Inflammatory Properties of Neutrophil-Activating Protein-1/Interleukin 8 (NAP-1/IL-8) in Human Skin: A Light- and Electronmicroscopic Study

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Neutrophil-activating protein-1/interleukin 8 (NAP-1/IL-8), purified to homogeneity from lipopolysaccharide-stimulated human peripheral blood monocytes, was injected intracutaneously into human skin. Sequential biopsy specimens were taken in order to investigate the sequence of ultrastructural changes induced by the cytokine.

Whereas intracutaneous injection of 100 ng of NAP-1/IL-8 per site caused no macroscopic changes, by histology infiltration with polymorphonuclear leukocytes (PMN) and monocytes was present within 1 h and increased at 3 and 5 h. No lymphocyte infiltration was noted.

The first ultrastructural changes (30 min) consisted of the presence of cytoplasmic 7-nm microfilament bundles, as well as numerous protrusions of the luminal plasma membrane of endothelial cells (EC). As a striking feature, multiple 100- to 160-nm electron lucent vesicles could be observed in the EC cytoplasm. These structures differed from plasmalemmal vesicles and suggest secretory activity. When PMN and monocytes appeared in the vascular lumen (1 h and later), the number of 100-160-nm electron-lucent vesicles had decreased significantly.

In contrast to C5a-injected skin sites, mast cell degranulation was absent. J Invest Dermatol 96:682–689, 1991

Recenty, we have purified and sequenced protein cytokines with potent neutrophil-chemotactic properties in vitro from LPS-stimulated monocytes [1], lectin-stimulated T-lymphocyte-preparations [2], LPS-stimulated umbilical cord endothelial cells [3], as well as IL-1- or TNF-α-stimulated dermal fibroblasts [4]. The predominant form of this cytokine, termed neutrophil activating protein 1 (NAP-1) or Interleukin 8 [5,6], was structurally characterized in our laboratory [7] as well as in others [8–10]. NAP-1/IL-8 exists in several aminoterminal modified variants [3,4,10–12].

In contrast to other well-characterized chemoattractants like FMLP, C5a, LTBI, or PAF, NAP-1/IL-8 attracts neutrophils (PMN), but not monocytes [11,13] or eosinophils [1]. However, lymphocytes [5,14] as well as basophils [15] were attracted by this cytokine in vitro, although with a considerably lower efficacy compared with PMN.

Because we could isolate large amounts of NAP-1/IL-8 as well as structurally related neutrophil attractants from psoriatic scales [16], we were interested in knowing the ultrastructural changes induced by intradermal injection of NAP-1/IL-8 into human skin.

MATERIALS AND METHODS

Reagents Neutrophil-activating protein-1/interleukin 8 (NAP-1/IL-8) was prepared from supernatants of bacterial lipopolysaccharide-stimulated peripheral blood monocytes according to the purification protocol described by Schröder [17].

Briefly, supernatants of LPS-stimulated monocytes were separated by preparative reversed-phase (RP 8) HPLC. Fractions containing NAP-1/IL-8 were further purified by analytic cyaenopopyl-HPLC. NAP-1/IL-8 eluted as a single peak giving a single line upon SDS-PAGE analysis. Aminoterminal amino acid sequence analysis revealed the sequence Ser-Ala-Lys-Glu-Leu-Arg, indicating the presence of the 72-residue form of NAP-1/IL-8 as the only polypeptide. NAP-1/IL-8, stored in 50% acetonitrile containing 0.1% (v/v) TFA, was lyophilized and the residue was solubilized in sterile and pyrogen-free 0.9% NaCl just prior to use at desired

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concentrations. All NAP-1/IL-8 preparations were free of endotoxin, as revealed by the limulus amoebocyte lysate assay (Sigma).

**Patients** Twelve patients hospitalized for treatment of facial skin tumors or varicose veins agreed to participate in this study, giving their written consent after being informed. Studies were performed on the flexor aspects of the forearm. The skin sites were not affected by any disease and none of the subjects had taken systemic drugs within at least 2d preceding the study.

