

## **Rapid Secretion of Prestored Interleukin 8 from Weibel-Palade Bodies of Microvascular Endothelial Cells**

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### **Summary**

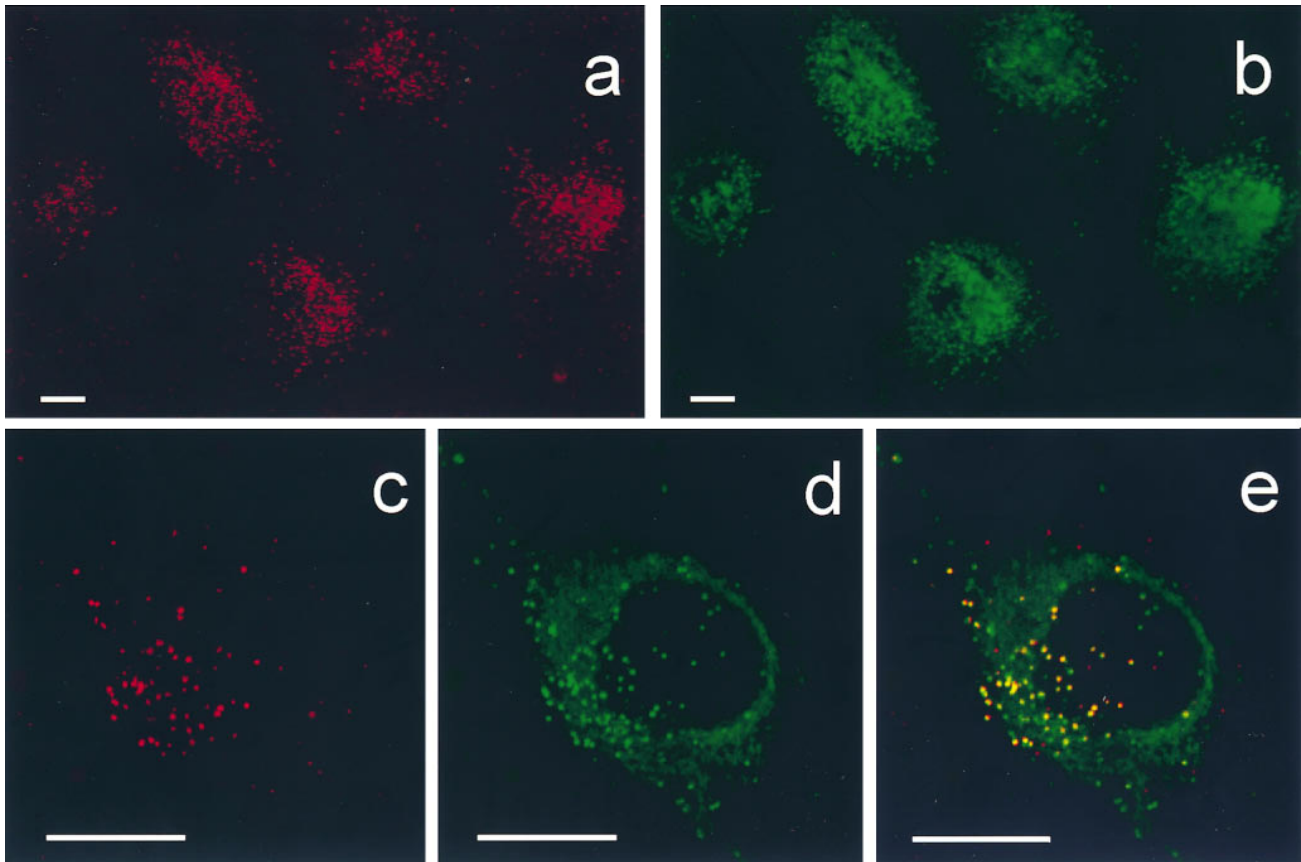
Interleukin (IL)-8, a C-X-C chemokine, activates integrin-mediated adhesion of neutrophils. Presentation of IL-8 on the endothelial cell surface may promote leukocyte extravasation. We found that cultured human microvascular endothelial cells from the intestine (HIMEC) and from nasal polyps (PMEC), but not human umbilical vein endothelial cells (HUVEC), contained IL-8 in intracellular granules that coexpressed von Willebrand factor (vWf). This observation was corroborated by the immunohistochemical observation of double-positive granules (IL-8<sup>+</sup>vWf<sup>+</sup>) in vessels of small and large intestine, nasal mucosa, and skin, whereas umbilical cords revealed no endothelial IL-8. After treatment of HIMEC or PMEC with histamine or thrombin, a dramatic increase in supernatant IL-8 concentration was observed within 3 min, whereas no increase in IL-8 was detected in supernatants of identically treated HUVEC cultures. Histamine or thrombin treatment also caused IL-8-containing granules to rapidly disappear from HIMEC. In HUVEC, IL-8-containing granules were inducible by treatment with recombinant human IL-1 $\beta$  for 24 h; additional histamine treatment doubled IL-8 secretion from HUVEC in the same rapid manner observed for mucosal EC. These data suggested that IL-8 prestored in microvascular endothelial cells may provide a rapid pathway for specific activation of neutrophil adhesion at sites of acute inflammation.

