Functional aspects of the interaction between interleukin-8 and sulfated glycosaminoglycans

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During the immune response, the cytokine interleukin 8 (IL-8, CXCL8) functions as a strong chemoattractant for polymorphonuclear leukocytes helping to direct these cells to infected/injured sites. This review focuses on the interaction of IL-8 with sulfated glycosaminoglycans expressed on cell surfaces and the extracellular matrix. This interaction contributes to the recruitment of polymorphonuclear cells from blood, penetration of these cells through the vessel wall, and their directed migration to inflammatory sites. Regulatory aspects of the interplay between IL-8 and heparan sulfate, the most abundant glycosaminoglycan, are highlighted. In this field, the large natural heterogeneity of glycosaminoglycans represents a great challenge that impedes the modeling of IL-8 functions. The interaction of IL-8 with newly developed artificial sulfated hyaluronan derivatives is also considered as these artificial substrates are an important tool for development of new materials in regenerative medicine.

Introduction

Chemokines are small proteins participating as important players of the immune system in recruitment of leukocytes, cell communication and activation. In humans, more than 40 different chemokines are known for their specific functions in innate and acquired immunity. They are divided into four main classes (C, CC, CXC and CX$_3$C chemokines) according to position and spacing of cysteine(s) near the N-terminus. The cytokine interleukin-8 (IL-8, also called CXCL8 according to the chemokine nomenclature) released from fibroblasts, monocytes/macrophages, endothelial and epithelial cells at inflammatory sites is a strong chemoattractant agent mainly toward polymorphonuclear leukocytes (PMNs).[1] These cells are rapidly recruited to infected and/or injured tissue and represent the first line of immune response in our body. In addition to IL-8, the infiltration

of PMNs to inflammatory sites is also highly regulated and supported by adhesion molecules, other cytokines, and extracellular matrix components in both blood vessel wall and adjacent tissue.[2,3] Polymorphonuclear leukocytes contribute to pathogen defense, regulation of the inflammatory process and to tissue injury by releasing special proteins and generating reactive metabolites. The interplay between apoptotic and necrotic PMNs as well as macrophages at infected/injured sites mainly determines the outcome of inflammatory response.[4-9] However, fine mechanisms important to switch from a pro- to an anti-inflammatory state including the termination of IL-8 responses are only scarcely known and remain to be further evaluated.

Here we briefly review the physiological functions of IL-8 in the stepwise recruitment of PMNs with special consideration of the interaction of IL-8 with polysaccharides of the extracellular matrix. The binding of IL-8 to sulfated glycosaminoglycans (GAGs) is a crucial step for the regulation of cell adhesion, migration and inflammation. Due to the great structural diversity of GAGs, a number of important questions concerning the matrix interaction of IL-8 remain unsolved. Answers to these questions will help to better understand regulation of inflammation, but also to develop new therapeutic strategies to treat conditions of dysregulated innate immune functions.

Structure of IL-8 and Isoforms

The three-dimensional structure of IL-8, composed of 72 amino acids, is well described.[10] The monomer consists of a conformational flexible N-terminus, which passes through a loop region and a three stranded antiparallel β-sheet, arranged in Greek key manner, into a C-terminal α-helix (residues 57–72).[11-14] Two disulfide bridges are necessary for the anchorage of the N-terminus to the protein core and for the maintenance of the tertiary structure.[12,15] Important structural details of human IL-8 are given in **Figure 1**.

The short, more disordered N-terminus contains cleavage sites for proteolytic enzymes. After secretion as a monomeric protein, IL-8 is processed at the N-terminal end by extracellular proteinases to yield several active forms. The most prominent forms consist of 77 or 72 amino acid residues.[16] Proteinase 3, thrombin,

