Heparin Decreases the Blood Clearance of Interferon-γ and Increases Its Activity by Limiting the Processing of Its Carboxyl-terminal Sequence*

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Interferon-γ (IFN-γ) binds with high affinity to heparan sulfate and heparin molecules through its carboxyl-terminal domain. In vivo, IFN-γ is eliminated from the bloodstream with a half-life (t½) of 1.1 min, due to binding to heparan sulfate. Unbound IFN-γ is cleaved rapidly at the carboxyl-terminal side, a process that removes at least 18 amino acids and inactivates the cytokine. When bound to heparin, the plasma clearance of IFN-γ is decreased greatly (t½ = 99 min), and the area under the curve obtained with IFN-γ alone represented only 15% of that obtained with injected IFN-γ bound to heparin. Furthermore, the binding of heparin to IFN-γ limits the extent of its carboxyl-terminal domain degradation to less than 10 amino acids. Importantly, this process increases the cytokine activity by as much as 600%. These data demonstrate that the blood clearance of the cytokine is a non-receptor-mediated process and that in vivo the local concentration of heparan sulfate/heparin-like molecules regulates IFN-γ activity by a unique mechanism involving a controlled processing of its carboxyl-terminal sequence.

Cytokines have pleiotropic and overlapping activities and mediate cell to cell communication (1). Many cytokines, like their signaling receptors, are distributed widely (2) and are effective at very low concentrations (nano- to picomolar range). Therefore, specific stimulation of a defined cell population by a cytokine for a limited time period requires a regulatory system (3). For example, latent cytokines can be processed extracellularly either to be inactivated or to yield a mature form. Extracellular matrix has been shown to sequester cytokines and thus to provide a means for continued and/or localized stimulation of cells (4, 5). The concept that cytokines and growth factor activity is governed not only by their binding to specific cell surface receptors, but also by other cell surface or extracellular matrix components is now generally recognized.

For example, IFN-γ, a T-cell secreted cytokine that displays pleiotropic activities (6, 7), binds to extracellular matrix and cell surface heparan sulfate proteoglycan (8, 9), as do a number of other cytokines, growth factors, and chemokines (10–12). Therefore extracellular matrix has been thought to provide a local concentration of IFN-γ in nearby cells. Proteolytic cleavage of IFN-γ also represents a possible form of regulation of the cytokine activity. In particular, the carboxyl-terminal part of IFN-γ, the integrity of which is critical for biological activity, is highly susceptible to proteolytic cleavages, and it has been postulated that this domain could be a regulatory element of the cytokine (13). Using IFN-γ with a defined number of missing carboxyl-terminal amino acids, it has been found that if a limited number of amino acids (less than 10) is removed, the activity is enhanced, whereas the removal of 14 or 18 amino acids results in a loss of biological activity (14). These studies suggested a central role for the basic sequence KRKR (amino acids 128–131), found in the carboxyl terminus of IFN-γ, since the beginning of its removal correlates with the reduction in activity.

Previous studies have demonstrated that IFN-γ specifically interacts with specific sequences of heparan sulfate containing heparin-like domains (15). Interestingly, the amino acids involved in this binding are located in the carboxyl-terminal domain of the cytokine, within a consensus sequence for heparin recognition and mainly involve the KRKR sequence (16). Given the data accumulated on the importance of the KRKR domain for IFN-γ activity, we have postulated that heparan sulfate or heparin may participate in regulating the cytokine activity. To discriminate between the IFN-γ receptor and the heparan sulfate molecules, both of which are high affinity binding sites, we injected into rats the human cytokine, which does not bind to the murine receptor (7) but binds to the murine heparan sulfate (8). Based on previous studies (9, 15) showing that heparin and heparan sulfate compete with one another to bind to IFN-γ, we also injected IFN-γ bound to heparin to block the heparan sulfate binding site of the cytokine. We report here that in vivo, the binding of IFN-γ to heparin-like/heparan sulfate molecules is not only involved in the plasma clearance of the cytokine, but also limits the extent of the carboxyl-terminal degradation, which in turn enhances the cytokine activity. In contrast, unbound IFN-γ is inactivated rapidly by more extensive cleavages of its carboxyl-terminal domain.

