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Transcytosis and Surface Presentation of IL-8 by Venular Endothelial Cells

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Summary

Chemokines have been convincingly implicated in actuating inflammatory leukocyte emigration. To affect the circulating leukocytes, tissue-derived chemokines have to traverse the endothelial cells (ECs). This was thought to be accomplished by chemokine diffusion through the intercellular gaps. On the contrary, we show by electron microscopy that the prototype chemokine IL-8 is internalized by venular ECs abluminally and transcytosed to the luminal surface. Here, it is presented to the adherent leukocytes on the EC membrane, predominantly in association with the EC projections. The intact C terminus of IL-8, the molecule's "immobilization" domain, is required for the EC binding, transcytosis, and consequently, the in vivo proemigratory activity of IL-8, indicating that the described subcellular interactions of IL-8 with the ECs are functionally relevant.

Introduction

Inflammatory diseases are characterized by the egress of leukocytes from the circulation across the walls of small blood vessels into the affected tissues. Chemoattractants, including the prototype chemokine (chemotactic cytokine) interleukin-8 (IL-8), have conventionally been thought to drive leukocyte emigration (Baggiolini et al., 1994; Schall, 1994; Strieter et al., 1996). The entire process of inflammatory neutrophil recruitment can be emulated by the injection of IL-8 into the tissues (Colditz et al., 1990; Leonard et al., 1991; Swensson et al., 1991). A prerequisite of neutrophil emigration is their attachment to the EC surface. According to the current paradigm, this interaction involves a stepwise engagement of juxtaposed neutrophil- and EC-adhesion molecules: first selectins and their sugar counterligands, which mediate neutrophil tethering and rolling, followed by β2 integrins and immunoglobulin-like intercellular adhesion molecules, which mediate firm neutrophil adhesion (Butcher, 1991; Carlos and Harlan, 1994; Springer, 1994; Imhof and Dunon, 1995). The induction of the integrin-mediated firm neutrophil adhesion to the ECs requires rapid activation of β2 integrins. This can be achieved by stimulation of leukocytes with chemoattractants, including IL-8 (Carveth et al., 1989; Detmers et al., 1990). The most prominent in vitro effect of chemoattractants is the induction of directed leukocyte migration, i.e., chemotaxis, which by definition is a response to gradients of soluble molecules (Rot, 1992a). Therefore, leukocyte-EC adhesion and emigration have also traditionally been thought to be induced by soluble chemoattractants (reviewed by Colditz, 1985; Rot, 1992a). However, it has been noted that soluble chemoattractant gradients cannot persist on the blood-EC interface: they are likely to be washed away by the blood flow (Colditz, 1985; Rot, 1992c; Tanaka et al., 1993b). Also, soluble chemoattractant gradients would activate leukocytes in circulation before their initial selectin-mediated adhesive interaction with the ECs. Such activation results in the loss of leukocytes' ability to initiate EC adhesion and emigrate (Ley et al., 1993). Due to these theoretical considerations, the observation of the in situ IL-8 binding to the ECs in human skin (Rot, 1992b), and the ability of the immobilized IL-8 to attract leukocytes in vitro (Rot, 1993a), it was suggested that IL-8 and other chemokines may promote the leukocyte-EC adhesion in vivo more effectively while being immobilized on the EC membrane (Rot, 1992c; Tanaka et al., 1993b). Whereas this idea has been rapidly accepted and is featured in several comprehensive reviews (Carlos and Harlan, 1994; Springer, 1994; Imhof and Dunon, 1995; Schall and Bacon, 1994; Bacon and Schall, 1996; Butcher and Picker, 1996), the hypothesis has not been formally proven: neither chemokine immobilization on the luminal EC membrane nor its presentation to the adherent leukocytes has been observed experimentally. To study the subcellular events that underlie chemokine-induced leukocyte-EC adhesion and emigration, IL-8 and ^{125}I-IL-8 injections in rabbit skin have been followed by immunoelectron microscopy (IEM) and electron microscopic autoradiography (EMA), respectively. These experiments allowed us to address the subcellular fate of IL-8 directly in the process of leukocyte emigration and lead to the observation of chemokine presentation on the luminal EC membrane and also to the description of EC transcytosis of IL-8 in the abluminal-to-luminal direction.

Results and Discussion

EC Surface Presentation of IL-8

After intradermal injection in rabbits, IL-8 immunoreactivity was detected by IEM at all the time points studied in the walls of postcapillary venules and small veins, but not any other blood vessels. This is the segment of the circulatory tree in which leukocytes adhere to the ECs and emigrate in clinical and experimental inflammatory lesions and in response to injections of chemoattractants (Colditz et al., 1990; Rot, 1992a; Ebnet et al., 1996). IL-8 bound to the ECs, pericytes, and other perivascular

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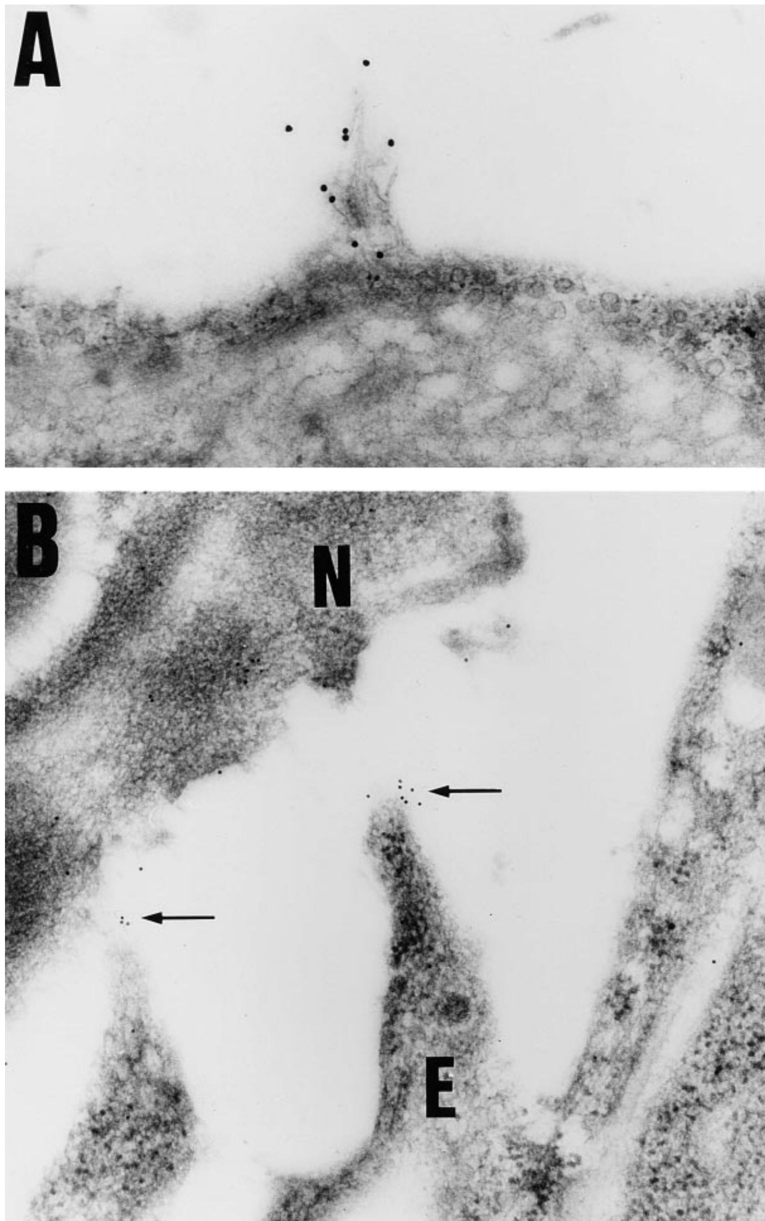