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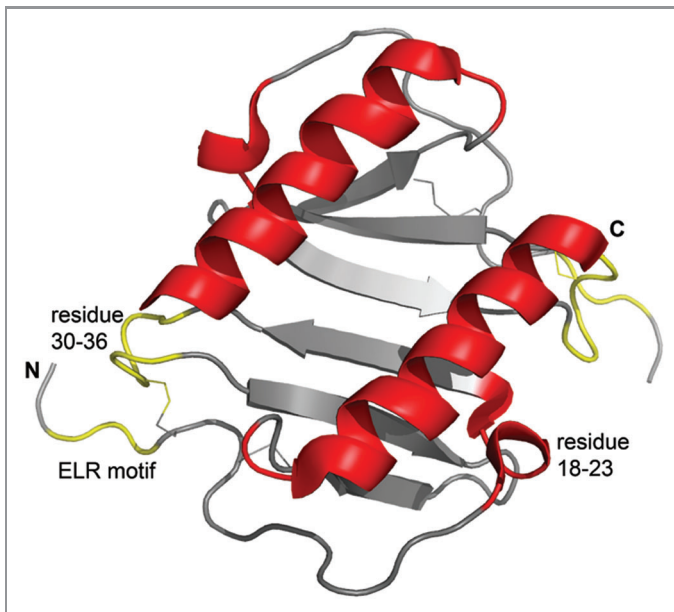Email: juergen.arnhold@medizin.uni-leipzig.de

Figure 1. Three-dimensional structure of human IL-8 in solution as determined by NMR spectroscopy. The figure was constructed using the coordinates deposited in the Protein Data Bank (accession code pdb1IL8) by the pymol software (version 0.99). Due to the high concentrations used in structural studies IL-8 is present as a dimer, where the two α-helices are arranged in antiparallel fashion on top of a six stranded β-sheet. The receptor binding site, including the N-terminal receptor binding motif ELR (Glu-Leu-Arg) and the loop region from residue 30–36, is shown in yellow. GAG-binding regions, shown in red, comprise the residues 18–23 in the proximal loop and the α-helical domain. Of note, receptor- and GAG-binding sites are spatially separated. Further details are given in the text.

plasmin, CD13, cathepsin L and several matrix metalloproteinases are known to be involved in the processing of IL-8. Elongation and deimination are further modification procedures of natural occurring IL-8.[17,18]

As many other cytokines, IL-8 monomers tend to form dimers. Apparently, the monomeric form dominates at nano- and low micromolar concentrations, i.e., the concentration range commonly used in receptor binding studies. At higher concentrations, important for structural studies, the dimer is preferred.[19] However, little is known about the existence of these forms, their functions and the equilibrium between them in vivo. Genetic engineering allows the production of stable IL-8 monomers and dimers that can be used to evaluate the role of this cytokine in inflammatory models.[20]

The receptor binding motif ELR (Glu^4-Leu^5-Arg^6 of the 72 amino acid form of IL-8) is localized near the N-terminus and arranged in a conformation favorable for receptor binding by interaction with the residues 30–36 of the neighboring loop region.[21-24] Apparently, this ELR motif is also sterically protected from proteolytic attack. Most, but not all, posttranslational modifications of IL-8 are accompanied by a higher activity of this cytokine in receptor-mediated responses in PMNs or receptor-transfected cells.[18,25] In IL-8, the receptor binding ELR motif is immediately adjacent to the first cysteine of the CXC motif that is an important structure characteristic of CXC chemokines.

On the surface of IL-8, there are several positively charged epitopes involved in the binding of sulfated glycosaminoglycans.[26-28] The two main binding regions for heparin and heparan sulfate are localized on the C-terminal α-helix and the proximal loop around residues 18–23.

Thus, binding sites for receptors and GAGs are well separated, allowing IL-8 to interact with both components at the same time. While the removal of the entire C-terminus reduces partially the biological activity of IL-8, the elimination of the N-terminus with the receptor-binding motif completely prevents cytokine binding to the receptor.[12,21,29,30] Citrullination of the N-terminal arginine in IL-8 has only small effects on the receptor-binding capacity, but weakens considerably binding to heparan sulfate.[17]

Receptors for IL-8

Neutrophils as well as other leukocytes are equipped with two kinds of G protein-coupled receptors for IL-8 called CXCR1 and CXCR2.[31,32] Both integral proteins have seven transmembrane domains. They bind IL-8 with high affinity as indicated by low interaction constants.[1] While CXCR1 is highly specific to IL-8, CXCR2 is known to interact with a number of other cytokines bearing an ELR group.[33]

Receptor activation by IL-8 causes phosphorylation of protein kinase B, calcium influx, formation of F-actin, and cytoskeletal rearrangement. These events are very important for directed chemotactic movement of PMNs.