MATERIALS AND METHODS

Animal Preparations, Injections, and Blood Sampling—Adult male Sprague-Dawley rats weighing 500 g were obtained from IFFA-Credo France. The carotid was exposed on one side in anesthetized animals and blood samples were withdrawn through the cannula at the indicated times and mixed with sodium citrate (3.8%). Plasma was separated immediately by centrifugation and stored frozen (−80 °C) until analysis. Recombinant human IFN-γ (2.107 units/mg of protein) was radiodinated by the chloramine-T method (17) to a specific radioactivity of 6.5 × 10⁶ cpm/μg. This material was 90% trichloroacetic acid-precipitable and was fully active (data not shown). The rats were injected intravenously with 20 μg of IFN-γ containing 4 × 10⁹ cpm as a tracer, alone or in combination with heparin (2,000 units/kg of body weight). The heparin used had a molecular mass of 15 kDa on average. Blood samples (200 μl) were withdrawn at the indicated times, and the trichloroacetic acid-precipitable radioactivity associated with the plasma was determined.

Pharmacokinetic Analysis—Plasma concentration-time data were
Heparin Regulates IFN-γ Activity and Blood Clearance

**RESULTS**

The Heparan Sulfate Binding Site of IFN-γ Is Involved in the Blood Clearance of the Cytokine—To investigate whether the fate of IFN-γ in vivo depends on possible interactions with heparan sulfate, we injected intravenously the labeled cytokine alone or in combination with heparin (to block the heparan sulfate binding site) and measured the amount of circulating 125I-IFN-γ. Blood samples were withdrawn at various times after the injection and the plasma trichloroacetic acid-precipitable radioactivity was counted (Fig. 1). More than 85% of the radioactivity was shown to be trichloroacetic acid-precipitable in all samples. Injected alone, 125I-IFN-γ was eliminated from the bloodstream by a biexponential process in which almost 90% of the cytokine disappeared within the first 5–10 min with an elimination half-life (t1/2) of 1.1 ± 0.05 min. The remaining 10% (the slow component of the biexponential curve) was eliminated from the bloodstream much more slowly, with a t1/2 of 94 ± 15 min. Coinjected with heparin, the 125I-IFN-γ plasma concentration-time profile was quite different. The data fitted now to a monoeponential curve, characterized by an elimination half-life of 99 ± 13 min. In addition, heparin injected 8 min after the administration of IFN-γ (Fig. 1, arrow) immediately released some of the previously bound cytokine into the bloodstream (Fig. 1, dashed line). Once released into the bloodstream by the heparin injection, 125I-IFN-γ was eliminated in a monoeponential manner with a t1/2 of 90 ± 6.3 min. Together these data suggest that the heparan sulfate binding site of IFN-γ was involved in the clearance of the cytokine. Interestingly, the elimination half-life of 125I-IFN-γ, whose heparan sulfate binding site had been blocked by heparin (90–99 min), was very similar to that of the slow component elimination half-life of IFN-γ alone (94 min). This might suggest that the cytokine that remained in circulation after the injection of 125I-IFN-γ alone (open square from 10 to 120 min; Fig. 1) had bound to circulating endogenous heparin-like molecules or had lost the ability to bind to heparan sulfate (see below).

Pharmacokinetic Analysis of the Plasma Concentration-Time Data—To demonstrate further that IFN-γ injected alone or bound to heparin behaved differently, several pharmacokinetic parameters were determined (Table I). Assuming that the blood volume represents 8% of the body weight (21) and therefore should be 40 ml, 10^6 cpm/ml was expected at the time of injection, in close agreement with the experimental data and the applied model. Furthermore, the blood volumes (Vb) calculated from the exponential equation coefficients were also in the same range (40–43 ml; Table I). More interestingly the volume of distribution at steady state was calculated to be 262 ml/kg. To analyze better this parameter, the cytokine was labeled with 125I before or bound to heparin. This was done in order to increase the number of binding sites available for the cytokine. The results obtained are summarized in Table I. As expected, the value of the blood volume (Vb) was increased when the cytokine was injected with heparin, and the value of the area under curve (AUC0–l) was also increased.

**Table I**

| Parameter | IFN-γ alone | IFN-γ/heparin |
|-----------|-------------|---------------|
| Vb (ml)   | 40          | 43            |
| Vdss (ml) | 262         | 43.5          |
| Clp (ml/min) | 2.1        | 0.32          |
| AUC0–l (cpm.min.ml⁻¹) | 1.93 × 10⁶ | 1.25 × 10⁷ |
| t1/2 (min) | 1.1         | 99            |
| t2/3 (min) | 94          | NA            |

*Not available when data are described by a monoeponential equation.*
Together these data suggest that a large proportion (90%) of the injected IFN-γ was sequestered within a few min by heparan sulfate/heparin-like molecules, whereas bound to heparin, the cytokine remained in the bloodstream from which it was eliminated slowly. We could exclude the possibility that the IFN-γ receptor participated in these interactions because in this case we used the human cytokine, which does not bind to the rat receptor (7).

