were initially quasilinear, but with increasing times they become flattened at higher cytokine levels as the absorbances increase (Fig. 1). This indicates that exhaustion of one or more components in the detection system may be occurring. Thus, the shape of the curves cannot necessarily be taken as representing saturation of the IL-12 binding sites. rmIL-12 also shows very similar dose-dependent binding to the immobilized heparin complex, with a strong response being readily detectable with 1 ng of cytokine (Fig. 1C). The large absorbances obtained in these various binding curves are in marked contrast to similar binding experiments performed with rhIL-1α, rhIL-1β, and recombinant human granulocyte-macrophage CSF, in which no or negligible binding responses were obtained (data not shown). When the heparin-BSA complex synthesized in this laboratory was substituted with the same amount of a commercially obtained counterpart, an essentially identical binding curve for rhIL-12 was obtained (data not shown).

To confirm that interaction with the heparin chains in the immobilized complex is responsible for the observed binding responses, we performed competitive assays in which rhIL-12 was mixed with soluble heparin before addition to the immobilized complex. As may be seen in Fig. 2A, the strong binding of rhIL-12 to the heparin complex is completely inhibited by <10 μg/ml heparin, with an IC₅₀ of ~0.1 μg/ml. As shown in Fig. 2B, an essentially identical displacement curve is obtained with mIL-12, giving a similar IC₅₀ value. The potent competition obtained with soluble heparin demonstrates that the binding of both human and murine IL-12 to the heparin-BSA complex is due to interaction with the immobilized heparin chains.

To examine the specificity of the interaction between hIL-12 and heparin, we employed various different glycosaminoglycans as soluble competitors. As may be seen in Fig. 3A, in addition to heparin, fucoidan, a highly sulfated fucose polymer isolated from a marine algae, is an effective inhibitor of the binding of rhIL-12 to the immobilized heparin complex. By contrast, neither chondroitin sulfate A, nor chondroitin sulfate C gives significant inhibition of binding (p = 0.05, by ANOVA with posthoc analysis on...
data from three independent experiments). In contrast, chondroitin sulfate B (dermatan sulfate) and HSBI do cause significant inhibition ($p = 0.05$). However, these latter two glycosaminoglycans are weak inhibitors, as under the conditions employed they give inhibition in the range 10–20% compared with the substantial inhibitions observed with heparin and fucoidan.

The specificity of rhIL-12 interactions with heparin was further examined using a range of HS from different sources. As shown in Fig. 3B, HSBI and HSK both significantly inhibit rhIL-12 binding ($p = 0.05$, by ANOVA of data from three independent experiments), although in each case the inhibition obtained is less than that with the same concentration of heparin. By contrast, HSA from porcine intestinal mucosa gives no significant inhibition ($p = 0.05$), whereas HSE, a more highly sulfated preparation from the same source, is a relatively potent inhibitor of binding.

The inhibitory activity of HSBI and HSE were investigated further by preincubating a fixed concentration of cytokine with increasing concentrations of these soluble glycosaminoglycans. As may be seen in Fig. 4, HSBI is confirmed as a weak inhibitor. At concentrations between 2 and 10 $\mu$g/ml, some 20% inhibition of binding is observed with no clearly apparent concentration-dependent increase. The same concentration range of heparin gives >95% inhibition of rhIL-12 binding. The highly sulfated HS, HSE, which in Fig. 3A gave 50% inhibition, is seen in Fig. 4 to give 75% inhibition. Indeed, some quantitative variation in the inhibition obtained with a particular glycosaminoglycan was found between individual experiments. However, as may be seen in Fig. 4, this level of inhibition is not concentration-dependent as the titration curve attains a plateau between 2 and 10 $\mu$g/ml. Thus, these partial inhibitors appear to be unable to provide complete inhibition of IL-12 binding to the immobilized complex even when added at concentrations 10-fold higher than 1 $\mu$g/ml, at which >95% inhibition is attained with soluble heparin (Figs. 2 and 4). One implication of this is that the efficacy of partial inhibitors cannot be compared by using $IC_{50}$ values because some inhibitors, for example HSBI, do not routinely achieve 50% inhibition.