The duffy antigen receptor for chemokines (DARC) is also able to bind IL-8 and other chemokines with high affinity. This receptor is involved in transepithelial transport of IL-8.[34]

In addition to proteolytic processing, several other factors are known to modulate the binding of IL-8 to its receptors. Alpha-1 antitrypsin and IL-8 form a complex that is unable to interact with CXCR1.[35] Oligomerization of chemokines affects also their interaction with receptors. While both monomeric and dimeric IL-8 forms are capable of inducing cell recruitment, the dimeric form seems to induce a stronger initial migration of neutrophils.[20] Binding to sulfated glycosaminoglycans promotes oligomerization of IL-8.[36]

Biochemistry of the IL-8/Glycosaminoglycan Interaction

Structural diversity of glycosaminoglycans. Glycosaminoglycans are complex, linear polysaccharides of the extracellular matrix.[37,38] They are composed of repeating disaccharide units that can be additionally modified by epimerization and/or sulfation. Most important GAGs in the extracellular matrix are the sulfated polysaccharides heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate as well as the non-sulfated hyaluronan.[3] Mast cells contain and release heparin, a polysaccharide closely related to heparan sulfate. Basic structural properties of these GAGs and potential sites for postsynthetic modifications are listed in **Table 1**.

Table 1. Glycosaminoglycans of the extracellular matrix

Glycosaminoglycan	Repeating sugar units	Postsynthetic modifications		
		Epimerisation of GlcA to IdoA	O-Sulfation	Modification of N-acetyl groups
Hyaluronan	→3)βGlcNAc(1→4)βGlcA(1→	—	—	—
Dermatan sulfate	→3)βGalNAc(1→4)βGlcA(1→	+	GalNAc: 4-O-SO$_3^-$	—
Keratan sulfate	→4)βGlcNAc(1→3)βGal(1→	—	GlcNAc: 6-O-SO$_3^-$	—
Chondroitin-4-sulfate	→3)βGalNAc(1→4)βGlcA(1→	+	GalNAc: 4-O-SO$_3^-$	—
Chondroitin-6-sulfate	→3)βGalNAc(1→4)βGlcA(1→	+	GalNAc: 6-O-SO$_3^-$	—
Heparan sulfate/heparin	→4)βGlcNAc(1→4)αGlcA(1→	+	GlcNAc: 3-O-SO$_3^-$ 6-O-SO$_3^-$ GlcA/IdoA: 2-O-SO$_3^-$	Deacetylation, N-sulfation

GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; Gal, D-galactose; GlcA, D-glucuronic acid; IdoA, L-iduronic acid.

Different kinds of postsynthetic modifications result in a great structural diversity of polymers especially in the sulfated forms. For example, 48 different disaccharide units can principally be formed in heparin/heparan sulfate. Even though only 23 of these variants really exist due to steric restrictions,[39] the resulting large variety of these polysaccharides is enormous. Moreover, there are also large differences in the degree of modifications in repeating units along the polysaccharide chain. In heparan sulfate, highly N-sulfated regions are alternating with unmodified N-acetyl-rich domains.[40] The latter domains contain a considerable fraction of 6-O-sulfate residues.[41] This large structural heterogeneity limits considerably our understanding of interacting mechanisms between cytokines and glycosaminoglycans.