In Vivo, Heparin Increases the Specific Activity of IFN-γ—To determine whether the binding to heparin also influences the cytokine function, another set of experiments was conducted in which the antiviral activity of IFN-γ was measured in blood samples 2, 10, 60, and 120 min following the injection. When IFN-γ was injected alone, 4,220, 860, 365, and 190 units/ml were measured at 2, 10, 60, and 120 min postinjection, respectively (Fig. 3a). This represents a 22-fold decrease in activity during that period of time. Since there was only a 5-fold decrease in the circulating amount of IFN-γ during that same time, this indicates that the cytokine was progressively inactivated in the bloodstream, as demonstrated by the shift in its specific activity from 28.5 units/ng at 2 min to 6.7 units/ng at 120 min postinjection (Fig. 3b). When IFN-γ bound to heparin was injected, as much as 60,300 units/ml were detected 2 min postinjection. The activity decreased with time to 9,200 units/ml after 2 h (Fig. 3a). Since the blood volume was approximately 40 ml, no more than 10,000 units/ml could have been expected in the blood samples, and therefore the cytokine activity has been increased. This is shown in Fig. 3b (hatched bars), where the specific activity of IFN-γ drops from 20 units/ng before the injection to 125 units/ng at 2 min after the injection. The specific activity then decreased with time, but after 2 h the cytokine was still twice as active as the parent molecules. Considering the increase in specific activity and the increase in the amount of circulating IFN-γ due to heparin, there is a total increase in activity in the blood samples of 14-fold at 2 min, which goes up to approximately 50-fold at 10, 60, and 120 min postinjection. To investigate whether the sequestered IFN-γ was also activated, the antiviral activity released into the bloodstream by the heparin injection was measured. We found that, consistent with the release in the bloodstream of IFN-γ (see Fig. 1), the heparin injection also released biological activity (compare Fig. 3a, IFN-γ and IFN-γ+Hp). More importantly we found that after being released into the circulation, the cytokine was also twice as active as the

In Vivo, Heparin Regulates the Extent of the Carboxyl-terminal Degradation of IFN-γ—To investigate whether the modulation of the cytokine activity observed in vivo might be due to a processing of the carboxyl-terminal domain, we set up a competitive radioimmunoassay using two monodonal antibodies (293-4-45 and 13-16-2, described elsewhere; Ref. 22) which define two adjacent domains (D1 and D2) of the carboxyl-terminal part of the cytokine, located within the heparin binding domain (D1; antibody 293-4-45, amino acids 125–134) and downstream of the heparin binding domain (D2; antibody 13-16-2, amino acids 132–138). Antibody 293-4-45 competes with heparin to bind to IFN-γ, but antibody 13-16-2 does not (data
The model depicts the structure of the IFN-γ (units/kg of body weight) used to obtain the results described. After which the two epitopes were no longer detectable in the bloodstream (2h; Fig. 4, panel a). IFN-γ was processed rapidly and lost the D1 (dotted bar) and D2 (closed bar) carboxyl-terminal domains, indicating that it was shortened by cleavages upstream of the heparin binding site. In contrast, co-injected with heparin (panel b), the D1 domain was preserved although the D2 domain was removed within a few min. nd, not detected. The model depicts the structure of the IFN-γ carboxyl-terminal terminus. The two boxes represent the two clusters of basic amino acids, which are highly susceptible to proteolytic cleavages (14), the first one (125–131) being the heparin/heparan sulfate binding domain (16). The arrows indicate some of the possible enzymatic cleavage sites (14). The D1 and D2 domains defined by the two monoclonal antibodies that we used are also indicated. In the absence of heparin, the IFN-γ carboxyl-terminal part is cleaved readily (loss of D1 and D2); however, bound to heparin (HP), D1 is protected, and the cytokine is only shortened downstream of the heparin binding site (loss of D2 only).

