10. A novel leukocyte chemotactic and activating cytokine, interleukin-8 (IL-8)

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Inflammation occurs as a host defensive reaction to tissue injuries caused by various inciting stimuli and is inevitably associated with leukocyte infiltration into a site of inflammation. Limited types of leukocytes infiltrate, depending upon the type, degree, and timing of tissue injuries [1]. Several leukocyte chemotactic factors such as C5a, leukotriene B_4, and bacteria-derived formyl peptide have been long described [2]. However, these factors can induce chemotaxis of any type of leukocytes, thus suggesting the existence of a cell-type-specific leukocyte chemotactic factor(s) in addition to these nonspecific ones.

In the early 1980s, partially purfied interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) were once claimed to be chemotactic for monocytes as well as neutrophils [3,4]. However, subsequent studies revealed that either highly purified natural or recombinant IL-1α, β, or TNF-α lacked chemotactic activities against leukocytes. Moreover, it became evident that the neutrophil chemotactic factor present in lipopolysaccharide (LPS)-stimulated human monocyte supernatants is distinct from IL-1 and TNF-α on a high-performance liquid chromatography [5]. In collaboration with Yoshimura, we purified the factor biochemically [6] and cloned the cDNA encoding this factor from a cDNA library of LPS-stimulated human monocytes [7]. We named this factor monocyte-derived neutrophil chemotactic factor (MDNCF). The cDNA for MDNCF was identical with the cDNA which had been cloned as an enterotoxin-inducible gene in human leukocytes without defining its biological functions [8]. Several other groups, including Baggiolini [9], Schröder [10], and Van Damme [11] subsequently purified the identical neutrophil chemotactic and activating factor from phytohemagglutinin (PHA)- or LPS-stimulated peripheral blood mononuclear cell culture supernatants. They called this factor neutrophil-activating factor (NAF), neutrophil-activating peptide (NAP), and granulocyte chemotactic peptide (GCP), respectively. Moreover, we [12] demonstrated that MDNCF was identical with T-lymphocyte chemotactic factor (TCF) described previously by Van Epp's group [13]. Since MDNCF has multiple target cells, mediates various important inflammatory and immune responses, and is produced by various types of cells, the factor has been renamed IL-8.

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MetThrSerLysLeuAlaValAlaLeuLeuAlaAlaPheLeuIleSerAlaAlaLeuCys
ATGACTTCCAAGCTGGCCGTGGCTCTCTTGGCAGCCTTCCTGATTTCTGCAGCTCTGTGT

*Glu*Gly*Ala*ValLeuProArg*Ser*Ala*Lys**Glu***LeuArg**Cys**Gln**Cys**IleLysThrTyr
GAAGGTGCAGTTTTGCCAAGGAGTGCTAAAGAACTTAGATGTCAGTGCATAAAGACATAC

SerLysProPheHisProLysPheIleLysGluLeuArgValIleGluSerGlyProHis
TCCAAACCTTTCCACCCCAAATTTATCAAAGAACTGAGAGTGATTGAGAGTGGACCACAC

CysAlaAsnThrGluIleIleValLysLeuSerAspGlyArgGluLeu**Cys**LeuAspPro
TGCGCCAACACAGAAATTATTGTAAAGCTTTCTGATGGAAGAGAGCTCTGTCTGGACCCC

LysGluAsnTrpValGlnArgValValGluLysPheLeuLysArgAlaGluAsnSer *
AAGGAAAACTGGGTGCAGAGGGTTGTGGAGAAGTTTTTGAAGAGGGCTGAGAATTCATAA

Figure 1A. Nucleotide sequence and predicted amino acid sequence of human IL-8. The end of the amino-terminus is marked with outlined letters while those of intermediate and truncated forms are marked with italics. Presumed signal peptides are underlined while cysteines are marked with bold letters.

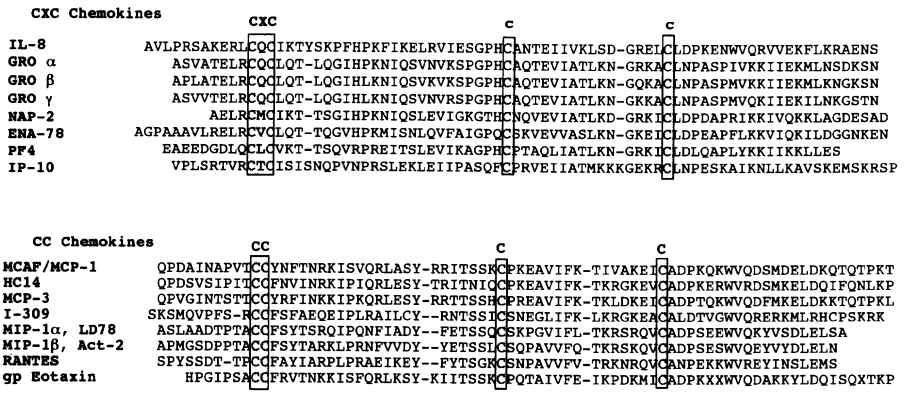

Figure 1B. Comparison of amino acid sequences of chemokines. Amino acid sequences are aligned according to their cysteines.

IL-8 is a prototype of a novel family of basic heparin-binding polypeptide cytokines with a molecular mass of 8–10 kDa. These cytokines have 4 cysteine residue at the well-conserved positions and are presumed to possess a similar structure [14] (Figure 1). Since it has become evident that most of these types of cytokines have chemotactic activity for specific types of leukocytes, the name of chemokine (chemotactic cytokine) has been proposed for this novel cytokine family [15]. Chemokines can be subdivided

into 2 groups, depending on whether the first 2 cysteines are separated by a single amino acid or in an adjacent position. The former are called C-X-C chemokines, and their genes are located on chromosome 4q12–21 in humans, while the latter are called C–C chemokines, and their genes reside on chromosome 17q11–12 in humans [14,15]. In this review, the biological, biochemical, and physicochemical properties of IL-8, properties of IL-8 receptors, molecular regulation of IL-8 production, and clinical relevance of IL-8 will be extensively discussed. Properties of other chemokines will be minimally described in this review; the reader may refer to recent reviews by us [14,16] and by others [15,17] for more extensive discussion of these properties.

Molecular and structural characterization of IL-8

The precursor form of IL-8 consists of 99 amino acids with a putative signal peptide sequence as shown in Figure 1A [6,7]. The amino-terminal end of the active form of IL-8 predominantly starts at serine of reside number 28. Intermediately processed forms (79- and 77-amino acids long) have been also purified from culture supernatants of leukocytes [18]. These intermediate forms can be easily cleaved to 72 amino acid form by thrombin in vitro [19]. In addition, biologically active truncated form (69-amino acids long) has been purified from culture supernatants and has been detected in body fluids such as urines from patients with urinary tract infections [20]. There are 14 basic amino acids (lysine and arginine) in the mature IL-8 molecule, contributing to the basic characteristics of the protein (pI 10). The sequence Lys-Phe-Leu-Lys-Arg at the carboxyl-terminal region is the most probable heparin-binding site. The mature form of IL-8 has 4 cysteine residues, and the first cysteines are separated by 1 amino acid, glutamine, as are other members of C-X-C chemokine family. Since there is no putative N-glycosylation site and purified natural IL-8 has been completely sequenced by the Edman degradation method, the IL-8 molecule is probably not glycosylated.