Sulfated GAGs (most of all heparan sulfate) are often attached to proteins forming proteoglycans. **Table 2** contains important proteoglycans of the cell surface and the extracellular matrix. Syndecans and glypicans are expressed on the surface of endothelial cell and leukocytes. Agrin, perlecan and type XVIII collagen are components of the subendothelial basement membrane.[42] Several other proteoglycans on the cell surface acquire heparan sulfate chains only upon activation of the cell. Important members of this group are the hyaluronan receptor CD44, the type III transforming growth factor-β receptor betaglycan, and testican.[42-44]

Together with different collagens, proteoglycans form a tight network stabilizing tissues and contributing to various tissue-specific functions. In case of infection or injury, immune cells have to penetrate from surrounding blood vessels to inflammatory sites through this network to fulfill their specific immune functions. Interleukin-8, like other cytokines and a number of small proteins such as growth factors, lipases and proteases, is known to interact closely with sulfated proteoglycans.[45-49]

Structural aspects of the interaction of IL-8 with sulfated glycosaminoglycans. An obvious important question is the significance of particular postsynthetic modifications of sulfated GAGs in interaction with IL-8. Measurement of the tryptophan fluorescence of recombinant human IL-8 (human IL-8 contains one tryptophan residue at position 57)[11] in interaction with selected glycosaminoglycan hexasaccharides revealed high affinity binding for both chondroitin-6-sulfate (K$_D$ = 1.4 ± 0.4 μM) and heparin (K$_D$ = 2.0 ± 0.4 μM).[28,50] Binding constants for chondroitin-4-sulfate, dermatan sulfate and hyaluronan are considerably higher.[28] These data indicate the importance of 6-O-sulfate groups in these saccharides, which are only found in chondroitin-6-sulfate and heparin/heparan sulfate. The significance of other modifications still remains unknown.

Based on site-directed mutagenesis,[51,52] molecular modeling[52,53] and NMR spectroscopy,[26,50] H18, K20, R60, K64, K67 and R68 were identified as the essential residues in IL-8, participating in the interaction with heparin and heparan sulfate. These basic amino acids, located in the proximal loop and the C-terminal α-helix, are the driving force for electrostatic interaction with negatively charged carboxylic and sulfate groups of the GAGs. It is therefore not surprising that IL-8 possess the same binding

Table 2. Important proteoglycans of cell surfaces and extracellular matrices

Proteoglycan	Location			Glycosaminoglycan side chains		Full-time expression
	Cell surface	Basement membrane	Extracellular matrix	Heparan sulfate	Chondroitin sulfate	
Syndecan	+			+	+	+
Glypican	+			+		+
Agrin		+	+	+		+
Perlecan		+	+	+		+
Type XVIII collagen		+	+	+		+
CD44	+			+		
Betaglycan	+			+	+	

regions for heparin and monosulfated GAGs like chondroitin sulfate and dermatan sulfate. Nevertheless, depending on the structure of the GAGs not only different binding constants were determined, but also differences in the involved IL-8 amino acid residues were observed. For example, using NMR spectroscopy, an interaction of chondroitin-6-sulfate with the residues R60, K67, R68 of IL-8 was demonstrated, while this was not the case in the interaction of IL-8 with chondroitin-4-sulfate.[28]

Although an interaction of hyaluronan with IL-8 was demonstrated in diverse binding studies,[28,54] no specific binding sites could be determined by means of NMR spectroscopy.[28]

According to computational prognoses, it is assumed that in case of monomeric IL-8, binding of GAGs occurs parallel to the C-terminal α-helix under involvement of residues 18–23 of the N-terminal loop.[28,52] However, the orientation of GAG molecules in an IL-8 dimer is controversial. For instance, it is supposed that, by a perpendicular orientation of GAGs to the helix axis, the two IL-8 monomers are bridged with each other.[27,53] This conflicts with the assumption that longer GAGs bind dimeric IL-8 in a horseshoe fashion, in which respective GAG residues are again aligned in parallel to the α-helix.[28,52,55]

Published studies have shown that the interaction of GAGs with a protein can be improved by sulfation.[27,28,50] However, in addition to the overall number of sulfate groups, the relative positions of sulfate groups also play an essential role.[50,55] Hence, the interaction between GAGs and IL-8 is not only a purely electrostatically driven process, but steric interactions as well as the formation of hydrogen bonds are also crucial for its specificity.