We next sought to determine the size requirements for binding to rhIL-12. To this end we first employed three different clinical low m.w. heparins, Fragmin, Fraxiparine, and Tinzaparin. These typically have number average $M_r$ in the range of 3–5.5 kDa, corresponding to ~10–17 hexose residues in length. When used as soluble competitors at 2 $\mu$g/ml, these three preparations gave inhibitions of binding between 75 and 85%, thus showing little or no diminution from the 85% inhibition obtained with unfractonated, normal heparin, ~16 kDa (data not shown). Therefore, to examine the inhibitory activity in even smaller heparin fragments, we investigated oligosaccharides generated from heparin by digestion with heparinase I. As may be seen in Fig. 5A, under the conditions employed tetradecasaccharides give ~90% inhibition, a value which approaches that obtained with unfractionated heparin in the same experiment. With each stepwise disaccharide decrease in size there is a progressive loss of inhibition such that decasaccharides give ~50% inhibition, whereas with tetrasaccharides inhibition is essentially undetectable. A similar study using heparin-derived oligosaccharides obtained by depolymerisation with nitrous acid also showed that small fragments compete for the binding of rhIL-12. As may be seen in Fig. 5B, the tetradecasaccharides obtained in this way, like those produced with heparinase I, give almost complete inhibition of binding under the conditions employed. Again, with decreasing oligosaccharide size there is a stepwise decrease in activity, with the tetrasaccharides and hexasaccharides being weak inhibitors. However, with the nitrous acid fragments, the decrease in activity between the dodecasaccharides and the octasaccharides is less marked compared with that seen with the heparinase I products. Thus, the nitrous acid-derived octasaccharides give rise to >50% inhibition of binding, whereas its enzymically produced counterpart does not. The greater activity of the nitrous acid-cleaved fragment was examined by titration of the inhibitory activity of both octasaccharides over the range 0–10 $\mu$g/ml. With the
Discussion

We establish here that rhIL-12 and mIL-12 bind strongly to heparin. This binding is specific in that no interaction with two other sulfated glycosaminoglycans, chondroitin sulfates A and C, is detectable. However, chondroitin sulfate B, dermatan sulfate, is found to be a significant, albeit weak, competitor of the interaction of IL-12 with heparin. Unlike the other chondroitin sulfates, the B variant contains iduronate residues formed by epimerization at the carbon-5 position of glucuronate residues. This postincorporation modification, which also occurs in heparin and HS biosynthesis, confers conformational flexibility on the glycosaminoglycan chain and is thus likely to facilitate interactions with proteins (19). We demonstrate here that IL-12 binding also extends to HS, a class of glycosaminoglycan with close structural relationship to heparin. However, the range of HS examined shows varying abilities to compete with heparin for IL-12. Thus, HSK is a weak competitor compared with HSBI. Furthermore, of the two fractions of HS isolated from porcine intestinal mucosa, the more highly sulfated preparation, HSE, is a strong competitor, whereas the less elaborated fraction, HSA, gives no significant inhibition. However, none of the HS examined compete as strongly as heparin, which has a higher ratio of iduronate:glucuronate residues and is more intensely sulfated than HS. Nonetheless, our data shows that at least some HS have appreciable affinity for IL-12, and, given the wide tissue availability of HS in biological tissues (20), this indicates that IL-12 could be retained by at least some of the HS chains distributed in extracellular matrices and on cell surfaces. In our previous study, using the same experimental approach to investigate the interaction of rhIL-2 with heparin, HSBI as well as HSA and HSE showed no or negligible competition for heparin binding. Thus, rhIL-12 appears to be less selective in that it interacts with a wider range of HS. Fucoidan, a sulfated polysaccharide of non-mammalian origin, is shown here to be a strong competitor. It is generally the case that heparin-binding proteins also interact with fucoidan due to its high sulfate density and branched, comb-like structure, which contrasts with the linear structure of mammalian glycosaminoglycans (21).

Our study shows that relatively small heparin-derived oligosaccharides compete for binding to the intact heparin chains. Substantial inhibition of binding, exceeding 50% in the case of nitrous acid-derived oligosaccharides, is obtained with fragments as small as octamers. Given the considerable sequence heterogeneity of heparin chains, each oligosaccharide size pool will contain a large range of different sequences, the diversity of which will increase with size. The two heparin cleavage methods employed here have different linkage specificities, with heparinase 1 selectively cleaving between N-sulfated glucosamines and 2-O-sulfated iduronates (22), whereas nitrous acid is less selective. Thus the generation of less active octasaccharides by the enzymic route compared with nitrous acid suggests a role for N-sulfated glucosamine-2-O-sulfated iduronate disaccharides in the binding of IL-12. Such disaccharides have already been implicated in FGF-2 binding (23–25). This disaccharide is relatively abundant in heparin, but less common in HS, particularly where this has a low overall sulfate content (26). Thus, our finding that the competitive activity is in the order heparin > high sulfated HS > low sulfated HS is entirely consistent with a role for sequences containing this disaccharide.