The three-dimensional structure of human IL-8 has been determined by nuclear magnetic resonance (NMR) [21] as well as by X-ray crystallography [22,23], using purified recombinant human IL-8 expressed in *Escherichia coli*. These structural studies revealed that IL-8 is a dimer at a high concentration with an overall topology that is nearly identical to that observed by the X-ray crystallography of bovine platelet factor 4 which is a member of C-X-C chemokine [24] (Figure 2). Two intermolecular disulftide bonds are formed between Cys7-Cys34 and Cys9-Cys50. Triple-stranded β-sheet is formed between residue 7 and 54, while α-helix is formed in the carboxyl-terminal region. The dimer is primarily stabilized by hydrogen bonds between the first β-strand (residues 23–29) in each molecule.

Clore et al. [21] speculated that the IL-8 dimer may interact with its

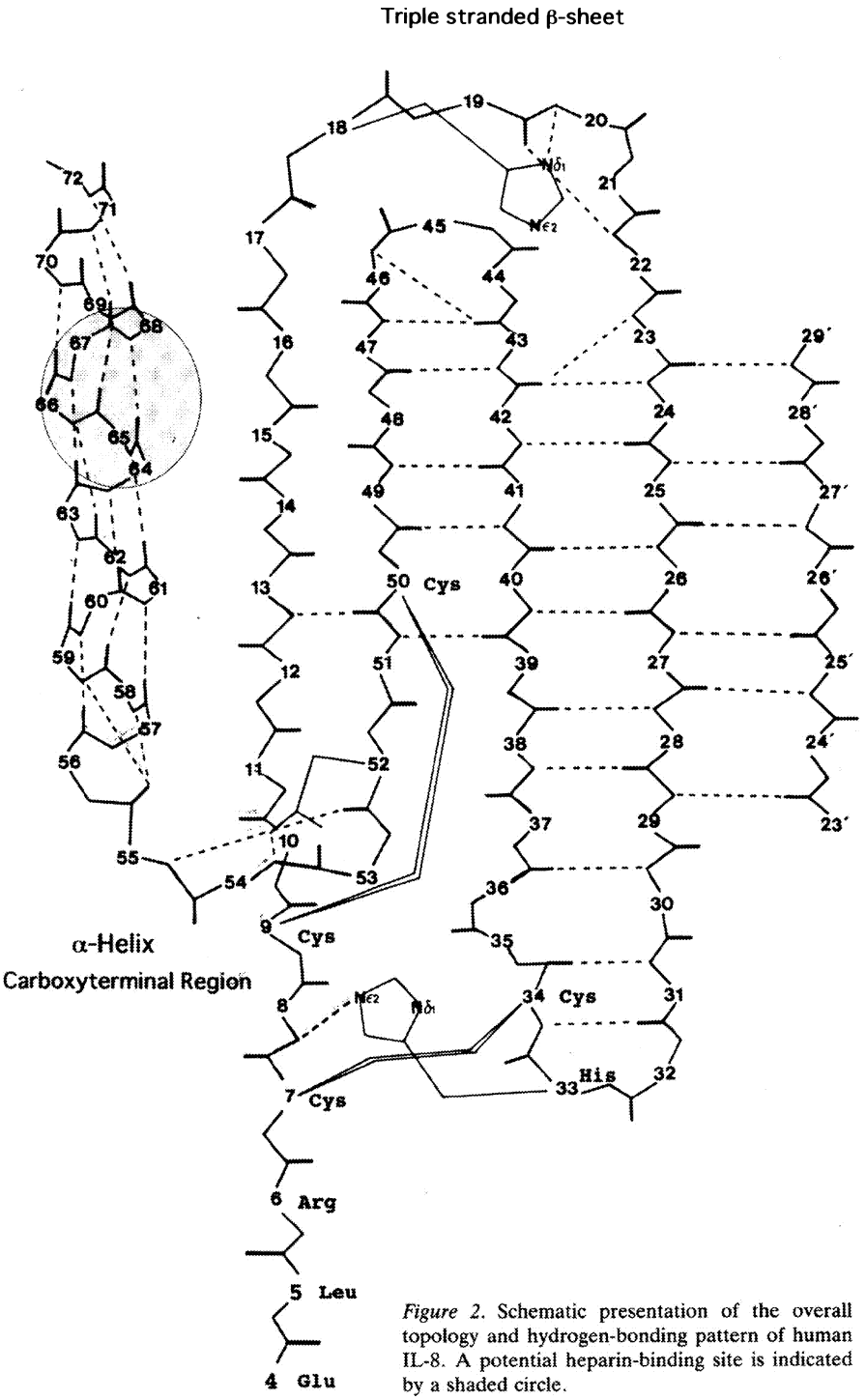

Figure 2. Schematic presentation of the overall topology and hydrogen-bonding pattern of human IL-8. A potential heparin-binding site is indicated by a shaded circle.

receptor through the 2 helices by analogy with HLA-A2. Since the biological activity of IL-8 is maximal at 10 ng/ml, significant concentrations of monomeric IL-8 may be present under physiological conditions. An IL-8 analog was chemically synthesized by Clark-Lewis et al. [25], with the amide nitrogen of leucine-25 methylated to selectively block formation of hydrogen bonds between monomers and thereby dimerization. The IL-8 analog, which was shown to be a monomer as assessed by analytical ultracentrifugation and NMR, was equivalent to native IL-8 in assays of neutrophil activation [25]. These observations indicate that the monomer is a functional form of IL-8.

In order to elucidate the role of α-helices in receptor binding, we prepared a chimeric molecule which consisted of amino-terminal 54 residues of IL-8 and the carboxyl-terminal 20 residues of MCAF. The chimeric molecule retained potent neutrophil chemotactic activity without monocyte chemotactic activity [our unpublished data]. These data and truncation mutants identified by Lindley et al. [26] suggest that the helices are primarily involved in forming and stabilizing the hydrophobic core.

The scanning mutagenesis demonstrated that the sequence Glu4-Leu5-Arg6 are essentially involved in the receptor binding [27]. Platelet factor 4, which lacked binding capacity to IL-8 receptors on neutrophils, obtained the ability to bind to neutrophil IL-8 receptor when its amino-terminus is modified with Glu-Leu-Arg [28], further supporting the notion. In addition to this region, the region around the β-turn at His33 is also well conserved among human and rabbit IL-8 and gro's (Figure 1B), all of which can bind to the IL-8 receptor on human neutrophils. The structural studies demonstrated that amino-terminus and β-turn at His33 are near each other in 3 dimensions and extend from the β-edge [23]. In addition, the lesser potency of longer intermediate forms (79- and 77-amino acid form) [18] suggests that an extended amino-terminus near the active site ligands acts to block receptor binding. Collectively, the residues near the turn at His33 are likely to comprise the ligand which binds to the cell-surface receptor.