Figure 1. IL-8 Binding to the Luminal Surface of the Venular ECs in Rabbit Skin

(A) IL-8, seen as electron-dense grains, appears on a microvillous-like extension of the luminal EC surface (magnification ×68,800). (B) Chemokine presentation by the EC (E) to an adherent neutrophil (N). IL-8 (arrows) is concentrated on the tips of EC projections; in the region of direct cell contact, IL-8 is also visible on the neutrophil surface (×53,680).

cells; IL-8 was also seen in the dermal macrophages and in the extracellular matrix, predominantly around the blood vessels, in part associated with collagen fibers and in part with the ground substance. Already 30 minutes after its injection, IL-8 immunoreactivity was observed on the luminal EC surface where, at all time points studied, it was particularly concentrated on the microvillous processes (Figure 1A) and tips of larger projections that extended into the luminal space (Figure 1B). Approximately ten times more IL-8 immunoreactivity was present on luminal projections than on the smooth EC membrane, with two out of three observed microvilli harboring IL-8 (Table 1). Similar preferential distribution to the tips of leukocyte microvilli has been described for several adhesion molecules involved in primary juxtacrine interaction with the ECs, e.g., L-selectin, P-selectin ligand, E-selectin ligand, and $\alpha4$ integrins (Picker et al., 1991; Berlin et al., 1995; Moore et al., 1995; Steegmaier et al., 1997). It is possible that microvilli and EC projections may represent analogously salient sites, since, like the leukocyte microvilli, they may preferentially harbor adhesion molecules, e.g., a counterligand of L-selectin, sialomucin CD34 (Fina et al., 1990), and constitute the initial points of contact between the endothelium and the adherent leukocytes. At the points of such direct neutrophil-EC contact, IL-8 was also associated with the neutrophil membrane (Figure 1B). This embodies the conjectural EC presentation of chemokine immobilized on the EC surface (Rot, 1992c; Tanaka, 1993b). There are indications that the localization to the microvilli is of great importance for the in vivo function of adhesion molecules. For example, the L-selectin-CD44 chimera that is excluded from the leukocyte microvilli, despite its ability to initiate EC adhesion under static

Table 1. Distribution of IL-8 Immunoreactivity on the EC Surface in Rabbit Venules

Venule	Time (min)	Flat Membrane Length (μm)	Flat Membrane Number of Grains	Flat Membrane Grains/ 10 μm	Microvillous Membrane Length (μm)	Microvillous Membrane Number of Grains	Microvillous Membrane Grains/ 10 μm	Number of Microvilli Immunopositive	Number of Microvilli Immunonegative
1.	30	18.2	4	2.2	53.3	28	5.3	3	3
2.	30	30.5	4	1.3	30.8	17	5.5	5	0
3.	60	36.1	1	0.3	22.2	6	2.7	4	3
4.	60	13.1	0	0	46.8	8	1.7	3	1
5.	60	9.4	0	0	25	38	15.2	5	2
6.	60	45	9	2.0	3.3	14	42.0	2	0
7.	60	19	3	1.6	24.1	11	4.6	2	3
8.	120	30.4	4	1.3	31.1	39	12.5	8	4
9.	120	47.1	2	0.4	34.1	10	2.9	1	2
10.	120	34.7	0	0	62.2	6	1.0	2	2
11.	120	40.3	2	0.5	20	14	7.0	3	0
12.	120	25.1	0	0	22.8	10	4.4	2	0
Mean ± SEM	—	29 ± 3.6	2.4 ± 0.8	0.8 ± 0.2	31 ± 4.6	16.8 ± 3.4	8.7 ± 3.2	3.3 ± 0.6	1.7 ± 0.4

IL-8 was injected into rabbit skin and detected using IEM. The length of cell membrane of microvilli and projections (microvillous membrane) and of the flat portions was measured using an electronic measuring device (onLine 5, Kasper and Richter, Germany). The distribution of gold grains on the luminal membrane was assessed in 12 random venules that contained more than 5 luminal grains. The concentration of grains on microvillous membrane (grains/μm) was significantly higher than on flat portions ($p < 0.05$ in t-test). Significantly more microvilli harbored IL-8 than did not ($p < 0.05$ in t-test).

conditions, fails to tether leukocytes under the conditions of lateral shear stress (von Andrian et al., 1995). It is attractive to postulate that the localization of chemokines to the EC microvilli, described here, may be equally important for their successful presentation to the rolling leukocytes and their in vivo proemigratory activity.

EC Transcytosis of IL-8

In inflammation, ECs fulfill the task of conveying multiple molecular signals from the tissues to the circulating leukocytes that lead to their adhesion and emigration. Two alternative ways of "information transfer" by the ECs can be envisaged: the direct posting of soluble mediators derived from the surrounding tissue and the production by the ECs of secondary cell surface–expressed and soluble effector molecules, including IL-8 (Ebnet et al., 1996). To show unequivocally that it was the injected and not secondary, EC-produced chemokine that we have observed by IEM on the luminal cell surface, radiolabeled IL-8 was injected into the rabbit skin and its subcellular localization was followed by EMA. This method yielded similar results to those obtained by IEM (Figure 2), indicating that injected IL-8 rapidly crosses the vessel wall and appears on the luminal EC surface. Likewise, the IL-8 produced by extravascular cells (e.g., macrophages, fibroblasts, epithelial cells, etc.) to exert its proemigratory effect must traverse the EC barrier. Theoretically, there are several ways in which this could be achieved: transcellular transport, pericellular diffusion between the ECs, or movement in the plane of the cell membrane. However, it has been generally assumed that chemokines reach the circulation by diffusion through the gaps between the ECs (reviewed by Furie and Randolf, 1995; Ebnet et al., 1996). The IEM study of the IL-8-injected rabbit skin allowed us to observe the mechanism of chemokine transport. In addition, IL-8 was injected in vitro into intact viable human skin that was removed during elective surgery. In both experimental systems, we observed that IL-8, following its binding to the abluminal EC surface, was internalized and incorporated into smooth membrane-bound plasmalemmal vesicles measuring 40–70 nm (Figures 3A–3C) that contained caveolin (Figure 3F), and accumulated primarily in basal and apical portions of the EC cytoplasm. Such vesicles have previously been shown to increase in number following intradermal injection of IL-8 (Schubert et al., 1989). In addition, IL-8 was contained in larger electron-lucent vesicles of greater than 100 nm diameter (Figure 3D). Upon fusion of both types of vesicles with the apical cell membrane, IL-8 was observed on the luminal surface (Figure 3E). At different time points after its injection, the subcellular localization of IL-8, quantitative differences aside (Table 2), was similar. No IL-8 was detected at the junctions themselves or in the luminal clefts between the cells adjacent to the junctions.