Interaction of IL-8 with artificially modified hyaluronan. Hyaluronan is released in large amounts from different cells at infected and/or injured sites in response to various pro-inflammatory stimuli. This long, unbranched and non-sulfated polysaccharide enlarges the space between cells and matrix components in tissues. This provides better conditions for penetration of immune cells, and pathogenic and injured material will be fixed for a given time allowing their removal by phagocytes. However, high molecular weight hyaluronan features predominantly immunosuppressive and anti-inflammatory activities even at sites of inflammation and was shown to promote healing.[56-58]

Although hyaluronan does not contain sulfated residues, the future application of artificially modified hyaluronan species in three-dimensional scaffolds, in novel tissue replacement approaches and in pads for treatment of chronic wounds is an important tool in regenerative medicine. A crucial chemical modification of hyaluronan is the incorporation of sulfate groups providing the molecule with cytokine, chemokine and growth factor binding capabilities. Specific interaction of sulfated hyaluronan with human bone morphogenetic protein-4 and transforming growth factor-β1 has been demonstrated.[59,60] We tested the adsorption of IL-8 to sulfated hyaluronans that were immobilized on a collagen matrix. Indeed, we detected substantial adsorption of IL-8 to the matrices only when they contained sulfated hyaluronan derivatives (**Fig. 2A**). In our assay we used two different hyaluronan derivatives (**Fig. 2B**), one sulfated in the C-6' position (low sulfated hyaluronan) and one sulfated in the C-6' position and additionally in the C-4' and/or C-2 position

(highly sulfated hyaluronan).[59,61] In these samples, average degree of sulfation per dianhydro sugar unit was 1.0 and 3.1 for the low and high sulfated hyaluronan, respectively. Intriguingly, IL-8 adsorption to highly sulfated hyaluronan was significantly higher than to the low sulfated form indicating a role for 4-O-sulfation and/or 2-O-sulfation in the binding of IL-8 to artificially modified hyaluronan.

Evidence for Biological Relevance of the IL-8/glycosaminoglycan Interactions

Internalization of IL-8. At the site of infection, IL-8 is produced by extravascular cells as well as stimulated endothelial cells. After binding to heparin sulfate chains and to DARC at the abluminal surface of the endothelium, this cytokine is internalized and transcytozed to the vessel lumen.[34,62] The immobilization of IL-8 on the cellular glycocalyx modulates the PMN recruitment.[63]
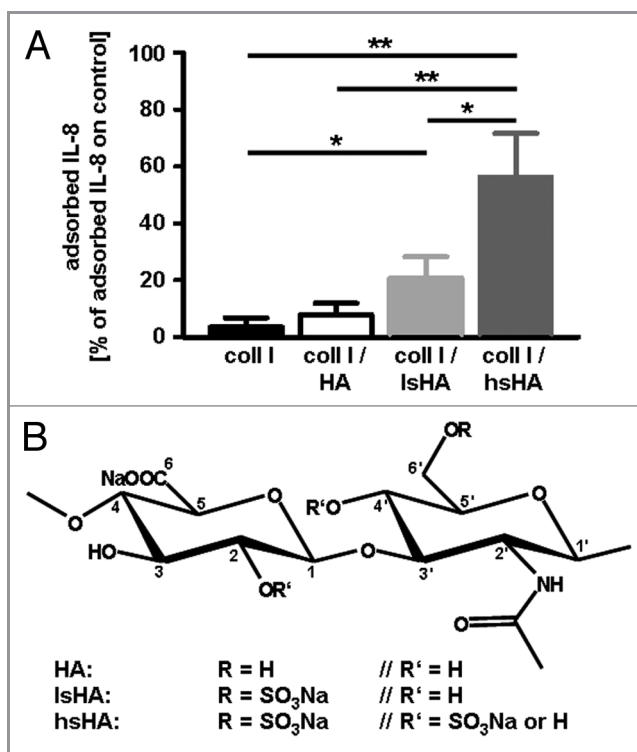