Molecular characteristics of IL-8 receptors

The presence of specific binding sites on human neutrophils has been demonstrated by several groups including ours [29,30,31]. We observed that blood neutrophils possess about 20,000 specific binding sites per cell with a single type of high-affinity binding site ($K_d = 8 \times 10^{-10}$ M), although some donor difference was observed [29]. Similar results were reported by Grob et al. [30], whereas Basemer et al. reported the presence of binding sites with 2 different affinities [31]. The molecular mass of IL-8 receptor was estimated to be 67 and 59 kDa based on cross-linking experiments [29]. Among numerous members of a chemokine family, gro/melanoma growth stimulating activity (MGSA), and, to a lesser degree, neutrophil-activating peptide-2 (NAP-2), could displace the binding of IL-8 to neutrophils [32].

Table 1. Biological functions of IL-8

Target cells	Effects
In vitro	
Neutrophils	chemotaxis (+), shape change (+) degranulation (+), respiratory burst (+) release of lysosomal enzyme (+) adherence to unstimulated endothelial cells (+) adherence to stimulated endothelial cells (−) transendothelial migration (+) expression of adhesion molecules (+) activation of 5-lipoxygenase release of leukotriene B_4 and 15-HETE (+) growth inhibtion of *Candida albicans*
T cells	chemotaxis (+)
B cells	IL-4-induced IgE production (−)
Basophils	chemotaxis (+) release of histamine and leukotriene (+)
Monocytes	adhesion to endothelial cells (+)
Melanoma cells	haptotactic effects (+) growth promotion (+)
Fibroblasts	collagen gene expression (−) replication of cytomegalovirus (+)
Keratinocytes	growth promotion (+)
In vivo	neutrophil and lymphocyte infiltration (+) permeabiity in the presence of prostaglandin E (+) neutrophilia destruction of synovial membrane prolideration of endothelium

(+) and (−) indicate positive and negative effects, respectively.

In addition to neutrophils, several additional types of cells possess specific binding sites for IL-8 (Table 2) [29]. Normal T cells express only 300 binding sites per cells, although they respond to 10-fold lower concentrations of IL-8 than neutrophils. This may mean that only limited subsets of T cells express receptors or that small number of receptors are sufficient for signal transmission. An Epstein–Barr virus-transformed cell line, FMO, expresses IL-8 binding sites (1,900 sites/cell). This may be related to the inhibitory effect of IL-8 on IL-4-induced IgE production [33]. Recently, we observed that a majority of $CD16^+$ lymphocytes were positively stained with specific antibodies to either type of IL-8 receptor [our unpublished data]. In addition, IL-8 has effects on several types of non-leukocytic cells, including haptotatic effects on melanoma cells [34], inhibition of collagen gene expression by fibroblasts [35], and enhancement of cytomegalovirus replication in lung fibroblasts in vitro [36]. The receptors on these types of cells, however, are not well characterized biochemically.

IL-8 itself downregulates its own receptor very rapidly within 10 minutes

at 37°C, and is followed by the internalization of ligand-receptor complex [37]. The internalized complex is transported to lysosomes where IL-8 is degraded by lysosomal enzymes and released extracellularly. IL-8 receptors reappears within 10 minutes once free ligand is removed. The recycling of receptors did not require protein synthesis. However, lysosomotropic inhibitors and ATP synthesis inhibitors inhibited recovery of downregulated receptors but not binding of the ligand and ligand internalization. Furthermore, there is a good correlation between the effective dose of a lysosomotropic inhibitor, ammonium chloride, blocking the recycling of the IL-8 receptors and that inhibiting the response to neutrophil chemotactic activity [37]. Collectively, IL-8 receptor may be regulated by its own ligand, and rapid recycling may be essential for signal transmission by IL-8.

Two groups independently cloned cDNA encoding human IL-8 receptors (Figure 3) [38,39]. Both of them are presumed to contain 7 transmembrane domains as receptors for other chemotactic factors, including fMet-Leu-Phe [40], C5a [41], and platelet-activating factor [42]. Based on the deduced amino acid sequence, both types of IL-8 receptors are presumed to be associated with G-protein(s), although the precise nature of associated G-protein(s) is not clarified yet. The cDNA of IL-8 receptor reported by Holmes et al. encodes a mature protein with 350 amino acids with a single open reading frame [38] and is now designated type I or type A. When the COS cells were transfected with this clone, the cells expressed specific binding sites for IL-8 with K_d = 3.6 nM. Murphy et al. independently reported the other cDNA encoding IL-8 receptor [39], now called type II or type B. At first, this clone was presumed to encode a low-affinity receptor reported by Basemer et al. However, subsequent studies demonstrated that the transfection with this clone resulted in the expression of IL-8 receptors with a high affinity [43].

The first and third extracellular domains of both type I and type II receptors contain potential N-glycosylation sites. They show a striking sequence similarity with each other at the amino acid level, reaching more than 80% homology except amino terminal portion (Figure 3). Both genes reside in chromosome 2p35 accompanied with 1 pseudogene which showed significant homology with both types of receptors, particularly type II [44], suggesting that these genes arose by gene duplication.

When each type of IL-8 receptor is transfected into COS cells [43], IL-8 binds to both types of receptors with a similar high affinity, whereas gro binds to only type II receptors with a high affinity [43]. Furthermore, the transfection with chimeric receptors revealed that an amino terminal portion of each receptor determines its binding specificity [45]. We generated specific antibodies which can recognize the amino terminal portion of each type and can bind to each type in a specific manner. Analysis using these antibodies demonstrated that all the mature neutrophils express both type I and type II receptors (Table 2B) [our unpublished data]. These antibodies failed to inhibit the binding of IL-8 to receptors, suggesting that additional extracel-

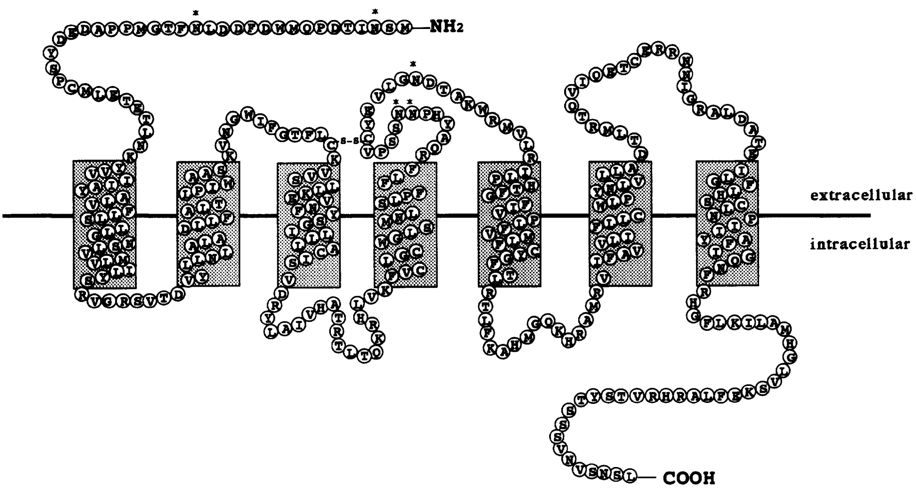

Figure 3A. Schematic structure of human IL-8 receptor type I. Presumed N-gycosylation sites are indicated with astericks.