The localization of IL-8 within the vesicular compartment of the EC cytoplasm and the time course of its accumulation on the luminal EC surface (Table 2) is consistent with the transcytotic route of chemokine transport. Plasmalemmal vesicles (caveolae) are known to serve as vehicles of EC transport of several molecules, though primarily in capillaries and arteries and in the luminal-to-abluminal direction (Vasile et al., 1983; Ghitescu et al., 1986; Milici et al., 1987; Parton et al., 1994; Pedrescu et al., 1994, Schnitzer and Oh, 1996). In other polar cells, e.g., epithelial cells, transcytosis is an important mechanism of molecular transport across the cellular barriers and takes place, with some exceptions, primarily in the basal-to-apical direction (Mostov, 1994). Even cells that are strewn in the tissues and do not form confluent barriers, e.g., macrophages and osteoclasts, transcytose their metabolites rather than allow them to diffuse pericellularly (Nesbitt and Horton, 1997; Salo et al., 1997).

The exact subcellular sequelae of caveolae-driven EC transcytosis are subject to ongoing debate. Two alternative, mutually nonexclusive mechanisms of EC transcytosis have been envisaged. The first implies an active shuttle-like movement of cargo-loaded vesicles between the EC surfaces (Ghitescu et al., 1986; Parton et

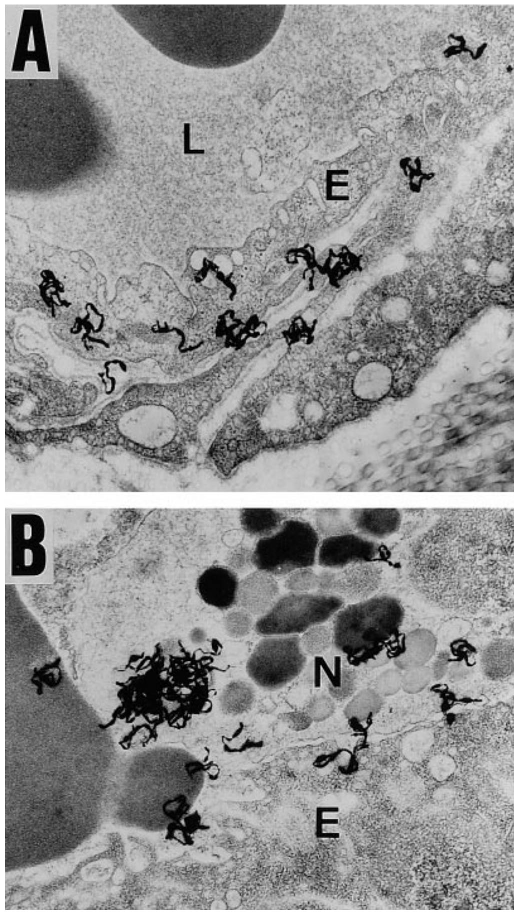

Figure 2. Autoradiographic Localization of ^{125}I-IL-8 in Rabbit Skin

(A) Radiolabel is observed within the venular EC (E) and at the luminal surface (L), as well as in the vessel wall, on the surface of pericytes, and in the endothelial basement membrane (×17,420).

(B) Adherent neutrophil (N) in close contact with the luminal surface of an EC (E). ^{125}I-IL-8 is present at the interface between the two cells and has been internalized by the neutrophil (×17,420).

al., 1994; Pedrescu et al., 1994; Schnitzer et al., 1996). Recently, it has been shown that caveolae contain the molecular machinery required for budding, movement, and docking with the cell membrane (Schnitzer et al., 1995) and that their fission from the cell surface is induced by the hydrolysis of guanosine triphosphate (Schnitzer et al., 1996). An alternative mechanism implies that molecules may diffuse passively through the pores that form by fusion of the individual vesicles. In the microvascular ECs, caveolae were described to fuse with each other and with larger vacuoles to form grape-like conglomerate structures, the vesiculo-vacuolar organelles, which, according to the three-dimensional reconstruction studies, can span the EC cytoplasm and connect luminal and abluminal EC surfaces (Kohn et al., 1992; Dvorak et al., 1996; Feng et al., 1996). Under different circumstances, either of the two alternative mechanisms of transcytosis or both may take place; our data do not allow us to judge which one is used for the transport of IL-8 from the abluminal to the luminal EC surface.

Molecules Involved in EC Binding and Transcytosis of IL-8

Because chemokines bind heparin and related glycosaminoglycans, it was suggested that heparan sulfate (HS), which is abundantly present on the EC surface, immobilizes chemokines (Rot, 1992b, 1993b; Tanaka et al., 1993a, Webb et al., 1993; Witt and Lander, 1994). The digestion of frozen sections of IL-8-injected human skin by heparitinase, an enzyme that specifically hydrolyzes HS, reduced the EC-associated IL-8 immunoreactivity, including that on the luminal membrane (Figure 4). This supports the involvement of HS in IL-8 presentation and transcytosis by the EC. HS participates in the EC endocytosis and transcytosis of several other molecules including transferrin, lipoprotein lipase, and angiogenin (Omoto et al., 1990; Nakazono et al., 1991; Saxena et al., 1991; Moroianu and Riordan, 1994). HS molecules on the luminal EC surface, besides immobilizing chemokines, may play an additional role in the process of leukocyte emigration since they can contribute to several steps of leukocyte-EC adhesion by serving as counterligands for leukocyte L-selectin, the β2 integrin Mac-1, and PECAM-1 (DeLisser et al., 1993; Norgard-Sumnicht et al., 1993; Diamond et al., 1995).