In each volunteer a dose of 100 ng NAP-1/IL-8 in 100 μl saline was injected in each of up to four separate skin sites. Additionally, as a control 100-μl volumes of sterile pyrogen-free physiologic saline were injected (Figs 1A, 2A, 4A, 5A). After defined intervals (30 min, 1, 3, and 5 h) 4-μm punch biopsies were taken and subsequently prepared for light- and electron microscopy. Scandicain (2%) without epinephrine was used for local anesthesia.

**Light Microscopy** One half of the biopsied tissue was prepared for light microscopy, the other half for electron microscopy. Sections prepared from paraffin blocks were stained with hematoxylin and eosin (HE). In order to investigate mast cells and PMN, sections were stained histochemically for chloroacetate esterase.

Monocytes were identified by immunolabeling with the monoclonal antibody Ki-M1P in routinely processed paraffin sections by using the alkaline phosphatase method [18].

**Electron Microscopy** For transmission electron microscopy all biopsy specimens were immersed in cold (4°C) 5% phosphate-buffered glutaraldehyde (pH 7.8) for 2 h, repeatedly rinsed in cold phosphate buffer, and postfixed in 4% phosphate buffered osmotic acid for 2 h. After fixation these specimens were dehydrated in graded steps of acetone and then embedded in Araldit (Araldit Cy212, Sigma). One-μm sections were stained with toluidine blue for lightmicroscopical examination. Ultrathin sections (0.1 μm) were contrasted by use of saturated uranyl acetate solution (methanol) and lead acetate solution (4% in distilled water, pH 12.0). Tissue sections were examined with a TEM 201C electron microscope (Philips).

**RESULTS**

**Clinical Observations** After injection of 100-μl volume of NAP-1/IL-8 or physiologic saline, a transient wheal formed at the injection site with a maximal diameter of approximately 8 mm. This lesion disappeared completely within the following 15 min and no pruritus or pain was noted.

**Light Microscopy** Microscopically, some swelling of capillary walls was noted 30 min after injection of NAP-1/IL-8. At that time infiltrating leukocytes were not seen.

Biopsy sections stained histochemically for chloroacetate esterase showed intact tissue mast cells packed with numerous dark stained granules. Even at later phases (1, 3, and 5 h) there was no sign of mast cell degranulation.

One h after injection of NAP-1/IL-8 few PMN and monocytes appeared in the perivascular zone of the upper dermis.

Three h after injection significant emigration of inflammatory cells into the perivascular tissue had occurred. Leukocytic infiltration was confined to the perivascular regions and consisted of approximately 80% PMN and 20% monocytes. Inflammatory skin reactions were maximal 5 h after injection (Fig 1B). Approximately 60 leukocytes per cross-sectioned vessel could be counted. Interestingly, at this time the infiltrates consisted predominantly of monocytes (approximately 60%) rather than PMN (40%). Significant participation of lymphocytes was not observed at any point in time.

**Electron Microscopy** Early ultrastructural alterations were seen in the endothelial cells (EC) of postcapillary venules, whereas during the following hours extravasation of PMN and monocytes became more prominent.

All specimens (30 min, 1, 3, and 5 h) demonstrated resting mast cells with intact plasma membranes, characteristic peripheral villi, and numerous electron-dense granules (Fig 3A). There were no signs of granule discharge.

In specimens taken 30 min after injection, EC showed edematous swelling together with numerous protrusions of varying size and shape at the luminal plasma membranes. These protrusions were shaped irregularly and showed broad stalks connecting them with the EC (Fig 2B). In addition, large bundles of 7-nm actin filaments were noted within the cytoplasm of most EC (Fig 4B,C).

Furthermore, two categories of electron-lucent vesicles were present in the cytoplasm of nearly every EC. First we observed numerous plasmalemmal vesicles with a diameter of 50 to 100 nm, located adjacent to the plasma membranes (Fig 2C and 3A), which resulted in a band-like appearance of these vesicles at the luminal as
Figure 2. Electron micrographs of postcapillary venules 30 min after injection of sterile pyrogen-free saline (A) and NAP-1/IL-8 (B), (C), respectively. A, Cross sectioned postcapillary venule with smooth surfaced endothelial cell (EC) layer and a characteristic multilayered basal lamina (bl). Note that the adjacent mast cell (MC) is not degranulated. L: vascular lumen. Magnification × 6,100. B, The luminal side of the endothelial cell (EC) plasma membrane shows numerous irregularly shaped protrusions (small arrows). In the cytoplasm multiple electron-lucent vesicles of varying size (100–160 nm) (large arrows) are obvious. bl: multilayered basal lamina, E: erythrocyte, L: vascular lumen. Magnification × 6,000. C, Bandlike formation of small 50–100-nm plasmalemmal vesicles (arrows) adjacent to the luminal as well as abluminal endothelial cell (EC) membrane. L: vascular lumen, n: nucleus. Magnification × 14,000.
Three and 5 h after injection of the stimulus, leukocytes at various stages during the process of extravasation could be observed. Intact PMN (Fig 6) as well as monocytes were seen engulfed by EC or appeared extraluminal in the vicinity of postcapillary venules.