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                                 1
                   10         20         30         40         50
hIL-8R1   -3 ...MSNITDP QMWDFDD-LN FTGMPPADED YSPCMLETET LNKYVVIIAY    47
hIL-8R2    1 MESDSFEDFW KGEDLSNYSY SSTLPPFLLD AAPCEPESLE INKYFVVIIY    50

                   60         70         80         90        100
hIL-8R1   48 ALVFLLSLLG NSLVMLVILY SRVGRSVTDV YLLNLALADL LFALTLPIWA    97
hIL-8R2   51 ALVFLLSLLE NSLVMLVILY SRVGRSVTDV YLLNLALADL LFALTLPIWA   100
                  2
                  110        120        130        140        150
hIL-8R1   98 ASKVNGWIFG TFLCKVVSLL KEVNFYSGIL LLACISVDRY LAIVHATRTL   147
hIL-8R2  101 ASKVNGWIFG TFLCKVVSLL KEVNFYSGIL LLACISVDRY LAIVHATRTL   150
                                                  3
                  160        170        180        190        200
hIL-8R1  148 TQKRHLVKFV CLGCWGLSMN LSLPFFLFRQ AYHPNNSSPV CYEVLGNDTA   197
hIL-8R2  151 TQKRYLVKFI CLSIWGLSLL LALPVLLFRR TVYSSNVSPA CYEDMGNNTA   200

                  210        220        230        240        250
hIL-8R1  198 KWRMVLRILP HTFGFIVPLF VMLFCYGFTL RTLFKAHMGQ KHRAMRVIFA   247
hIL-8R2  201 NWRMLLRILP QSFGFIVPLL IMLFCYGFTL RTLFKAHMGQ KHRAMRVIFA   250
                                         4
                  260        270        280        290        300
hIL-8R1  248 VVLIFLLCWL PYNLVLLADT LMRTQVIQET CERRNNIGRA LDATEILGFL   297
hIL-8R2  251 VVLIFLLCWL PYNLVLLADT LMRTQVIQET CERRNHIDRA LDATEILGIL   300

                  310        320        330        340        350
hIL-8R1  298 HSCLNPIIYA FIGQNFRHGF LKILAMHGLV SKEFLARHRV TSYTSSSVNV   347
hIL-8R2  301 HSCLNPLIYA FIGQKFRHGL LKILAIHGLI SKDSLPKDSR PSFVGASSGH   350

                  360        370        380        390        400
hIL-8R1  348 SSNL*..... .......... .......... .......... ..........   397
hIL-8R2  351 TSTTL*.... .......... .......... .......... ..........   400
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Figure 3B. Alignment of the amino acid sequences of type I (type A) (upper) and type II (type B) human IL-8 receptors (lower panel). Extracellular portions are indicated by bars and numbered from the amino-terminal one. The positions occupied by conserved sequences are indicated by filled boxes.

Table 2A. Distribution of IL-8 receptors on various types of cells as revelaed by ^{125}I-labeled IL-8 binding assay

Cell type	Receptor number	K_d (nM)
Neutrophils	20,000	0.8
Lymphocytes	300	not determined
Monocytes	9,000	not determined
FMO (EB-virus transformed cell line)	2,000	not determined
DMSO-treated HL60 (differentiate into neutrophils)	7,000	1.2

Table 2B. Distribution of each type of IL-8 receptor on various types of leukocytes as revealed by immunofluorescence analysis

Cell type	type I receptor	type II receptor
Neutrophils	++	++
Eosinophils	−	−
$CD16^+$ lymphocytes	+	+
$CD3^+$ lymphocytes	+	+
$CD20^+$ lymphocytes	−	−
Monocytes	+	+

lular portion(s) participates in the ligand binding as well as the amino acid terminus, as previously claimed by other group [46].

IL-8 receptor mRNA expression on neutrophils is upregulated by LPS or granulocyte colony stimulating factor (G-CSF) and is downregulated by TNFα [47]. Recent cloning of genomic DNA for human IL-8 type II receptor revealed that the gene consists of 3 exons and 2 introns [47]. Functional analysis of promoter region of IL-8 receptors will clarify the regulatory mechanisms of IL-8 receptor gene expression.

In addition to the above-mentioned receptors, the presence of IL-8-binding protein on erythrocytes has been reported [48]. This protein can bind IL-8 with a high affinity (K_d = 5 nM), numbering 1,000 to 9,000 sites/cell. Moreover, with a similar affinity, this receptor-like protein can bind several additional chemokines including gro, RANTES, and MCAF/MCP-1. Furthermore, the receptor-like molecule on erythrocytes has been identified as Duffy antigen, which was presumed to be the entry site of *Plasmodium vivax* into erythrocytes [49]. However, since Duffy antigen is also expressed on endothelium of postcapillary venule in various tissues, additional pathophysiological roles of this protein remain to be determined.

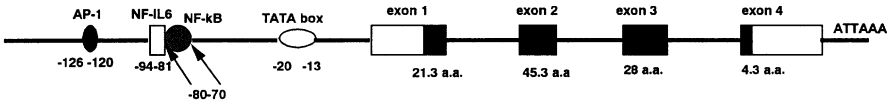

Figure 4. Schematic structure of human IL-8 gene. Open and closed boxes indicate untranslated and translated portions of exons, respectively. The position of potential binding sites for transcription factors, including AP-1, NF-IL6, and NF-κB, are shown along with that of TATA box. The numbers of lower line indicate the numbers of amino acids which each exon encodes.

Regulatory mechanism of IL-8 gene

Mechanism of IL-8 gene activation

Various types of cells express IL-8 mRNA rapidly and massively when stimulated with pro-inflammatory cytokines such as IL-1 or TNF-α [14,16]. A protein synthesis inhibitor, cycloheximide, did no inhibit, but rather enhanced IL-8 mRNA expression induced by IL-1 [50], suggesting that IL-8 mRNA induction does not require de novo protein synthesis. Nuclear runoff assays revealed that the mRNA induction by IL-1 or TNF is at least partly ascribed to the activation of transcription in most types of cells [16]. In astrocytoma U373 cells, the stability of IL-8 mRNA is also increased by stimulation with IL-1 [51]. The involvement of reactive oxygen intermediates [52] or sulfatides [53] in IL-8 mRNA accumulation has been documented. However, at the moment, direct evidence is lacking on the roles of these molecules in regulating IL-8 gene transcription.

The determination of the entire sequence of genomic IL-8 including 1.5 kb 5′-flanking region revealed that IL-8 genomic DNA consists of 4 exons and 3 introns with a single 'TATA'- and 'CAT'-like structure [54]. Moreover, the 5′-flanking region contains several potential binding sites for known transcription factors (Figure 4). The sequence from −94 to −71 bp, consisting of NF-IL6 and NF-κB binding sites, was identified to be minimally required for the responsiveness to IL-1 in a glioblastoma cell line, T98G, as revealed by the analyses using chloramphenycol acetyl transferase (CAT) assays [55]. The same combination of cis-elements was necessary for IL-8 gene activation in a human fibrosarcoma cell line, 8387, stimulated with IL-1 or TNF-α [56].