However, HS is not the sole EC molecule with chemokine binding properties; other molecules may contribute to the EC transcytosis of chemokines. Duffy antigen/receptor for chemokines (DARC), which was initially described on the erythrocyte surface (Darbonne et al., 1991; Horuk et al., 1993), has recently been found on the ECs of postcapillary venules (Hadley et al., 1994; Peiper et al., 1995). It binds members of both α- and β-chemokine subfamilies, but not MIP-1α (Neote et al., 1993; Szabo et al., 1995). This chemokine binding profile is mirrored in situ by the ECs. RANTES, a β-chemokine, cross-competes with IL-8 for the EC binding (E. H. and A. R., unpublished data) and localizes to the same subcellular EC compartments as IL-8 (Figure 5). In contrast, we were not able to observe MIP-1α localization to the EC after its intradermal injection, while it has been detected in resident macrophages (Figure 5F). Also, MIP-1α failed to associate with ECs in the in situ binding assay (E. H. and A. R., unpublished data). Since, akin to other chemokines, MIP-1α binds heparin (Koopmann and Krangel, 1997), it is the "fingerprint" of DARC's specificity that can be recognized in the pattern of chemokine binding by the ECs. Future experiments should decide whether DARC plays a role in chemokine transcytosis and presentation by the ECs and how DARC and HS may cooperate in these two functions.

IL-8 Immobilization Is Required for Its EC Transcytosis and In Vivo Activity

IL-8 has two spatially parted functional domains: the N terminus, which is required for the elicitation of the leukocyte responses through the G protein–coupled receptors (Clark-Lewis et al., 1991), and the C-terminal α helix, which serves as an "immobilization" domain mediating the binding of IL-8 to HS (Webb et al., 1993; Witt and Lander, 1994) and possibly other molecules. We used a chemically synthesized, C-terminally truncated IL-8 analog IL-8(1–63) that has retained neutrophil-activating characteristics (Clark-Lewis et al., 1991)

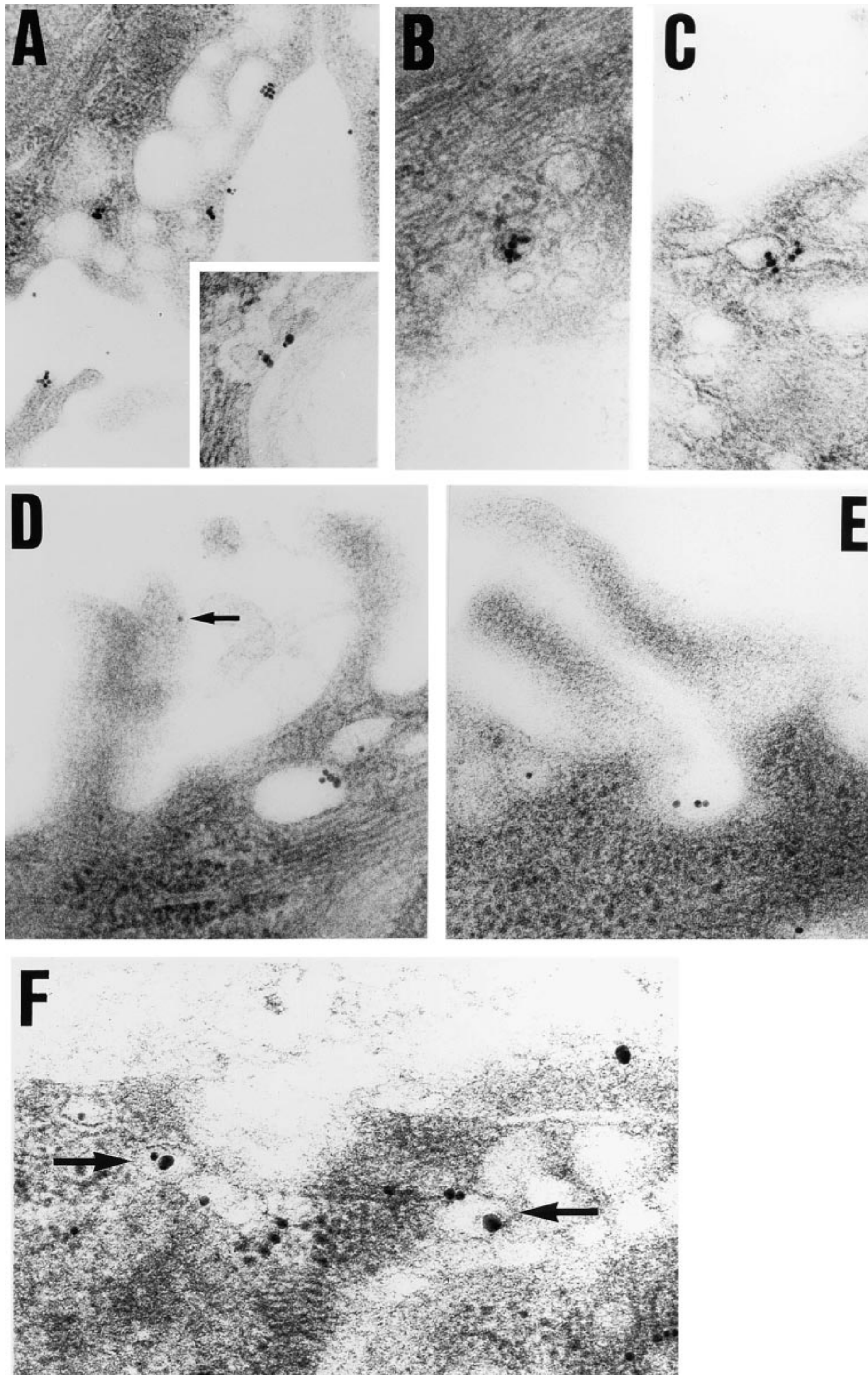

Figure 3. Transcytosis of IL-8 by Venular ECs in Human Skin

(A) IL-8 immunoreactivity at the abluminal surface of the EC. The inset shows the uptake of IL-8 into a plasmalemmal vesicle at the abluminal EC membrane.

(B and C) IL-8 in plasmalemmal vesicles in apical and basal portions of EC cytoplasm, respectively.

(D) IL-8 in large electron-lucent vesicles and on the cell surface (arrowed).

(E) Appearance of IL-8 on the luminal surface of the endothelium.

(F) Colocalization of IL-8 and caveolin in uncoated plasmalemmal vesicles. IL-8 and caveolin are visualized by 10 nm silver-enhanced gold and 5 nm silver-enhanced gold grains, respectively. Plasmalemmal vesicles that contain IL-8 and caveolin are arrowed. (Magnifications: [A], ×63,990; inset, ×113,400; [B], ×115,830; [C], ×91,530; [D], ×102,060; [E], ×103,680; [F], ×122,472.)