**DISCUSSION**

In this study it is shown that intradermal injection of NAP-1/IL-8 is followed by a characteristic sequence of vascular changes culminating in the emigration of leukocytes into the surrounding tissue. Whereas early changes consisted of structural alterations in vascular endothelium, resident cells of the upper dermis, notably mast cells remained unaltered. This is in contrast to the effects of C5a injected into skin. As reported by Yancey et al [24] and Schubert et al from our laboratory [25], this powerful chemotaxin causes a variety of cellular changes in human skin including early degranulation of mast cells (Fig 3B).

Also, intradermal injection of NAP-1/IL-8 causes no visible wheal-and-flare reaction, which again is in contrast to experiments using intradermal injection of C5a (which induces an intense wheal-and-flare reaction [24]). The reason for these differing reaction patterns following injection of NAP-1/IL-8 and C5a, respectively, could be related to the capability of C5a to degranulate mast cells [26]. Mediators like histamine or peptidoleukotrienes released from mast cells [27,28] may account for macroscopically visible signs like wheal and flare.

NAP-1/IL-8 apparently is unable to degranulate mast cells. Neither light microscopic nor ultrastructural analysis revealed any sign of mast cell degranulation.

Moreover, the morphology of the EC seen after NAP-1/IL-8 differs substantially from C5a-treated skin. As shown [29,30], injection of C5a is soon followed by the formation of cell protrusions at the luminal site that become voluminous until they finally rupture towards the vascular lumen. Changes now induced by NAP-1/IL-8 appeared more subtle, consisting of membrane protrusions without signs of rupture and the formation of numerous plasmalemmal as well as 100–160-nm electron-lucent vesicles. These latter changes strongly indicate EC activation and probably represent secretory activities.

Interestingly, endothelial activation persisted throughout the observation period so that the protrusions of the plasma membrane together with the presence of cytoplasmic filaments were still prominent when PMN became located nearby (Fig 5B). However, the numbers of plasmalemmal vesicles as well as 100–160-nm vesicles remarkably decreased when leukocyte extravasation increased.

Although C5a and NAP-1/IL-8 are of comparable potency and efficacy in vitro [31], in vivo effectiveness of NAP-1/IL-8 appears to be lower when compared with C5a. Rampart et al [32] and Foster et al [33] observed that the injection of NAP-1/IL-8 into rabbit skin induced leukocyte accumulation only slightly when applied alone but caused substantial plasma leakage and leukocyte infiltration when injected in combination with the vasodilator substance PGE2. Injection of PGE2 alone caused neither plasma extravasation nor leukocyte infiltration.

C5a, in contrast to NAP-1/IL-8, is not only a chemoattractant for PMN but also induces remarkable vasodilation when injected into skin. The combination of these two properties might account for the greater in vivo effectiveness of C5a as compared with NAP-1/IL-8.

Moreover, the prevailing accumulation of PMN rather than monocytes within the vessels, as well as their close proximity in the early phases of leukocyte extravasation, suggests a prominent role for NAP-1/IL-8 as a chemoattractant for PMN.

Interestingly, NAP-1/IL-8 has recently been shown to increase PMN adherence to endothelial layers [34], although NAP-1/IL-8 apparently does not induce expression of adherence proteins in endothelial cells [31]. In PMN, however, NAP-1/IL-8 causes expression of CD11/CD18 integrins [35].