IL-1 induced the binding of NF-κB and NF-IL6 complexes to their corresponding cis-elements in T98G cells [55]. The NF-κB complexes were identified immunochemically to be composed of p50 and p65. Accumulating evidence indicates that NF-κB is associated with their inhibitor, IκB, in cytosol in a resting state, and that it dissociates from IκB, followed by nuclear translocation and binding to its cognate *cis*-elements upon the activation [57]. In T98G cells, IL-1 actually induced nuclear translocation of both p50 and p65 as revealed by Western blotting analysis [55]. Although

the dissociation of NF-κB from IκB is presumed to be preceded by the modification of IκB, including its phosphorylation [57] or protein degradation [58], it remains an open question how IL-1 treatment modifies IκB.

The translocated NF-κB complex induces the gene activation in co-operation with NF-IL6 since the mutation of either NF-κB or NF-IL6 binding site abolished the responsiveness to IL-1 [55]. This notion was further supported by the observation that the cotransfection with expression vectors of NF-IL6 and NF-κB subunits resulted in strong synergistic activation of IL-8-enhancer- as well as IL-6-enhancer-driven reporter gene [59]. Several lines of evidence indicate that NF-IL6 associates with NF-κB through the basic leucine zipper domain of NF-IL6 and the Rel homology domain of NF-κB subunits in vitro [59]. However, the direct evidence is lacking on the physical association of these 2 types of transcription factors in teh IL-8 gene.

We observed that IL-8 gene activation is mediated by AP-1 binding site (02126 to −120 bp in the IL-8 gene) in conjunction with NF-κB site in other types of cells, such as a human T-cell leukemia cell line, Jurkat cells stimulated with phorbol ester plus ionomycin [60], and a human gastric cancer cell-line, MKN45 cells, stimulated with TNFα plus interferon (IFN)-γ [61]. The requirement of a similar region was also documented on human lung epithelial cell-lines [62]. In MKN45 cells, we could not detect any complex formation using an NF-IL6 site in the IL-8 gene by EMSA [our unpublished data], suggesting the absence of NF-IL6. Moreover, when T98G cells were transfected with the CAT-expression plasmid containing a mutated NF-IL6 and an intact AP-1 and NF-κB sites, CAT activity was induced by the stimulation with IL-1 [55]. These observations prompted us to speculate on the relative role of these 3 types of transcription factors as follows: although the NF-κB is the most crucial factor for IL-8 gene transcription, the cooperation with other types of transcription factors is necessary for the IL-8 gene activation. NF-IL6 is the first choice as a cooperator while NF-κB chooses AP-1, either in the absence of NF-IL6 or in the presence of mutation of NF-IL6 site (Figure 5A).

Mechanism of IL-8 gene repression

Several agents inhibit IL-8 production, including 1,25-dihydroxycholecaliferol [63], glucocorticoids [55], FK506 [60], IL-4 [64], and IFN's [65]. Among these agents glucocorticoids, FK506, and IFN's inhibit IL-8 production at the transcriptional level.

A glucocorticoid, widely used as an anti-inflammatory agent, inhibits gene transcription of several cytokines including IL-2, IL-3, IL-6, IL-8, monocyte chemotactic and activating factor (MCAF), and IFNγ [55]. In contrast to previous reports that the repression of collagenase gene by glucocorticoids was ascribed to the inhibition of AP-1 complexes [66], neither mutation nor deletion of AP-1 site in the IL-8 gene abolished the IL-1-induced transcription and the gene repression by a synthetic glucocorticoid,

dexamethasone (DEX), in T98G cells [55], suggesting that AP-1 site is not a main target of DEX. However, CAT activities were induced by IL-1, and the induced CAT activities were inhibited by DEX when the cells were transfected with CAT-expression vectors which contains tandemly repeated NF-κB sequence and the basal promoter region. IL-1-induced formation of NF-κB (p50–p65) was diminished by DEX treatment without changing the component of the complex and the amount of nuclear-translocated p50 and p65 [55] (Figure 5B). Taken together, these results suggest that glucocorticoid interferes with the binding of the most essential transcription factor, NF-κB, to its cognate *cis*-element without inhibiting the nuclear translocation of the factor, thereby suppressing the transcription of IL-8 gene.

IFNα/β also inhibited TNF-induced IL-8 gene transcription in human fibroblasts through inducing the physical association of NF-κB with NF-IL6. The association inhibited the transcriptional activity of NF-κB and suppressed IL-8 gene transcription [65].

In the case of FK506-mediated IL-8 gene repression in a human T-cell line, Jurkat, the target *cis*-elements were the AP-1 site in addition to the

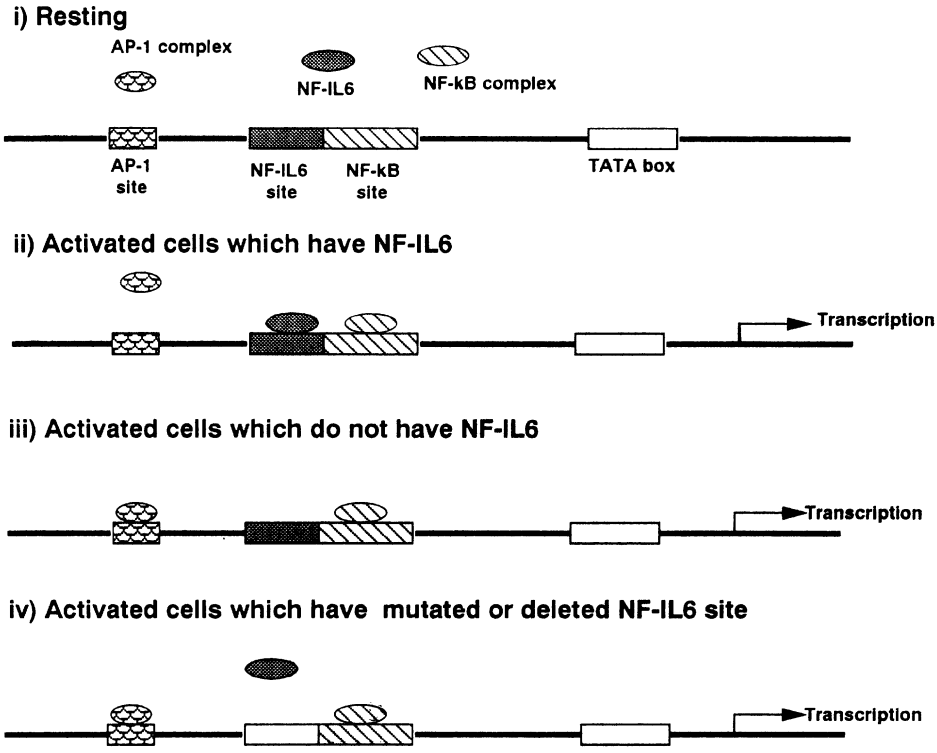

Figure 5A. Proposed mechanism of cell-type specific requirement for the transcription factors.