Table 2. Distribution of IL-8 and IL-8(1–63) Immunoreactivity in Venular ECs of Human Skin

Time	Chemokine	Abluminal Surface (particles/10 μm)	Intracellular (particles/10 μm²)	Luminal Surface (particles/10 μm)
0′	Uninjected	0.4 ± 0.3	1.8 ± 0.8	0.4 ± 0.2
60′	Uninjected	0.6 ± 0.3	1.3 ± 2.9	0.5 ± 0.3
0′	IL-8	1.6 ± 0.4	3.7 ± 1.0	1.1 ± 0.5
30′	IL-8	1.9 ± 0.6	9.8 ± 1.4	3.5 ± 0.7
60′	IL-8	5.8 ± 1.5	12.7 ± 3.0	4.4 ± 1.1
120′	IL-8	7.8 ± 1.1	23.7 ± 3.9	11.7 ± 2.0
0′	IL-8(1–63)	0.7 ± 0.4	4.2 ± 0.8	0.8 ± 0.3
30′	IL-8(1–63)	2.8 ± 1.1	5.6 ± 2.0	1.0 ± 0.5*
60′	IL-8(1–63)	1.5 ± 0.6*	5.3 ± 1.3*	0.2 ± 0.1*
120′	IL-8(1–63)	2.1 ± 0.7*	4.1 ± 1.1*	1.0 ± 0.4*

Chemokines were injected in human skin and detected using IEM. All values are after subtracting the individual background control values that were obtained using equimolar normal rabbit immunoglobulin instead of anti-IL-8. Control means were 1.9 ± 0.7, 1.5 ± 0.4, and 1.4 ± 0.6 for abluminal, intracellular, and luminal immunoreactivity, respectively. Asterisks mark IL-8(1–63) values significantly lower than corresponding IL-8 values ($p < 0.05$ in t-test).

but has impaired heparin binding (Webb et al., 1993) to evaluate the requirement of IL-8 immobilization for its subcellular interactions with the ECs and in vivo biological activity. IL-8(1–63) was injected into human skin, and its binding and transport by the ECs was compared using IEM to that of native IL-8 (Table 2). Initially, the amount of IL-8(1–63) that bound to the abluminal EC surface was not significantly different from that of IL-8. However, later, the levels of both abluminal and intracellular, vesicle-associated IL-8(1–63) were significantly lower than those of IL-8. The amount of IL-8 on the luminal surface increased with time; in contrast, IL-8(1–63) at all the time points was barely detectable on the luminal cell surface (Table 2). Thus, EC endocytosis of IL-8 is not happening by nonspecific fluid uptake via caveolae but requires binding to the EC membrane via the C terminus of the chemokine. The functional importance of IL-8 immobilization in the process of in vivo leukocyte emigration is underscored by the decreased proemigratory activity of IL-8(1–63) in comparison with native IL-8 (Figures 6A and 6B). This parallels the reduced potency of IL-8(1–63) observed in in vitro neutrophil migration assay (Figure 6C) and contrasts the similar potencies of the two chemokines for neutrophil enzyme release (Figure 6D), which is not dependent on IL-8 immobilization. The decreased in vivo and in vitro migratory activities of IL-8(1–63) directly correspond to its reduced (in comparison with IL-8) binding to HS (Figure 6E) and polycarbonate filter (Figure 6F), respectively. Thus, similarly to IL-8-induced in vitro leukocyte migration, which happens in response to surface-bound chemokine molecules by haptotactic mechanism (Rot, 1993a), in vivo leukocyte emigration also requires chemokine immobilization by the ECs. Furthermore, these data also suggest that our morphological observations of the subcellular IL-8 interactions in venular ECs reflect functionally relevant processes.

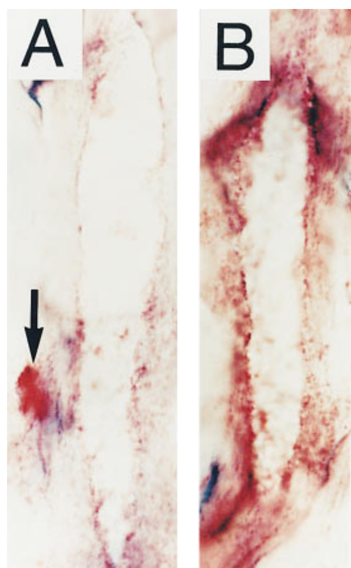

Figure 4. Reduction of the EC-Associated IL-8 Immunoreactivity by Heparitinase Treatment

Heparitinase treatment (A) diminished IL-8 immunoreactivity in venular ECs but not resident dermal cells (arrowed) in comparison with the chondroitinase-treated section (B) or buffer-treated control (not shown; [A] and [B], ×781).

Cultured ECs Do Not Bind IL-8

For studying the EC binding and endocytosis of IL-8, we had to rely completely on the in situ methods: all of the in vitro-propagated ECs tested by us, including those derived from skin microvasculature and umbilical veins, failed, in agreement with previous reports (Watson et al., 1995; Petzelbauer et al., 1995), to show specific IL-8 binding (E. H. and A. R., unpublished data). In contrast to microvascular ECs, which rapidly lose their IL-8-binding ability in the process of culture, ECs in intact umbilical veins do not bind IL-8 in situ (data not shown). These findings emphasize the heterogeneous nature of ECs in different organs and segments of the vascular tree and caution that the results of the studies that use cultured ECs to investigate their interaction with chemokines may not faithfully reflect the in vivo situation. Most in vitro experimental systems that model chemokine-induced leukocyte-EC adhesion fail to account for the fact that in vivo leukocytes encounter chemokines in complex with presentation molecules. The inability of