Figure 2. (A) Adsorption of human macrophage-derived IL-8 to modified hyaluronan containing different levels of sulfation. For adsorption of IL-8, native and differentially sulfated hyaluronan (kindly provided by Dr Moeller, INNOVENT e.V. Jena, Germany) immobilized on a collagen matrix were incubated with supernatants of LPS stimulated human monocyte-derived macrophages containing 138 ± 46 ng/ml IL-8. Tissue culture polystyrene was used as control substrate. After 24 h at 37°C, the supernatants were harvested and amounts of IL-8 determined by ELISA. Level of adsorbed IL-8 was calculated from the difference of the IL-8 amount determined in supernatants from the control substrate and of the IL-8 amount determined in supernatants from the different hyaluronan matrices, respectively. All data are mean (± SD) of three independent experiments. *p < 0.05; **p < 0.005 (t-test); (B) chemical structures of native HA and sulfated HA derivatives.[59] coll I, collagen I; HA, hyaluronan; lsHA, low sulfated HA; hsHA, high sulfated HA

However, the relevance of this immobilization is still a matter of controversy.

Effects of glycosaminoglycans on IL-8-mediated chemotaxis of PMNs. Several studies support the hypothesis that binding of IL-8 to surface endothelial GAGs is a crucial prerequisite for PMN attraction. A shortened IL-8 mutant, where the C-terminal GAG-binding domain has been deleted, failed to attract PMNs with the same extent as native IL-8 in vitro and in vivo.[62] In another study, IL-8 variants containing alanine mutations in GAG binding domains were instilled into the lungs of mice that led to higher plasma concentrations accompanied by an increased PMN infiltration.[64] The high plasma levels of this mutant IL-8 may be caused by its inability to bind to matrix components.

Divergent results were also obtained when chemotaxis of PMNs was assessed in vitro in Boyden chamber assays where two medium-filled chambers are separated by a porous filter membrane. In general, cells are allowed to migrate from the upper to the lower compartment containing chemotactic stimuli. In this experimental setting, the chemotactic activity of PMNs toward IL-8 was determined to occur at low nanomolar concentrations (ED_{50} = 0.4 ± 0.1 nM).[21,50] To achieve a more physiological situation, GAGs have to be included into the experimental setup. Two different strategies can be followed: GAGs can be added either to the cell suspension in the upper chamber or to IL-8 in the lower chamber. Co-incubation of IL-8 with heparin or dextran sulfate decreased the chemotaxis of PMNs,[50,65] while co-incubation with heparan sulfate either enhanced chemotaxis[66] or did not exert any effect.[65] When heparin, chondroitin sulfate or hyaluronan were co-incubated with PMNs, the migratory activity was unaffected.[50] While native hyaluronan had no impact on IL-8-induced PMN migration, both, low and high sulfated hyaluronan markedly reduced the chemotactic activity of PMNs upon co-incubation with IL-8 in the Boyden chamber assay (data not shown). These reports imply that investigating chemotaxis in vitro and interpreting the data demands great caution. Addition of GAGs to IL-8 potentially induces the formation of protein-GAG-aggregates that reduce the available amount of IL-8 to build up a chemotactic gradient and thereby hampers migration.[28] However, in vivo all three components (PMNs, IL-8 and GAGs) are present together and, thus, IL-8 may still stimulate chemotaxis. Moreover, due to dynamic equilibria between GAG-bound and soluble IL-8 as well as between monomers and dimers, different forms of IL-8 exist that may modulate PMN recruitment profiles spatiotemporal.[20]

Effects of IL-8 on integrin functions. During the recruitment of PMNs from blood to inflammatory sites, IL-8 acts in concert with selectins and integrins. The ligation of IL-8 to its G-protein coupled receptors CXCR1 and CXCR2 initiates a signaling cascade in PMNs that passes, among others, through the activation of phospholipase C and an inositol 1,4,5-triphosphate-mediated calcium release from internal stores.[67] For instance, IL-8 can induce the recruitment of secretory vesicles and to a minor extent of gelatinase granules to the plasma membrane, which both contain the integrin αMβ2 (CD11b/CD18).[68] In this way, the cell surface is furnished with additional adhesive proteins required for a firm attachment to the vessel wall.[63,69]