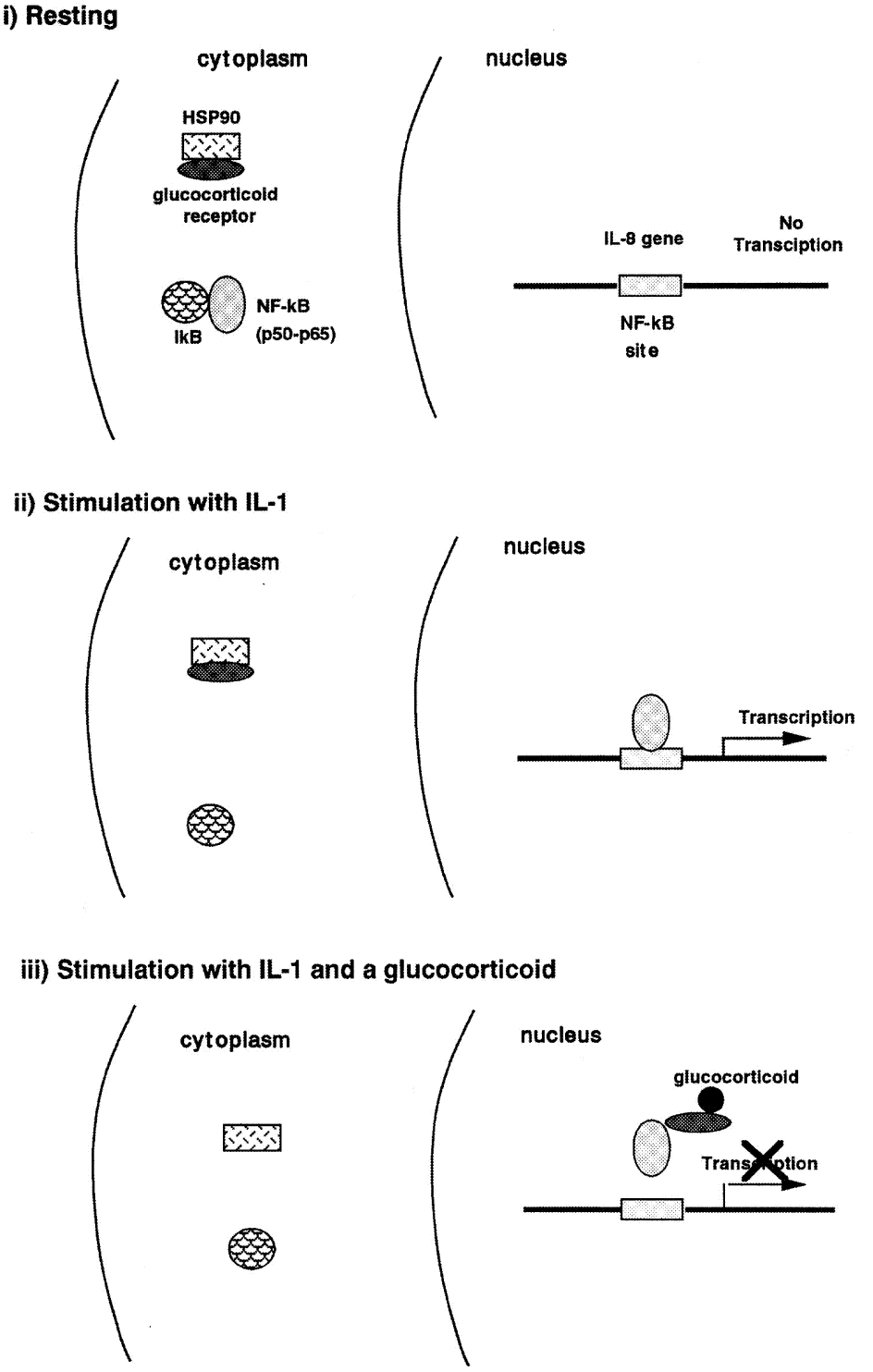

Figure 5B. Proposed mechanism of a glucocorticoid-mediated IL-8 gene repression in a human glioblastoma cell line, T98G stimulated with IL-1.

NF-κB site [60]. When Jurkat cells were transfected with the enhancerless IL-8 core promoter, which was linked with tandemly repeated AP-1 or NF-κB site, FK506 inhibited the activity reporter gene induced by Ca^{2+}-mobilizing stimuli but not by Ca^{2+}-non-mobilizing stimuli.

FK506, however, affected little if any AP-1 complexes induced by the stimuli, which was immunochemically identified to be composed of Jun-D and c-Fos. In contrast, FK506 changed the migration of complexes formed on the NF-κB site in the IL-8 gene similarly to the way EGTA did [60]. These results suggested that a Ca^{2+}-dependent pathway is involved in the complex formation of the IL-8 NF-κB site, and that FK506 affected the complex formation in a Ca^{2+}-dependent manner. The complexes in Jurkat cells using NF-κB site were immunochemically distinct from members of c-*Rel* family [60]. FK506 and cyclosporin A suppress the transcriptional activity of the NF-AT and NFIL2-A sites of the IL-2 gene by inhibiting the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, thereby inhibiting IL-2 gene transcription [67]. Moreover, recent cloning revealed that NF-AT proteins contains *Rel* domains [68]. Hence, it is tempting to speculate that NF-AT or its related protein(s) is involved in IL-8 gene activation in T lymphocytes and is a target of FK506.

Mechanism of signal transmission

IL-8 receptors interact with GTP-binding proteins of G_i type as originally suggested by the inhibition of neutrophil responses after pretreatment of *Bordetella pertussis* toxin [69]. Exposure of neutrophil plasma membrane to IL-8 enhances GTPase activity and the high-affinity binding of radiolabeled GTPγS [70], indicating that G-proteins are activated on IL-8 binding to the receptor.

IL-8 activates phosphatidylinositol-specific phospholipase C, resulting in the generation of 1,4,5- inositoltrisphosphaste (IP_3) [71]. IL-8 also stimulates phophatidylinositol-4-phosphate kinase [72], which synthesize phosphatidylinositol-4, 5-bisphosphate, the source of IP_3. The generated IP_3 induces release of $[Ca^{2+}]_i$ from intracellular stores [69, 71], which is sufficient to induce exocytosis and respiratory burst. However, the precise involvement of additional second messenger system(s) and signal-transduction systems working in other types of cells remains elusive.

In vitro biological activities of IL-8

Interleukin-8 (IL-8), originally purified as a neutrophil chemotactic factor [6], has profound effects on neutrophils, including induction of shape change [69], induction of respiratory burst [73], adhesion to endothelium [74], transendothelial migration [75], release of lysosomal enzymes [76], generation of superoxide [69], and generation of bioactive lipids [77] (Table 1). In

addition to the effects on neutrophils, IL-8 induces the chemotaxis of T lymphocytes [12], basophils [78], and lymphokine-activated killer cells [79]. Taub et al. once claimed that IL-8 lacked in vitro T-cell chemotactic activity based on a comparison of biological functions of several chemokines [80]. However, their subsequent study demonstrated that IL-8 exhibits in vitro chemotactic activity for freshly isolated T lymphocytes [81]. IL-8 also induces in vitro eosinophil chemotaxis when the cells were pretreated with either IL-3 or GM-CSF [82].