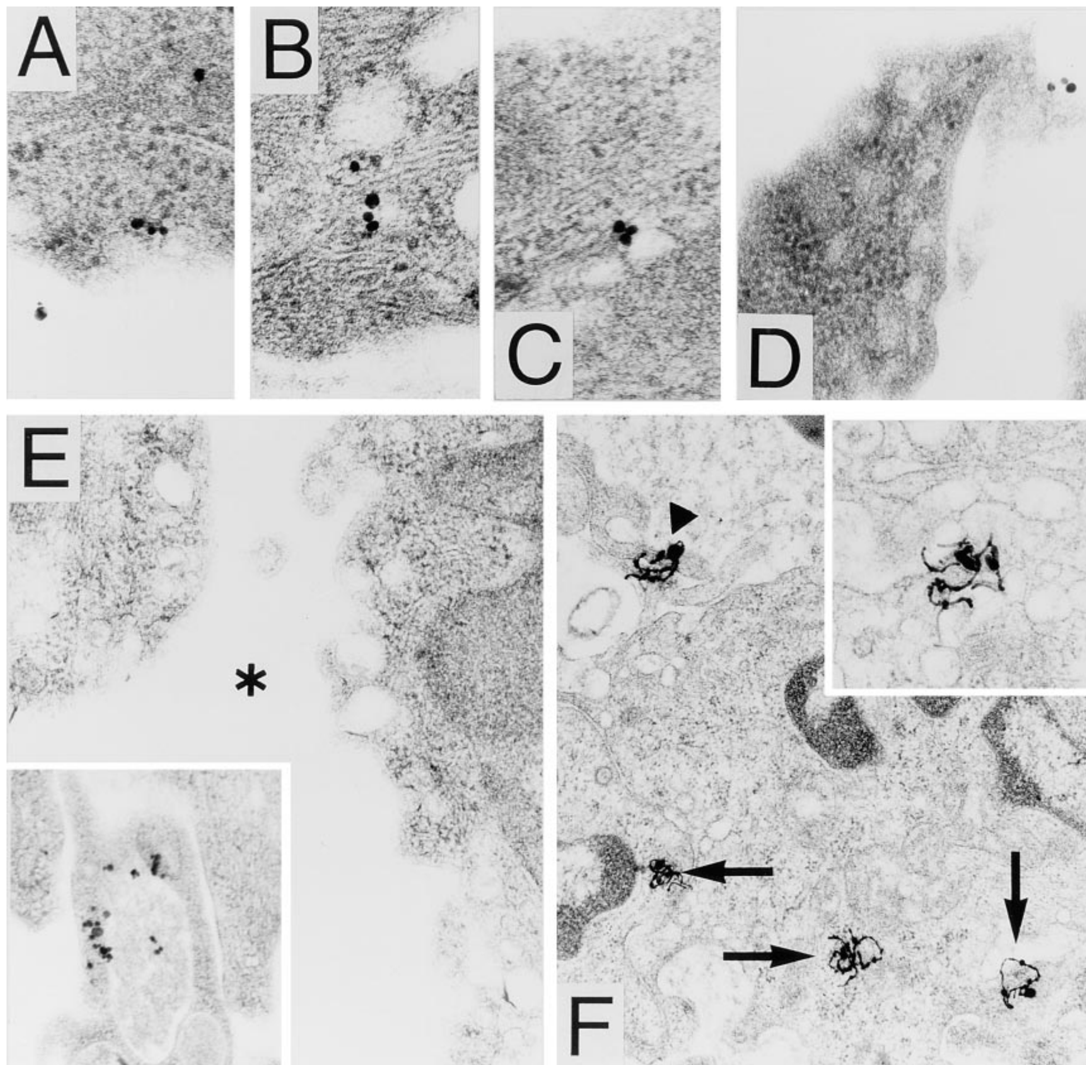

Figure 5. EC Transcytosis of RANTES

EC uptake of RANTES at the abluminal EC membrane (A); RANTES in plasmalemmal vesicles in apical and basal portions of EC cytoplasm ([B] and [C], respectively) and on the tip of luminal projection (D). Lack of EC-associated MIP-1α immunoreactivity following its injection in human skin ([E]; asterisk marks the lumen; inset shows a detail of an MIP-1α-positive macrophage. (F) RANTES EMA; vertical arrow, horizontal arrows, and arrowhead show abluminal, intracellular, and luminal radiolabel, respectively; inset shows detail of an intracellular label. ([A]–[C], ×98,900; [D], ×77,400; [E], ×54,040; inset, ×43,000; [F], ×27,520; inset, ×43,000).

the cultured ECs to couple chemokines with presentation molecules and display them on microvilli may explain why the EC adhesion of lymphocytes is not induced efficiently by chemokines in vitro (Butcher and Picker, 1996). The association with EC presentation molecules, HS in particular, may increase the chemotactic efficacy of chemokines (Webb et al., 1993).

In conclusion, we describe two novel subcellular mechanisms of IL-8 interaction with postcapillary venular ECs in the process of leukocyte emigration: EC transcytosis of chemokine and its luminal surface presentation to the adherent leukocytes on the tips of EC microvilli. We show that IL-8 immobilization via its C terminus is required for the induction of neutrophil emigration and postulate the cooperative involvement of HS and DARC in mediating chemokine interaction with the ECs.

Experimental Procedures

Intradermal Chemokine Injections in Rabbits

For IEM, human recombinant IL-8 (Novartis Forschungsinstitut [NFI], Vienna, Austria) was injected intradermally, 1 μg/site, in three Chinchilla rabbits (Charles River, Wiga, Germany); duplicate biopsies of the sites were taken 4 hr, 2 hr, 1 hr, 1/2 hr, and immediately after the injection (0 hr). Vehicle-injected sites sampled at the same time points and noninjected skin served as controls. For EMA, Chinchilla rabbits received 5 ng/site of either human recombinant ^{125}I-IL-8 or ^{125}I-RANTES (DuPont NEN, Vienna; specific radioactivity 250 μCi/μg) injected into eight intradermal sites. Duplicate sites were punch-biopsied at 4 hr, 2 hr, 1 hr, and 1/2 hr after the injection. Vehicle-injected sites sampled at the same time points and noninjected skin served as controls.

Ex Vivo Chemokine Injections in Human Skin

Pieces of normal human skin were obtained after informed consent from four healthy patients undergoing elective surgery. Immediately

upon removal, 100 ng/site of IL-8, IL-8 (1–63), RANTES, or MIP-1α (Peprotech, London, England) was injected into multiple sites and skin was incubated at room temperature in a RPMI1640 bath. Duplicate sites were cut out and processed for IEM 2 hr, 1 hr, 1/2 hr, and immediately after the injection (0 hr). Vehicle-injected skin samples and uninjected skin served as controls. For the enzyme digestion studies, the IL-8-injected human skin was snap-frozen 2 hr after the injection.

IEM

A modification of the published procedure (Fina et al., 1990) was used. Samples were fixed for 60 min in 2% paraformaldehyde with 0.05% glutaraldehyde in PBS, washed and dehydrated in gradually increasing concentrations of ethanol (30%, 50%, 70%, and 100%) at simultaneously decreasing temperatures (0°C, −15°C, −30°C, and −50°C), and infiltrated at -50°C with Lowicryl HM 20 (Agar Scientific, Stansted, UK) by gradually increasing its concentration (25%, 50%, and 75% for 60 min, 100% overnight). The blocks were polymerized under ultraviolet light for at least 48 hr at −50°C. Dehydration, infiltration, and polymerization were performed in Leica AFS (Leica, Vienna, Austria). Sections 100 nm thick were cut and collected on uncoated nickel grids that were placed in blocking buffer (PBS, 5% FCS, 0.8% BSA, 0.1% fish gelatin [pH 7.4]) for 30 min and then immersed overnight in anti-IL-8 mouse monoclonal or rabbit polyclonal antibodies (NFI). After repeated washes, sections were incubated for 90 min in 5 or 10 nm gold-labeled goat anti-mouse IgG (Amersham, Bucks or Biocell, Cardiff, both UK) and 5 nm gold-labeled goat anti-rabbit IgG (Amersham), respectively. Grids were washed and 5 nm gold conjugates were silver-enhanced in IntenSE (Amersham), followed by washing in ultrapure water. Sections were counterstained in uranyl acetate and lead citrate, and viewed in a Zeiss EM 10 transmission electron microscope at 80 kV. Control sections were incubated with purified mouse IgG instead of primary antibody. Subcellular localization of MIP-1α and RANTES was performed as described above using mouse monoclonal antibodies (Peprotech and a generous gift from Pete Nelson, respectively).