Key words: chemokine • inflammation • leukocyte adhesion • neutrophil recruitment • secretion

Adhesion of neutrophils to endothelial cells (EC) is an early, requisite event in the acute inflammatory response. Such binding of neutrophils can be rapid and transient, occurring within minutes under some conditions, or developing over hours depending on the factors that incite inflammation or tissue damage. According to the current paradigm, these interactions involve a stepwise engagement of juxtaposed neutrophil- and EC-adhesion molecules, first selectins and their counterreceptors, which mediate neutrophil tethering and rolling, then  $\beta_2$ -integrins and immunoglobulin-like intercellular adhesion molecules, which mediate firm neutrophil adhesion (1–3). The most rapid recruitment of neutrophils may be initiated by P-selectin, a transmembrane glycoprotein constitutively present in the Weibel-Palade bodies of EC (4, 5); this selectin is translocated within minutes to the cell surface membrane after stimulation with a number of secretagogues, including thrombin or histamine (6). On the EC surface, P-selectin supports the initial rolling of neutrophils both in vitro (7) and in vivo (8, 9). However, this rolling is of a transient nature, and firm adhesion through

integrin binding requires neutrophil activation through  $G_{\alpha_i}$ -linked receptors (7, 10, 11). Such activation can be accomplished by broadly acting leukocyte activators (3, 12) such as platelet-activating factor (PAF [13, 14]) or by activators that are more specific for neutrophils, such as the chemotactic cytokine (chemokine) IL-8 (15–18).

In fact, the entire process of inflammatory neutrophil recruitment can be emulated in vivo by injection of IL-8 into the tissues (19–22). Moreover, neutrophil influx is severely impaired after intraperitoneal injection of thioglycollate in mice deficient in the IL-8 receptor homologue (23). 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 (24–26). Therefore, the observation of in situ binding of IL-8 to EC in human skin (27), and the enhanced ability of immobilized chemokines to attract and activate leukocytes in vitro (28), led to the theory that chemokines bound to the EC membrane may effectively promote leukocyte-EC adhesion in vivo. Furthermore, tissue-derived IL-8 can be trans-



**Figure 1.** Expression of IL-8 and vWf in resting HIMEC. Two-color immunofluorescence staining for IL-8 (a, c, and e) and vWf (b, d, and e) on monolayer culture. Red, IL-8 staining; green, vWf staining; yellow, colocalized IL-8 and vWf staining. Scale bars, 10  $\mu$ m.

cytosol and presented on the surface of EC (29), but such translocation of IL-8 requires its release or production in the tissue and its subsequent transport to the EC surface.

Here we describe that IL-8 can also be released within minutes from Weibel-Palade bodies in resting (i.e., not previously exposed to proinflammatory reagents *in vitro*) cultured human intestinal microvascular EC (HIMEC) and nasal polyp-derived microvascular EC (PMEC) after stimulation with histamine or thrombin. Such storage of a chemokine may be a novel principle in the biology of leukocyte adhesion that would serve to rapidly increase local chemokine concentrations at the EC surface and enable a higher level of specificity than that provided by classical chemoattractants such as PAF.

## Materials and Methods

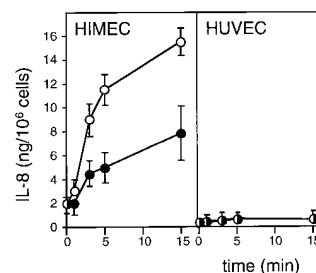
### Reagents

**Cytokines and Cell Culture Reagents.** Recombinant human (rh)IL-1 $\beta$  and rh basic fibroblast growth factor were obtained from R&D Systems Ltd. (Abingdon, UK). MCDB 131, cycloheximide, dibutyryl cAMP, histamine, thrombin, epidermal growth factor, and hydrocortisone were from Sigma Chemical Co. (St. Louis, MO), and Endothelial-SFM, FCS, and gentamicin from Life Technologies Ltd. (Paisley, UK).