Interleukin-8 is also involved in integrin activation.[70] In addition to CD11b/CD18, the integrin αLβ2 (CD11a/CD18) plays a central role in neutrophil migration. During IL-8-stimulated chemotaxis, CD11a/CD18 is bound to the lamellipodium, while CD11b/CD18 is redistributed to the rear of activated neutrophils.[71] This suggests that clustering is a possible mechanism by which IL-8 induces enhanced integrin activity. Another study indicates that CD11a/CD18 is required for the directional migration, whereas CD11b/CD18 mediates cell movement during IL-8 activation.[72]

Effects of IL-8 on oxidative activity of PMNs. Upon reaching the site of infection, PMNs use several mechanisms to exterminate invading pathogens. Phagocytosis, secretion of hydrolyzing enzymes, microbicidal proteins and myeloperoxidase from azurophilic granules into the phagosoms as well as the production of reactive oxygen species (ROS) belong to their cytotoxic arsenal. To minimize severe collateral damage, those mechanisms have to be activated temporally and spatially restricted.

Interleukin-8 mediates substantial production of ROS in PMNs only at micromolar concentrations as evaluated by conversion of the non-fluorescent dihydrorhodamine 123 into a fluorescent product.[50] This concentration range is about three orders of magnitude higher than that of IL-8-induced chemotactic movement. Thus, the main function of IL-8 is to direct PMNs to inflammatory loci and to cause a priming of these cells. Subsequently, full activation of PMNs performing microbicidal functions is achieved by other stimulating agents such as bacterial products (e.g., formylated tripeptides or lipopolysaccharides), components of the complement system and others.

With respect to the influence of GAGs on the ROS production induced by IL-8 only a few information are available. We recently published a study that investigated the relevance of heparin, chondroitin sulfate and hyaluronan for this mechanism. Intriguingly, heparin and chondroitin sulfate enhanced the respiratory activity of PMNs when used in combination with IL-8, while hyaluronan was ineffective.[50] Hence, IL-8-mediated stimulation of PMN defense mechanisms necessitates reconsideration, whereby the influence of IL-8/GAG binding has to be taken into account for a proper understanding of the system.

Conclusions

Interleukin-8 is an important chemokine directing polymorphonuclear leukocytes to inflammatory sites. Thereby it interacts closely with sulfated polysaccharides of proteoglycans of cell surfaces and the extracellular matrix, whereby this interaction may be disturbed by enzymatic digestion of polysaccharides.[26,28,36,73-75] Although many details of the interplay between IL-8 with its receptors and proteoglycans are well known, we are far from understanding this role of interaction in biological processes in vivo. This is, among others, caused by the complex set of modifications that modify IL-8 and GAGs. On the one hand, different forms of IL-8 exist due to processing and citrullination. On the other hand, the large, natural heterogeneity of postsynthetic modifications in heparan sulfate chains yields an enormous diversity of sulfation and charge distribution in the proteoglycan

layer already at short distances. Proteoglycan digesting enzymes that are present during inflammation further modulate the characteristics of the layer. Taken together, this makes it problematic to evaluate the significance of the IL-8-GAG-interaction in vivo.

In vitro experiments revealed the importance of 6-*O*-sulfation for binding of IL-8 to GAGs. However, it remains unknown which particular patterns of sulfation and dynamic alterations in these patterns contribute to the regulation of IL-8 functions in vivo.

The creation of artificially modified matrices is a fundamental tool in regenerative medicine to improve wound healing and immunological safety of graft materials. Thus, it is highly important to know how these materials interact with cytokines and immune cells. As shown on selected examples, the introduction of sulfate groups into a hyaluronan matrix significantly improves its binding of IL-8.

Thus, in immune responses IL-8 is a necessary component to direct immune cells to infected/injured sites. Hence, it is a high challenge to gain insight into further unknown details of functional and regulatory aspects of this chemokine.

Acknowledgments

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