EC, upon stimulation with bacterial LPS, IL-1, or TNF, express the NAP-1/IL-8 gene [36,37] and are able to secrete significant

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**Figure 3.** A, Postcapillary venule 30 min after injection of NAP-1/IL-8. Endothelial cells (EC) show irregularly shaped luminal protrusions (arrowheads), as well as two types of electron-lucent vesicles, 50–100 nm plasmalemmal vesicles (small arrows), and 100–160 nm vesicles (large arrows). Note that in the adjacent mast cell (MC) ultrastructural signs of degranulation are absent. Magnification ×5400. B, Perivascular located degranulated tissue mast cell (MC) 30 min after injection of C5a. Note the diminished number of mast cell granules and the reduced electron density of the remaining granules. E: erythrocyte. Magnification ×3800.

well as the abluminal site, suggesting increased transcellular transport activity [19–23].

Beyond this, multiple electron-lucent vesicles with a diameter of 100 to 160 nm could be observed. In contrast to plasmalemmal vesicles these structures were seen not only near EC plasma membranes but also close to the nucleus and the Golgi-area (Fig 2B and 3A). Coated pits were occasionally noted at the luminal plasma membranes. Furthermore, coated vesicles, 50 to 60 nm in diameter, were found next to the Golgi area or appeared to be associated with electron-lucent vesicles (Fig 4C).

In specimens biopsied during the subsequent hours PMN were noted within the vascular lumen (Fig 5B). They were tightly packed and appeared in close contact with portions of the protruding endothelial cell membranes. These PMN demonstrated moderate signs of activation consisting of the formation of coated pits, as well as endosomes and secondary lysosomes. Interestingly, at this time the intramembranous lining of 50–100 nm vesicles in EC had disappeared, as had the electron-lucent 100–160 nm vesicles. However, the membrane protrusions were still prominent.
amounts of biologically active NAP-1/IL-8 [3]. In addition, IL-1 has been shown to activate EC for increased adherence [38,39]. On the other hand, NAP-1/IL-8 was shown to inhibit PMN adherence to EC when these were activated by IL-1 [40]. Therefore, the inhibitory effect of NAP-1/IL-8 appears to depend on the state of activation of EC.

In contrast to the predominant PMN infiltration 3 h after injection of NAP-1/IL-8, at 5 h up to 60% of the inflammatory cells were monocytes. In vitro, NAP-1/IL-8 does not attract monocytes [1,5,13], although it does bind to monocytes [14]. Our in vivo observations therefore strongly suggest the participation of other cytokines in NAP-1/IL-8–induced tissue reactions. The late onset of monocyte migration into the tissue may point towards generation of monocyte chemotaxis (i.e., MCP-1 [41,42] or CAP-37 [43]) in response to the presence of NAP-1/IL-8. Although it is not known whether NAP-1/IL-8 is able to induce release of monocyte chemotactic protein in resident tissue cells, immigrating PMN may release the monocyte chemotactic protein CAP-37 [43].
Figure 5. A. Electron micrograph 3 h after injection of sterile pyrogen-free saline. Note the thin endothelial cells (EC) and their smooth plasma membranes. The intact adjacent mast cell (MC) is filled with electron-dense granules. Inflammatory cells are absent. E: erythrocyte, L: vascular lumen. Magnification ×5,100. B. Postcapillary venule 3 h after injection of NAP-1/IL-8. The capillary lumen is filled with tightly packed polymorphs (PMN), whereas the endothelial cells (EC) show numerous club-like membrane protrusions (small arrows) and are filled with microfilaments (mf). Coated pits (large arrows) are present on endothelial cells as well as on intravascular PMN, whereas granule contents of PMN appear unaltered. Magnification ×7,700. Inset, coated pit on the endothelial cell membrane (a) as well as on the PMN surface (b). Magnification ×24,000.
Figure 6. Postcapillary venule of upper dermis 3 h after injection of NAP-1/IL-8. Transtracellular diapedesis of neutrophils (PMN) takes place at this time. Note that one PMN (1) is nearly completely surrounded by flattened portions of endothelial cell (EC) cytoplasm (arrows), which together with basal lamina (arrowheads) separate the PMN from the perivascular tissue. The PMN below (2) has already reached the perivascular space. L: vascular lumen. Magnification ×6,000.

REFERENCES


ANNOUNCEMENT

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