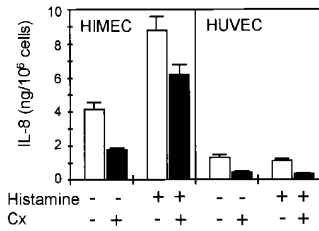
**Primary Antibodies and Secondary Conjugates.** mAbs to human IL-8 (clone NAP-1, IgG1; and clone LS04/2A2, IgG1) were gifts from Dr. C. Svanborg (University of Lund, Lund, Sweden) and Dr. C. Mackay (LeukoSite, Inc., Cambridge, MA), respectively; rabbit anti-human von Willebrand factor (vWf, IgG fraction) and FITC-labeled swine anti-rabbit IgG conjugate were purchased from Dako-patts A/S (Glostrup, Denmark); biotinylated horse anti-mouse IgG was from Vector Laboratories, Inc. (Burlingame, CA), and streptavidin-Texas Red conjugate from GIBCO BRL (Gaithersburg, MD).

### Cell Culture

HIMEC and PMEC were isolated from segments of small intestine or from nasal polyps and cultured as described (30, 31). In brief, cells were dispersed from minced tissue by collagenase/dispase treatment, plated, and grown to confluence in Endothelial-



**Figure 2.** Effect of histamine on release of IL-8. Time course of IL-8 release from confluent EC cultures in the presence (open circles) or absence (filled circles) of histamine (0.1 mM). ELISA measurements in supernatant fluid, representative results from one of four similar experiments (mean  $\pm$  SD of triplicate wells).



**Figure 3.** Effect of cycloheximide on histamine-induced IL-8 secretion. Confluent EC were incubated in the absence (white bars) or presence (black bars) of cycloheximide (Cx; 1 mg/ml, 24 h) and washed twice in fresh medium before histamine stimulation (0.1 mM, 15 min). ELISA measurements of

IL-8 in EC culture supernatants. Representative results from one of three similar experiments (mean  $\pm$  SD of triplicate wells).

SFM containing 2.5% FCS, 1  $\mu$ g/ml hydrocortisone, 0.5 mM dibutyryl cAMP, 50  $\mu$ g/ml gentamicin, and 0.25  $\mu$ g/ml amphotericin-B. EC were selected from primary cultures by using paramagnetic beads armed either with mAb to CD31 (positive selection) or with mAb to CD44 (negative selection [31]). HIMEC and PMEC were subsequently maintained in MCDB 131 as described for HUVEC (see below).

HUVEC were isolated as described by Jaffe et al. (32) and cultured in MCDB 131 containing 7.5% FCS, 10 ng/ml epidermal growth factor, 1  $\mu$ g/ml hydrocortisone, 50  $\mu$ g/ml gentamicin, and 0.25  $\mu$ g/ml amphotericin-B. All cultures were used at passage levels 1–8, were uniformly positive for vWf, and contained <1% contaminating cell types determined as described elsewhere (30, 31).

#### ELISA Experiments

HIMEC, PMEC, and HUVEC were grown to confluence in 96-well trays (Becton Dickinson, San Jose, CA). At the start of the experiment, resting or IL-1 $\beta$ -activated (100 U/ml, 24 h) EC monolayers were washed twice. Cells were subsequently incubated at 37°C in culture medium with or without histamine (0.1 mM) or thrombin (3 U/ml). Cell culture supernatant fluids were analyzed for IL-8 with an ELISA kit according to the recommendations of the manufacturer (R&D Systems Ltd.). In our hands, this assay provided an analytical sensitivity of 51 pg/ml.

#### Immunocyto/histochemistry

Confluent monolayers of EC grown on LabTek chamber slides (Nunc, Inc., Roskilde, Denmark) were fixed (10 min, pH = 7.4) in 0.5% periodate-lysine-paraformaldehyde (33) or in 4% paraformaldehyde (PFA). Tissue specimens from histologically normal small ( $n = 2$ ) and large ( $n = 2$ ) intestine, skin ( $n = 2$ ), nasal mucosa ( $n = 3$ ), and umbilical cord ( $n = 1$ ) were snap frozen and stored at  $-70^{\circ}\text{C}$ . Cryosections were cut at 4  $\mu$ m and fixed in 4% PFA for 5 min at 23°C. All subsequent incubation steps except the last washing step were performed with 0.1% saponin for permeabilization. Cell monolayers or tissue sections were first incubated with mAb to IL-8 (10  $\mu$ g/ml) for 1 h or overnight, respec-