The best characterized heparin-protein interaction, the high-affinity binding of heparin to antithrombin III, involves a defined pentasaccharide sequence (27). The minimal sequence for the cytokine FGF-2 binding is less certain as different studies have implicated oligosaccharides varying from hexasaccharide (23, 24) to
decasaccharide (25). However, in the case of IFN-γ, the HS-binding sequence appears to be 10 kDa, corresponding to over 40 hexose residues. This large fragment is envisaged as spanning across the two identical subunits in the homodimeric IFN-γ molecule, interacting with basic residues at the two C termini (12). Similarly, the tetrameric chemokine, platelet factor 4, is bound by a 42-residue HS fragment that is envisaged to wrap around the molecule interacting with all four polypeptide subunits (28). Taken overall, our observation that comparatively small oligosaccharides are effective competitors for heparin binding suggest that only a single binding site is involved in IL-12 binding. Advancement of this hypothesis requires further study, including definition of the minimal binding sequence.

We show here that recombinant p40 subunits possess heparin-binding properties indistinguishable from those of the heterodimeric IL-12 molecule. Lack of availability of appropriate recombinant reagents prevents examination in the same way of the possible interaction of p35 with heparin. However, our current data indicates that the heparin binding of IL-12 is at least largely localized in the larger subunit. Studies of human/mouse hybrid combinations of the p35 and p40 subunits implicate p35 as largely responsible for receptor interactions (5). This finding reflects the fact that this smaller subunit displays a low homology with IL-6 and granulocyte CSF (29), whereas p40 has extensive albeit weak homology with the receptors for IL-6 and ciliary neurotrophic factor (5, 30). It is also significant that IL-12 secretion appears to be associated with excess p40 secretion and that the excess p40 acting either singly or as homodimers may function as a receptor antagonist of heterodimeric IL-12 (3). Thus, our present observations suggest heparin binds to the relatively accessory subunit rather than the smaller subunit, which appears to be the more important in signal transduction.

Given the strongly acidic nature of heparin and HS, the basic amino acids arginine and lysine are major determinants of their binding sites on polypeptides. It is quite possible for secondary structural folding of a polypeptide to bring into proximity basic residues that in the primary sequence are well separated from each other, so as to constitute discontinuous heparin binding sites (31). However, it is notable that human p40 possesses near its C terminus a cluster of six basic residues within a nine-amino acid sequence, as may be seen in Fig. 7. This unusually dense basic sequence is a prime candidate for a heparin binding site. We have found that murine IL-12 also binds heparin, and interestingly although the C-terminal region of murine p40 has little overall homology with that of its human counterpart, it too has a sequence cluster of six basic residues (Fig. 7). These clusters are comparable to the C-terminal proximal clusters of 4–5 basic residues that give rise to the heparin-binding properties of IFN-γ (11), IL-8 (33), and other CXC chemokine family members (34).

IL-12 appears to bind to heparin with a particularly high affinity. In our previous studies of the heparin-binding properties of anti-

References

thrombin III, FGF-2, and IL-2 using the same ELISA technique, we found that soluble heparin competed with IC50 values of 1.0, 0.15, and 5 µg/ml, respectively (13). For antithrombin III and FGF-2, these corresponded well with literature values for the estimated Kd of heparin binding for these two proteins, 50 mM and 12 mM, respectively (35, 36). The IC50, 0.1 µg/ml, observed here for both human and murine IL-12 indicate that it has an affinity for heparin at least comparable with that of FGF-2, and an order of magnitude greater than that of antithrombin III.

Therefore, we establish here that IL-12 binds with high affinity to heparin/HS glycosaminoglycan. This interaction is likely to retain IL-12 close to its tissue sites of secretion, establishing local high concentrations. Heparin is a constituent of the cytoplasmic granules of mast cells, which as a result of mast cell degranulation at tissues sites of inflammation will become a component of the extracellular milieu (37). IL-12, in cooperation with IFN-γ and IL-2, has a major role in establishing immune responses of a Th1 type. Therefore, it is of considerable interest that both IFN-γ and IL-2 have previously been shown to bind to the same glycosaminoglycans (11–14). This, IL-2 activation of the cell-mediated immunity may well be exerted within local tissue compartments by the combined activity of these heparin-retained cytokines. It has recently been shown that splenic dendritic cells respond rapidly to microbial challenge by secreting large amounts of IL-12 and migrating into T cell areas of the white pulp (38). The resulting close proximity of these dendritic cells to the T cell population they stimulate clearly exemplifies the short distances over which IL-12 functions physiologically. Moreover, systemically administered rIL-12 is associated with several toxic side effects (39). If endogenously secreted IL-12 is indeed localized in tissue compartments by retention on heparin/HS glycosaminoglycan, this would explain why the systemic route of administration is inappropriate.

FIGURE 7. Sequences of the prominent cluster of basic amino acids in the p40 subunit of human and murine IL-12. All residues are represented by the one letter code, with the basic amino acids, arginine and lysine, underlined. The sequences are taken from Refs. 1 and 32, with numbering according to the predicted secreted polypeptide.