IL-8 induces the release of histamine and leukotrienes from human basophils [83]. The release largely depends on the pretreatment of the cells with IL-3, IL-5, or GM-CSF, since untreated basophils release only low levels of histamine but not leukotrienes. In contrast, IL-8, at low concentrations, inhibits histamine and leukotriene release induced in basophils by a 'histamine-releasing factor,' or IL-3 at concentrations 10–100-fold lower than those required for the induction of release [84]. Thus, the effects of IL-8 on histamine release, particularly in vivo, can be complicated.

Monocytes possess specific binding sites for IL-8 [29] and specific antibodies to either type of IL-8 receptor positively stain monocytes [our unpublished data]. IL-8 elicits a minor $[Ca^{2+}]_i$ in human monocytes [85]. IL-8 also induces the respiratory burst which can be enhanced by the pretreatment of the monocytes with concanavalin A [85]. IL-8 inhibited the production of IgE and IgG_4 production by human normal B cells stimulated with IL-4 [33].

A vigorous efforts has been made to study potential functions of IL-8 on non-leukocytic cells. Culture of primary or metastasizing melanoma produces constitutively IL-8 in addition to groα, which was originally purified from culture supernatants of melanoma cells [86]. Moreover, melanoma cells have been reported to migrate in vitro in response to IL-8 by haptotaxis [34]. IL-8 and groα also inhibit collagen expression in synovial fibroblasts [35]. IL-8 also enhanced the replication of cytomegalovirus in fibroblasts in vitro [36]. Chemotaxis of epidermal cells in response to IL-8 has been observed [87].

Human IL-8 has been reported to be angiogenic in the rat cornea and to induce the migration of umbilical vein endothelial cells in vitro [88]. These observations were further substantiated by the fact that the addition of neutralizing antisera to IL-8 attenuated both reactions [89]. However, it remains to be investigated whether Il-8 directly stimulates the prolferation of endothelial cells.

In vivo activities of IL-8 and clinical relevance of IL-8 production

Animal experiments

Intravenous injection of IL-8 caused rapid neutrophilia in rabbits, mouse, rats, and dogs [14,15]. Intradermal injection of IL-8 in rats or rabbits caused

plasma exudate and accumulation of neutrophils at 2–3 hour after injection followed by lymphocyte infiltration afterwards [12,90,91]. A potent vasodilator, prostaglandin E_2, increases the number of accumulated neutrophils and the volume of plasma exudate [91]. The action of IL-8 is direct without inducing a second mediator, since no change was observed on co-injection of actinomycin D [90]. Moreover, the action of IL-8 lasts longer than those of other chemoattractants such as C5a, f-Met-Let-Leu-Phe, leukotriene, and platelet-activating factor, which are rapidly metabolized. IL-8 enhances its chemotactic activity in vitro once it binds with heparan sulfate [92]. Hence, it is possible that IL-8, as a cationic peptide, binds to glycosamines of tissue matrix and cell membrane and persists in active from in vivo as reported on MIP-1α [93]. These observations raise the possibility that IL-8 is involved in the infiltration of leukocytes, particularly neutrophils.

Recently, Mulligan et al. reported that the injection of anti-human IL-8 antibody into rats prevented glycogen-induced accumulation of neutrophils and exhibited the protective effects against lung and dermal vascular injury after deposition of IgG immune complexes [94]. Due to the failure in this study to identify the antigen(s) which anti-human IL-8 recognized, the role of IL-8 in inflammatory reactions in rodents still remains elusive.

We recently observed that the previously generated anti-human IL-8 monoclonal antibody inhibited the binding of rabbit IL-8 to rabbit peritoneal neutrophils as well as the chemotactic activity of rabbit IL-8 to rabbit peritoneal neutrophils [95]. Hence, in the series of the experiments, we used this monoclonal antibody to explore the role of endogenously produced IL-8 in inflammatory reactions.

A single intra-articular injection of IL-8 induces neutrophil infiltration with subsequent infiltration of lymphocytes. The synovial lining cells become ovoid, pleomorphic, and multilayered [96], mimicking the pathological changes of rheumatoid arthritis. When LPS or IL-1 was administered into joint cavity of rabbits, infiltration of neutrophils and that of mononuclear cells were observed in the joint cavity at 4 hours and only at 24 hours or later after the injection, respectively [97]. Histology revealed the thickening of synovial lining and mild edema of subsynovial tissue. Anti-IL-8 antibody significantly reduced leukocyte infiltration in the early phase of the event and prevented the damage of synovial membrane [97]. However, the infiltration of mononuclear cells was not inhibited by the antibody. These results suggest that IL-8 is essentially involved in neutrophil-dependent tissue injury at the acute phase.

Reperfusion injury is characterized biocemically by the generation of reactive oxygen intermediates (ROI) and pathologically by massive neutrophil infiltration [98]. In experimental lung reperfusion, where the increase of neutrophil numbers in bronchial lavage fluids, massive neutrophil infiltration into lung interstitium, destruction of pulmonary architecture, and severe pulmonary edema were observed, IL-8 as produced locally by alveolar macrophages and ciliary cells of bronchiole [99]. Anti-IL-8 antibody inhibited

neutrophil infiltration into lung tissue and the increase of neutrophils in BALF. Furthermore, anti-IL-8 treatment prevented the destruction of pulmonary architecture [99]. Considering that ROI upregulate L-8 production in vitro [52], these results suggest that ROI, generated during reperfusion, induce local production of IL-8, which, in turn, induces infiltration and activation of neutrophils, thereby establishing injury.

Intradermal injection of LPS into rabbits induces skin redness at the injection site [95]. Histology revealed that neutrophils started to migrate to the LPS-injected site from 2 hours, increasing thereafter until 12 hours after the injection. Anti-IL-8 antibody prevented the neutrophil infiltration until 6 hours after LPS injection, suggesting that IL-8 is involved in neutrophil infiltration, particularly at the acute phase, through inducing migration and expression of adhesion molecules. Interestingly, this antibody also blocked the appearance of skin redness completely at the injecton site at 24 hours, despite the presence of neutrophil infiltration. This suggests that IL-8 is essentially involved in the activation of neutrophils as well as neutrophil migration in the early phase. This assumption was further supported by the observations on acute immune-complex glomerulonephritis.

Repeated injection of crystallized bovine serum albumin (BSA) with complete Freund adjuvant (CFA) into rabbit induced a significant number of neutrophils infiltrated into glomeruli and fusion of epithelial foot process, a charge barrier in glomerular capillary wall [100]. Consequently, urinary excretion of proteins and albumin markedly increased with concomitant increase of urinary IL-8 levels. Anti-IL-8 antibody prevented fusion of epithelial foot process and, surprisingly, reversed the urinary excretion of protein and albumin to normal levels, although neutrophil infiltration was only partially inhibited [100]. These results further support the notion that IL-8 is essentially involved in the activation as well as the infiltration of neutrophils, particularly at the acute phase.