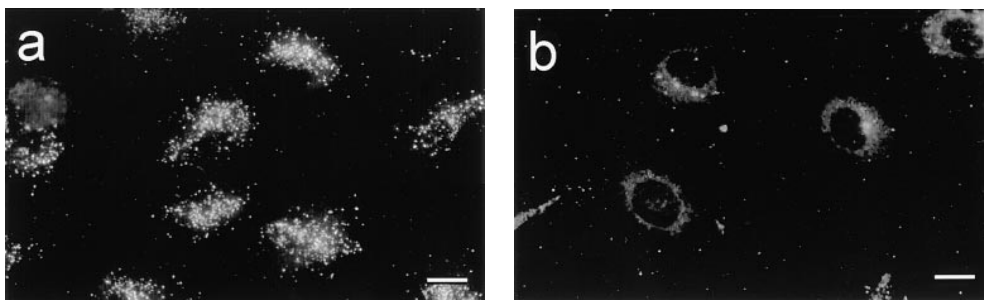
tively; then with rabbit IgG anti-human vWf (1:1,400) combined with biotinylated horse anti-mouse IgG (1:250) for 1 h; and finally with streptavidin-Texas red conjugate (1:200) combined with FITC-labeled swine anti-rabbit IgG (1:50) for 0.5 h. Negative controls were incubated with irrelevant isotype- and concentration-matched primary antibodies. All fixations and incubations took place at room temperature.

The immunostained slides were examined and analyzed with a confocal laser scanning microscope (MRC 600; Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) or a fluorescence microscope (model E800; Nikon, Tokyo, Japan) equipped with a cooled CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan).

## Results

*Resting Microvascular EC but not HUVEC Contain IL-8<sup>+</sup> Granules.* In contrast to HUVEC, unstimulated HIMEC (Fig. 1, a, c, and e) and PMEC (data not shown) contained IL-8 in intracellular vesicle-like granules as revealed by staining with mAbs LS04/2A2 and NAP-1. Staining of nonpermeabilized monolayers revealed no reactivity (data not shown). The observed subcellular localization of IL-8 was similar to that described for vWf (34) and P-selectin (4, 5), which both are stored in Weibel-Palade bodies. Therefore, we performed paired immunostaining with an antibody to vWf (Fig. 1, b, d, and e) and found coexpression of the two proteins in most granules. In addition, mAb NAP-1 revealed perinuclear IL-8 reactivity compatible with its presence in the Golgi apparatus (data not shown), as also reported for other cytokines (35), but this staining pattern was not observed with mAb LS04/2A2.

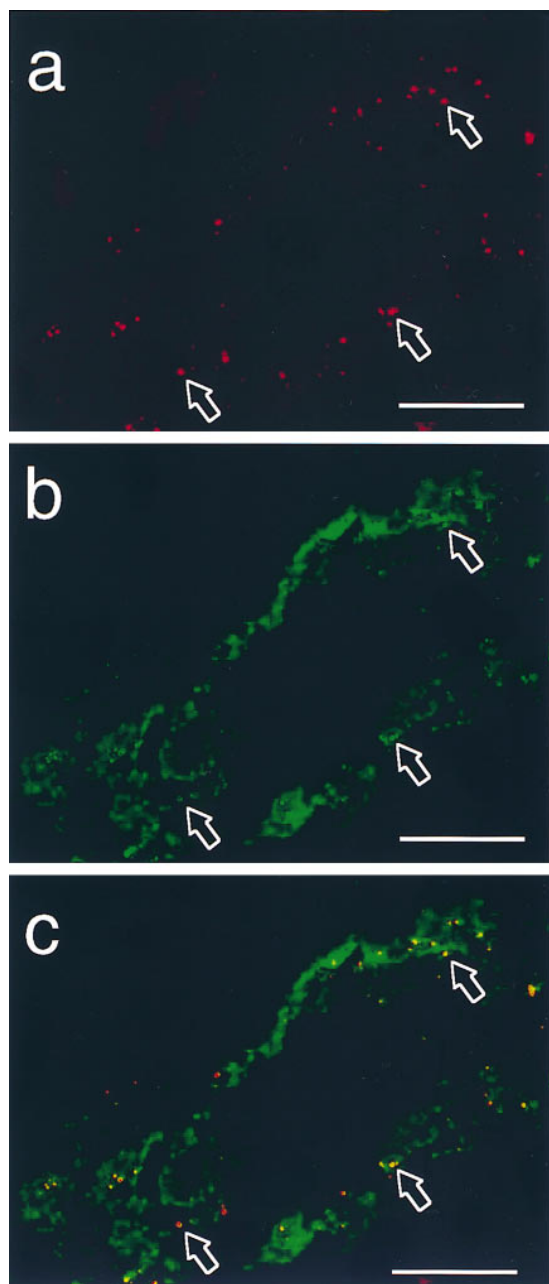
*Rapid Secretion of IL-8 from Microvascular EC Is Induced by Histamine or Thrombin.* We observed a higher constitutive secretion of IL-8 into the culture supernatant in unstimulated HIMEC and PMEC than in HUVEC (Fig. 2, and data not shown). However, treatment of HIMEC (Fig. 2) and PMEC (data not shown) monolayers with histamine (0.1 mM) or thrombin (3 U/ml, data not shown) more than doubled the level of IL-8 over unstimulated cultures within 5 min (Fig. 2). Consistent with the lack of detectable granule-associated IL-8 in HUVEC, these agents had no effect on IL-8 levels (Fig. 2). To exclude enhanced protein synthesis as a possible explanation for the increased levels of IL-8 in the supernatant, HIMEC and HUVEC were incubated with the protein synthesis inhibitor cycloheximide (1 mg/ml) for 24 h before histamine stimulation. De-