EMA

The tissue was processed conventionally for transmission electron microscopy (EM). It was fixed in 2.5% glutaraldehyde, post-fixed in osmium tetroxide, block-stained in uranyl acetate, dehydrated in ethanol, treated with propylene oxide, and embedded in Araldite. Ultrathin sections were coated with Ilford L-4 emulsion, exposed for 3–11 weeks, and developed in D-19 as described (Williams, 1978).

The Subcellular EC Localization of IL-8 and IL-8(1–63) in Human Skin

Both chemokines were injected in human skin and detected by IEM using the procedures described above and rabbit polyclonal anti-IL-8 antibody that recognized both IL-8 and IL-8(1–63). For both chemokines, the resulting gold granules were counted on the luminal and abluminal surfaces and in the cytoplasmic vesicles of the ECs from sixteen randomly chosen postcapillary venules in skin from two different individuals. Results were expressed as the number of

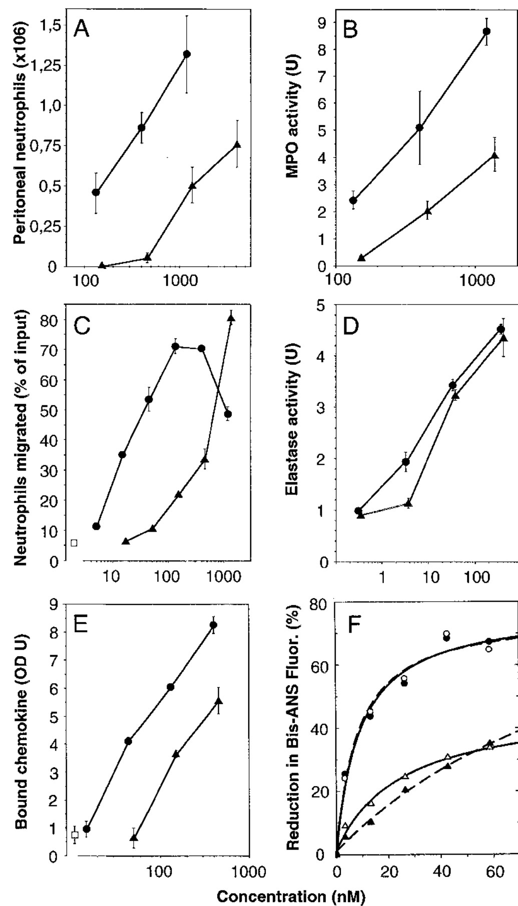

Figure 6. In Vivo and In Vitro Activities of IL-8 and IL-8(1–63)

(A) Chemokine-induced neutrophil emigration in mice. Balb/C mice received intraperitoneal injections of IL-8 and IL-8(1–63). After 3 hr, the peritoneal cavities were lavaged and leukocytes counted. Values are the mean ± SEM of three experiments. No neutrophils emigrated following vehicle injection.

(B) Chemokine-induced neutrophil emigration in primate skin. Two rhesus monkeys (Macacca mulata) received intradermal injections of IL-8 and IL-8(1–63) into triplicate sites. After 4 hr, the injection sites were punched out and homogenized, and the myeloperoxidase (MPO) activity was determined. The mean ± SEM in triplicate injection sites in one monkey are shown; MPO in vehicle-injected sites was 0.06 ± 0.03 U.

(C) Chemokine-induced in vitro neutrophil migration. Human neutrophils were isolated and their migration was tested in Boyden-type chambers. One of three experiments performed on cells from different donors is shown. Values are mean for duplicate wells; error bars show SD.

(D). Chemokine-induced in vitro release of elastase from human neutrophils was recorded by monitoring the hydrolysis of an elastase-specific substrate. The values are the mean ± SEM of determinations on neutrophils from three different donors. Vehicle induced the release of 0.9 ± 0.02 U of elastase.

(E) Binding of chemokines to polycarbonate filter. The amount of chemokines IL-8 and IL-8(1–63) that bound to the nucleopore filter during 20 min incubation was assessed using rabbit anti-human IL-8 antibodies. The values for IL-8(1–63) were multiplied by the detection correction factor of 1.23.

(F) Binding of HS to IL-8 and IL-8(1–63). A novel fluorescence assay allowed the quantitation of HS binding to IL-8 and IL-8(1–63) without labeling of the chemokines or HS. Dissociation of complexes between 1 μM IL-8 and 6 μM bis-ANS was induced by increasing concentrations of HS. The resulting reduction in bis-ANS intensity was detected either via fluorescence resonance energy transfer (FRET) from tryptophan 57 or by direct excitation and emission of bis-ANS. These were consistent with respective dissociation constants (Kd) of 8.0 ± 2.8 μM and 8.9 ± 3.3 μM. The same IL-8(1–63), bis-ANS, and HS stoichiometry revealed differences between the apparent Kds obtained by FRET (24.3 ± 8 μM), and direct bis-ANS fluorescence (118.7 ± 66 μM). This difference can be due to (a) considerable change in the relative position of the tryptophan's indol fluorophore due to restructuring of C-terminal a helix of IL-8(1–63) or (b) binding of bis-ANS at different positions within IL-8(1–63). In conclusion, IL-8(1–63) showed 3- to 14-fold lower affinity for HS than IL-8.

Circle, IL-8; triangle, IL-8(1–63); square, HBSS control; on (F), solid symbols, excitation 392 nm; empty symbols, −284 nm. Abscissa on (A)–(E), chemokine concentration, and on (F), HS concentration.

gold particles per unit surface length or per unit area of the cytoplasm. Sections of the uninjected sites had been processed and counted identically. The loss of antigenicity resulting from truncation of nine amino acids in IL-8(1–63) was quantified by drying equal amounts of both chemokines onto formvar-carbon-coated grids and processing them as for IEM. The gold particles were counted in 15 ×80,000 fields. The mean ± SEM of 47.4 ± 3.6 and 38.5 ± 5.3 gold particles for IL-8 and IL-8(1–63), respectively, resulted in the detection correction factor of 1.23 by which all of the values for IL-8(1–63) were multiplied.