DISCUSSION

We reported previously that human IFN-γ displays a high affinity for heparan sulfate (8). Considering the ubiquitous distribution of this molecule in vivo (23), we hypothesized that the biological functions of this cytokine might depend on such an interaction. Heparin, a molecule closely related to heparan sulfate, has been used to block the heparan sulfate binding site of IFN-γ (8, 9). The relatively high level of heparin (2,000 units/kg of body weight) used to obtain the results described has already been observed for other heparin-binding proteins, including hepatocyte growth factor, for which up to 10,000 units of heparin/kg of body weight have been used to lower the plasma clearance of the protein effectively (24). This is presumably due to the extremely high number of heparan sulfate molecules (25) present on most of the cell surface (up to 10^6/cell) or in the extracellular matrix. We found in the present study that in vivo there was a very rapid uptake of IFN-γ by heparin-like molecules. This uptake was almost quantitative since 90% of the injected IFN-γ was cleared from the bloodstream with a t_1/2 of 1.1 min (Fig. 1). Furthermore, the remaining 10% was eliminated slowly (t_1/2 = 94 min) because the heparan sulfate binding site had been removed (Fig. 4a), and the cytokine was no longer able to bind to heparan sulfate. This is also in line with the fact that the t_1/2 of IFN-γ from which the heparan sulfate binding site was blocked was in the same order of magnitude (90–99 min). The elimination half-life of biologically active IFN-γ has been reported for humans in other studies. It was shown that the t_1/2 depends on the amount of drug administered and varied from 2.6 to 31 min as the injected dose changed from 1.2 × 10^6 to 54 × 10^6 units (26). The differences in the plasma concentration time profiles following the injections of IFN-γ either alone or bound to heparin described here are also illustrated by the 6.5 times increase in AUC_0–∞ due to heparin (Table I). The fact that the volume of distribution at steady state of injected IFN-γ bound to heparin was similar to the blood volume indicates that the cytokine essentially remained in the bloodstream. In contrast, the volume of distribution at steady state of IFN-γ injected alone was 262 ml, indicating that in that case the cytokine was distributed in other tissues, a process that involved the heparan sulfate binding site of the cytokine. It is possible that part of this absorbed IFN-γ was immobilized by the heparan sulfate proteoglycans of the vascular endothelial cell surface rather than eliminated from the blood compartment, as suggested by the immediate release of biologically active cytokine following the heparin injection (Figs. 1 and 3a). This release of material by heparin injection has been already observed for other heparin-binding proteins such as tissue factor pathway inhibitor (27). The tissue distribution of IFN-γ has been investigated by the autoradiographic analysis of various tissues sections, and we found that local concentrations of heparin-like molecules may regulate the local accumulation of the cytokine (28). In addition to vascular structures, we found IFN-γ associated with kidney glomerulus following injection of the cytokine alone. The distribution of IFN-γ in this tissue was affected when it was co-injected with heparin. In particular, high levels of IFN-γ were found in the proximal tubules (28). This observation can rule out the possibility that the IFN-γ-heparin complex (50 kDa on average) was too large to cross the glomerular capillary wall and therefore that the observed changes in elimination were only due to size.

We also found in the present study that the IFN-γ-heparin interaction modulates the extent of the cytokine carboxyl-terminal cleavages, which in turn increase or decrease its activity. Using different enzymes in vitro, we verified that cleavages of the carboxyl-terminal sequence of IFN-γ downstream of the heparin/heparan sulfate binding site enhance the specific activity (up to 10-fold), whereas cleavages upstream of the heparin/heparan sulfate binding site strongly decrease the activity (data not shown). These data have been reported by others, and several enzymes that might act on the carboxyl-terminal part of IFN-γ and therefore modulate its activity have been suggested (14, 29, 30). In particular, the two basic domains of the cytokine, one of which is found within the consensus sequence for heparin recognition (16), are potential substrates for a
variety of serine proteases (14). This is in line with our present observation, showing that in vivo, heparin protects IFN-γ from cleavages within the KRKR domain, but not when it is downstream of this sequence. The observation that the natural cytokine is variously processed at the carboxyl terminus (6, 31) and lacks 9–16 amino acids reinforces the idea that the activity of endogenous IFN-γ might also be regulated by heparan sulfate present in the extracellular matrix or at the cell surface, by mechanisms similar to those described here.

These data help demonstrate that cell surface or extracellular matrix heparan sulfate is potentially able to modulate a variety of processes by affecting the compartmentalization, conformation, and reactivity of their ligands. It has been well established that heparan sulfate/heparin can modulate the activity of cytokines such as IFN-γ and lacks 9–16 amino acids reinforces the idea that the activity of endogenous IFN-γ might also be regulated by heparan sulfate present in the extracellular matrix or at the cell surface, by mechanisms similar to those described here.

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