**Figure 4.** IL-8<sup>+</sup> vesicles disappear rapidly after activation with histamine. IL-8 expression in the absence (a) or presence (b) of histamine (0.1 mM, 15 min). Immunofluorescence staining (clone NAP-1) of fixed and permeabilized HIMEC culture (scale bars, 10  $\mu$ m).

spite further reduction of the low level of constitutive IL-8 secretion observed in both cell types, cycloheximide had no effect on the amount of IL-8 released in response to histamine (Fig. 3).

*IL-8<sup>+</sup> Granules Disappear Rapidly after Stimulation with Histamine.* Immunocytochemical staining of resting and histamine-stimulated HIMEC (Fig. 4) revealed that the observed increase of secreted IL-8 was accompanied by disap-



**Figure 5.** In situ detection of IL-8 in human small intestine. EC in submucosal vein of human jejunum stained for IL-8 (a and c) and vWf (b and d). Red, IL-8 staining; green, vWf staining; yellow, colocalized IL-8 and vWf staining. Arrows, Double-positive granules; note variability of staining intensity for the two proteins. Two-color immunofluorescence staining of fixed and permeabilized tissue sections (scale bars, 20  $\mu$ m).

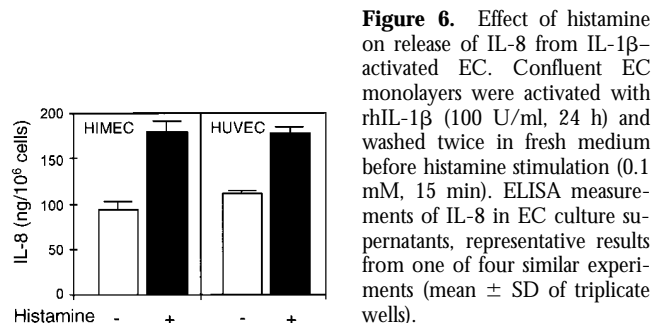
pearance of IL-8<sup>+</sup> granules, most apparent after 15 min (Fig. 4 b). Double staining also revealed a reduction of vWf<sup>+</sup> granules after histamine stimulation (data not shown).

*Microvascular EC, but not HUVEC, Contain IL-8<sup>+</sup> Granules In Situ.* This disparity between small vessel-derived EC and HUVEC observed in vitro was substantiated by immunohistochemical detection of IL-8 in small vessels of small intestine (Fig. 5, a and c) but not in EC of the umbilical vein (data not shown). The IL-8 staining of these vessels was observed in granules that costained for vWf (Fig. 5, b and d). Small vessels of the normal skin, nasal mucosa, and large intestine also contained IL-8<sup>+</sup> granules (data not shown).