Colocalization of IL-8 and Caveolin

Thin sections of ex vivo IL-8-injected human skin were incubated with monoclonal mouse anti-human IL-8 and polyclonal rabbit anti-caveolin antibodies (Transduction Laboratories, Lexington, KY) that were detected with goat anti-mouse 10 nm gold-labeled and goat anti-rabbit 5 nm gold-labeled secondary antibodies, respectively, as described above.

Susceptibility of EC Localization of IL-8 to Hydrolytic Digestion

Frozen sections (10 μm) of ex vivo IL-8-injected human skin were acetone-fixed for 10 min and incubated for 30 min at 37°C in either TRIS (pH 7.5), chondroitinase ABC, or heparitinase (both 50 U/ml; Seikagaku, Tokyo, Japan). Following the treatment, the slides were washed extensively in TRIS and incubated with mouse monoclonal anti-IL-8 antibody (Novartis Forschungsinstitut), binding of which was detected using APAAP kit (Dako, Glostrup, Denmark). The slides were studied under an Olympus BX60 microscope.

Chemokine-Induced Neutrophil Emigration in Mice

Balb/C mice (Charles River) received intraperitoneal injections of IL-8, IL-8(1–63), or vehicle control. After 3 hr, the peritoneal cavities were lavaged and total and differential counts of leukocytes in the lavage fluid were performed. The response to each tested concentration of chemokines was determined in three animals.

Chemokine-Induced Neutrophil Emigration in Primate Skin

Two rhesus monkeys (Macacca mulata) received intradermal injections of IL-8 , IL-8(1–63), and vehicle control into triplicate sites. After 4 hr, the injection sites were punch-biopsied, snap-frozen, and homogenized in a 50 mM potassium phosphate buffer (3 ml) containing 0.5% hexadecyltrimethylammoniumbromide (HTAB; Sigma-Aldrich, Austria) at a pH of 6.0. Samples were sonicated and snap-frozen three times before pelleting at 10,000 × g. Myeloperoxidase (MPO), representative of neutrophil numbers, was assayed by adapting the described procedure (Bradley et al., 1992). In a 96-well plate samples or MPO standard (stored as a stock solution of 5 U/mg protein at −80°C; Sigma-Aldrich) were diluted in a potassium phosphate buffer containing 0.5% HTAB at a pH of 5.4, so that each well contained 100 μl. The reaction was started by the addition of 80 μl of tetramethylbenzidine substrate and hydrogen peroxide as a ready-to-use liquid (Sigma-Aldrich). Plates were incubated for 10 min at 37°C, the reaction was stopped by addition of 90 μl of 4N sulfuric acid, and the resulting yellow color was measured spectrophotometrically at 450 nm.

Chemokine-Induced In Vitro Neutrophil Migration

Human neutrophils were isolated and their migration to IL-8 and IL-8(1–63) was tested in a modified Boyden-chamber assay as previously described (Rot, 1993a). Three experiments were performed on cells from different donors.

Chemokine-Induced Neutrophil Elastase Release

Chemokine-induced in vitro release of elastase from human neutrophils was measured essentially as previously described (Dewald and Baggiolini, 1987). Briefly, 10^6 neutrophils were resuspended in 50 μl HBSS, 2.5% BSA. After the addition of cytochalasin B (50 μl at 5 μg/ml) and the elastase-specific substrate N-succinyl-L-alanyl-L-prolyl-L-valine-7-amido-4-methylcoumarin (50 μl at 1.5 mg/ml), the cells were incubated in a microtiter plate for 15 min at 37°C. The elastase secretion was initiated by adding different concentrations of IL-8 and IL-8(1–63) and a buffer control. After 30 min, the amount of elastase released was measured; it was proportionate to the hydrolysis of nonfluorescent substrate that resulted in a fluorescent product, the amount of which was measured on a Fluoroscan II (Flow Laboratories, Irvine, Scotland) equipped with 335 nm excitation interference filter and 460 nm emission filter. Each experiment was performed on neutrophils from three donors.

Binding of Chemokines to Polycarbonate Filter

The amount of IL-8 and IL-8(1–63) that bound to the nucleopore filter during a 20 min incubation at 37°C was assessed using a rabbit-anti-human IL-8 antibody exactly as described before (Rot, 1993a). The values for IL-8(1–63) were multiplied by the detection correction factor of 1.23.

Binding of HS to IL-8 and IL-8(1–63)

The affinity of chemokine binding to HS was estimated based on the ability of HS to inhibit the hydrophobic interaction of 1,1′-bi(4-anilino)naphtalene-5,5′-disulfonic acid (bis-ANS; Rosen and Weber, 1969) with IL-8 and IL-8(1–63). Chemokine (1 μM) in PBS was incubated for 30 min with 6 μM bis-ANS in 0.06% DMSO and was then competed off by HS. Steady-state fluorescence measurements were performed on a SLM 8000C spectro-fluorometer equipped with JD-490 photomultipliers and a 450 W Xenon Arclamp (SLM Instruments, Urbana, IL) at 25°C in a 1000 TI quartz cuvette (10 mm optical pathlength). Spectral bandwidths were set to 8 and 16 nm for excitation and emission, respectively. The relative reduction in bis-ANS fluorescence intensity was detected either via fluorescence resonance energy transfer (FRET) from tryptophan 57 in IL-8 or IL-8(1–63) or by direct excitation and emission of bis-ANS in magic angle setting with the excitation wavelengths set to 284 nm and 392 nm, respectively. The data were corrected for primary inner filter effects using an empirical exponential equation (Birdsall et al., 1983). The fluorescence emission intensity of bis-ANS was integrated between 420 nm and 600 nm. The spectra were corrected for Raman scattering from the buffer solution, for IL-8–bis-ANS complex fluorescence, for a small fluorescence enhancement of bis-ANS interaction with HS, and for fluorescence of the HS (Sigma-Aldrich). For each HS concentration, the spectra were averaged over three recordings with an integration time of 2 sec and a step resolution of 2 nm. The reduction of the integrated fluorescence intensity of the chemokine bis-ANS was plotted as a function of the HS concentration (average molecular weight of 20,000 g/Mol) and fit to the equation $F = F_{min} + ((F_{max} - F_{min}) * (([IL\text{-}8] + [HS] + Kd) - (([IL\text{-}8] + [HS] + Kd)^2 - 4*[IL\text{-}8]*[HS])^{1/2})) / 2 * [IL\text{-}8]$, to result in apparent dissociation constants for chemokine interactions with HS.

In Situ Binding Assay

Normal human umbilical cords were obtained after informed consent and immediately cut into 2 mm slices with a scalpel blade. The in situ binding of ^{125}I-IL-8 (DuPont) to intact cord pieces was evaluated as described previously (Rot, 1992b).

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