Targeted deletion of murine IL-8 receptor homolog

Since murine homologue of IL-8 has not yet been discovered, murine homologue of IL-8 receptor gene was disrupted by homologous recombination to elucidate the physiological role of IL-8 or IL-8-related molecule(s) [101]. Gene disruption of IL-8 receptor resulted in the loss of response of neutrophils to human IL-8 and mouse MIP-2. However, neutrophils possess normal killing activities against baceteria. $\gamma\delta^{+}$T cells markedly increased in both spleen and lymph nodes. Increases of neutrophils and megakaryocytes at every stage, as well as that of plasma cells, were observed in bone marrow, spleen, and lymph nodes, leading to the enlargement of these organs. Neutrophil number increased in peripheral blood. These results suggest that IL-8 may have effects on early hematopoietic progenitor cells, as previously claimed by other groups [102,103]. IgE as well as IL-6 levels in sera elevated in the targeted mice, suggesting that IL-8 or its related

Table 3. Disease sassociated with elevated levels of IL-8 in body fluids

Condition	Body fluids with elevated IL-8 levels
Endotoxinemia	blood
HIV infection	blood
Bacterial meningitis	spinal fluid
Urinary tract infections	urine
Relapsing fever	blood
Acute peritonitis on CAPD patients	peritoneal fluids
Pleural mepyema	pleural fluid
Gouty arthritis	joint fluid
Rheumatoid arthritis	joint fluid
Psoriasis	skin lesion
Contact dermatitis	skin lesion
ARDS	BALF
Idiopathic pulmonary fibrosis	BALF
Acute myocardial infarction	blood
Rejection after transplantation	blood, urine
Kawasaki disease	blood
Inflammatory bowel diseases	blood
Glomerulonephritis	urine
Parturition	amniotic fluid

Abbreviations used in this table are as follows: ARDS, adult respiratory distress syndrome; BALF, bronchial alveolar lavage fluid; CAPD, continuous ambulatory peritoneal dialysis; HIV, human immunodeficiency virus.

chemokines also has a wide variety of effects on immunoregulation, under physiological as well as pathological conditions.

Role of IL-8 in diseases (Table 3)

Most cases of acute bacterial infections are associated with either neutrophilia or neutrophil infiltration into the lesions. Intravenous injection of LPS caused elevation of plasma levels of pro-inflammatory cytokines, including IL-6, IL-8, TNFα, but not IL-1 [104]. Urinary levels of IL-8 increased in most cases of urinary tract infections, reaching 1 ng/ml without elevation of serum IL-8 levels [20]. Moreover, anti-IL-8 antibody reduced the neutrophil chemotactic activity in urines, suggesting that the increase of neutrophils in urine is ascribed to local production of IL-8 in urinary tract. The local production of IL-8 at the sites of infection was also documented on patients who experienced peritonitis during the course of continuous ambulatory peritoneal dialysis [105]. These observations suggest that the determination of IL-8 levels may be useful for diagnosis and monitoring of infectious diseases.

Urinary IL-8 levels increase in patients with several types of glomerulonephritis, including IgA nephropathy, acute glomerulonephritis (AGN), purpura nephritis, membranous proliferative glomerulonephritis (MPGN), and lupus nephritis, which are characterized pathologically by the infiltration

of polymorphonuclear cells and/or mononuclear cells and proliferation of mesangial cells [106]. The local production of IL-8 was confirmed by immunohistochemical analyses. Moreover, urinary IL-8 levels decreased upon resolution of clinical symptoms.

Several independent studies documented the occurrence of elevated levels of IL-8 in synovial fluids of rheumatoid and gouty arthritis [96, 107, 108]. The highest IL-8 levels are generally observed in sero-positive rheumatoid arthritis [109]. However, there is no apparent correlation between IL-8 levels and clinical parameters of the disease [110].

The role of IL-8 in psoriasis has been established by the observation that IL-8 was overproduced at the skin lesion of psoriasis [111]. However, the cellular source of IL-8 in psoriatic lesion is controversial at the moment [112,113].

Serum IL-8 levels increased during the course of acute myocardial infarction [114]. There was, however, no apparent correlation between the serum IL-8 levels and severity of myocardial infarction. Several independent groups reported that IL-8 levels in BALF elevated in patients with adult respiratory distress syndrome (ARDS) [115]. Moreover, the patients who show elevated IL-8 levels in BALF at the admission are prone to be complicated with ARDS than those whose IL-8 levels in BALF did not increase [115].

Conclusions

To date, the number of members of chemokine superfamily is reaching more than 15. IL-8, a member of this family, acts on various types of cells as well as neutrophils. Accumulating evidence indicates that IL-8 is essentially involved in inflammatory reactions, particularly acute ones. Moreover, gene targeting of murine IL-8 receptor suggests its potential role in hematopoiesis and immune responses. Thus, the exploration of the action of the factor and the subsequent development of its agonist or antagonist will pave the way of novel types of therapeutic agents for hematological and inflammatory diseases.

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11. Interleukin-9: Structural characteristics and biologic properties

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Originally, Interleukin-9 (IL-9) was described as a murine T-cell growth factor produced by activated T_H2 lymphocytes and characterized by a narrow specificity for certain T-Helper clones [1]. In the presence of IL-9 containing supernatants, stable T-cell lines could be derived in the absence of feeder cells and antigen. The growth factor present in such supernatants was further purified, its corresponding cDNA was cloned, and provisionally designated P40 [2].

Independently, Hültner and colleagues reported that a factor produced by activated splenocytes was able to enhance the proliferation of mast-cell lines induced by IL-3 or IL-4 [3,4]. This activity, designated MEA (Mast-cell growth Enhancing Activity) was also found in supernatants from a murine T-Helper cell line derived by Schmitt and collaborators, who had observed that these cells produced a T-Cell growth factor which was called TCGF-III [5]. The molecular cloning of a murine P40 cDNA and the availability of recombinant protein led to the demonstration that the same factor, namely P40/IL-9, was responsible for all these biological activities [6].

In humans, IL-9 was initially identified and cloned by Yang and colleagues as a mitogenic factor for a human megakaryoblastic leukemia [7], while the same human cDNA was isolated by cross-hybridization with the mouse IL-9 probe [8]. More recently, IL-9 targets were found to encompass erythroid progenitors [9], human T cells [10], B cells [11,12], foetal thymocytes [13], thymic lymphomas [14], and immature neuronal cell lines [15].

Molecular and structural characterization of IL-9

The purification of mouse IL-9 from the supernatants of activated helper T cells was made possible by the use of stable factor-dependent T-cell lines, derived from normal antigen-dependent clones. The purified protein, originally designated P40 on the basis of its apparent size in gel filtration, was characterized by an elevated pI (~10) and a high level of glycosylation [1]. The amino acid sequence has been obtained by the complete sequencing

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