*IL-8<sup>+</sup> Granules Are Inducible in HUVEC.* After activation with rhIL-1 $\beta$  (100 U/ml) for 24 h, IL-8<sup>+</sup> granules co-expressing vWf appeared in HUVEC (data not shown), and strongly elevated IL-8 secretion levels were observed for both HIMEC and HUVEC after such treatment (Fig. 6). In HIMEC, IL-1 treatment enhanced the staining intensity of IL-8<sup>+</sup> granules (data not shown). We next tested if histamine would effect the release of IL-8 from cytokine-activated EC and found almost doubling of the level of released IL-8 in both cell types within 15 min (Fig. 6).

## Discussion

This study reports that the chemokine IL-8 is stored in resting microvascular EC and can be rapidly released to the luminal EC surface after activation by secretagogues such as histamine and thrombin. This availability may play a crucial role in providing rapid recruitment of neutrophils because prestored P-selectin, which is also made available at the endothelial surface after such activation (6), merely permits transient rolling of neutrophils (7). Subsequent firm adhesion appears to require the activation of  $\beta$ 2-integrins, which can be achieved by stimulation of leukocytes with broadly acting classical chemoattractants or with leukocyte type-specific chemokines affording a higher level of specificity (7, 10, 11). The classical chemoattractant PAF is the only known adhesion-activating factor that can be made available at the endothelial surface within minutes of EC stimulation (18). However, PAF activates several types of leukocytes that can both roll on P-selectin and adhere firmly to integrin counterreceptors on EC (3, 12, 14). On the other hand, the ability of IL-8 to activate integrin-mediated adhesion appears restricted to neutrophils, and its



**Figure 6.** Effect of histamine on release of IL-8 from IL-1 $\beta$ -activated EC. Confluent EC monolayers were activated with rhIL-1 $\beta$  (100 U/ml, 24 h) and washed twice in fresh medium before histamine stimulation (0.1 mM, 15 min). ELISA measurements of IL-8 in EC culture supernatants, representative results from one of four similar experiments (mean  $\pm$  SD of triplicate wells).

rapid secretion from previously resting microvascular EC as described here may provide a higher level of specificity in the earliest phases of neutrophil recruitment.

Our data convincingly show that human microvascular EC in intestinal and airway mucosa, as well as in skin, contain IL-8 in intracellular granules that also contain vWf, clearly indicating that these granules are Weibel-Palade bodies (34). Indeed, immunoelectron microscopy of IL-1-activated HUVEC revealed IL-8 in the Weibel-Palade bodies (36), thus supporting our conclusion. Moreover, histamine or thrombin induced a rapid release of IL-8 from HIMEC and PMEC, in a time course virtually identical to that described for translocation of P-selectin to the EC surface (6), whereas resting HUVEC failed to release IL-8 above background levels. The observed level of IL-8 released to the culture supernatant probably underestimated the true concentration obtainable in the microenvironment between the surface membranes of EC and adhering leukocytes. First, EC in vivo possess binding sites for IL-8 (24) that are grossly lost upon culture of HIMEC (our unpublished data) or EC from other tissue sources (37, 38). Second, the recently described presentation of transcytosed IL-8 on EC microvilli (29), paralleling that of  $\alpha 4\beta 7$  and L-selectin on leukocytes (39, 40), raises the interesting question as to whether prestored endothelial IL-8 may be presented in the same manner, thereby enhancing the availability of IL-8 to its leukocyte receptors. On the other hand, in vitro studies have suggested that as few as 300

molecules of IL-8 per  $\mu\text{m}^2$  are sufficient for activation of adhesion to occur (Campbell, J.J., personal communication).

The constitutive secretion of IL-8 measured at various time points also indicated a difference in the resting state between microvascular EC and HUVEC, possibly pointing to a fundamental biological difference between small and large vessels. However, after activation with rhIL-1 $\beta$ , both HUVEC and HIMEC secreted elevated levels of IL-8; in this case, IL-8<sup>+</sup> vesicles appeared in HUVEC, as also described by Wolff et al. (36). The biological significance of the latter finding is uncertain, but it may reflect a common sorting signal in the IL-8 sequence, whereas the absence of IL-8 inside unstimulated HUVEC might merely reflect the low constitutive level of IL-8 synthesis. To this end, it is interesting to note that the microvascular EC described in this study all derive from organs or tissues that border the external environment and may thus experience chronic inflammatory stimulation, possibly resulting in a higher level of constitutive IL-8 expression. The molecular mechanisms that allow such constitutive differences to persist over weeks in vitro remain to be elucidated.

In conclusion, we describe a novel mechanism by which IL-8 can be made available at the EC surface within minutes of activation. Such presentation may provide a rapid pathway for specific activation of neutrophil adhesion at sites of acute inflammation.

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