Time- and Stimulus-Dependent Secretion of NAP-1/IL-8 by Human Fibroblasts and Endothelial Cells

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The neutrophil-activating peptide 1/interleukin 8 (NAP-1/IL-8) has in the past been extensively characterized biochemically as well as functionally. Effects of NAP-1/IL-8 on inflammatory cells like neutrophilic granulocytes and lymphocytes, as well as its production by several different cell types, point towards an important role in different inflammatory processes. Recently, monoclonal antibodies have helped to establish immunoassays for detecting the peptide. Using such antibodies, we have performed *in vitro* studies on the time- and stimulus-dependent production of IL-8 by endothelial cells as well as fibroblasts. Tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) efficiently induced both focal intracellular expression as well as secretion of the peptide when tested by immunocytochemistry and enzyme-linked immunosorbent assay (ELISA). After stimulation with phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), such effects were seen only in endothelial cells, whereas interferon (IFN)-γ did not induce any pronounced effect on either of the cells tested. These studies demonstrated *in vitro* release of IL-8 by different cells upon specific stimulation, thus underlining the significance of the *in vivo* secretion of this peptide, as noted in recent studies. Key words: IL-8 – ELISA/IL-8 – immunocytochemistry/*in vitro* stimulation. *J Invest Dermatol 101:573–576, 1993*

In 1986 a novel peptide now generally referred to as neutrophil-activating peptide 1/interleukin 8 (NAP-1/IL-8) was detected and described by virtue of its near-selective activity on human neutrophilic granulocytes [1–3]. Originally it was noted to be present in scale material from patients with psoriasis [4].* Eventually it was purified from supernatants of stimulated human mononuclear cells [1,2,3,5], but in the meantime, apart from monocytes, fibroblasts [6,7], endothelial cells [8,9], and keratinocytes [7]† were shown to be producers of this cytokine. Although lymphocyte chemotactic [10] and keratinocyte mitogenic activity‡ have been detected as well, activation of neutrophilic granulocytes remains the most prominent feature. This comprises degranulation with release of myeloperoxidase, β-glucuronidase, and elastase [1,2,4,5], generation of toxic oxygen radicals [1,2,3,5], as well as chemotaxis [1,5].

In these studies a number of functional assays have been established in conjunction with biochemical techniques in order to detect either of these activities [1–5]. Often such assays are time-consuming or hampered by additional substances present in the samples to be tested, which show either similar biologic or inhibitory activity. In contrast, immunologic assays using monoclonal anti-NAP-1/IL-8 antibodies appear feasible for quick and easy measurement of NAP-1/IL-8 in any biologic fluid.

In our laboratory we have now established a sandwich enzyme-linked immunosorbent assay (ELISA), which is both specific and sensitive for the peptide. We have examined the time- and stimulus-dependent production and secretion of interleukin (IL)-8 peptide by human fibroblasts and endothelial cells using IL-8 antibodies [11] in both immunocytochemistry and ELISA. A distinct pattern of reactivity was found for each of these cell types, proving them to be most efficient producers of the pro-inflammatory peptide.

MATERIALS AND METHODS

Primary Cell Culture of Human Dermal Fibroblasts and Umbilical Vein Endothelial Cells Human fibroblasts were obtained from human dermis after heat separation of the epidermis [6]. Endothelial cells were harvested from human umbilical veins following collagenase digestion [8]. Both cell types were cultured as described before [6,8] and regularly passaged after tryptic digestion (0.05% w/v trypsin, 0.02% w/v ethelenediaminetetraacetic acid [EDTA]). For stimulation experiments, cells were grown on plastic coverslips in 24-well culture plates. Incubation with respective stimuli (human recombinant interleukin-1α [hrIL-1α] 100 U/ml, hr tumor necrosis factor [TNF]-α 100 ng/ml, hr interferon [IFN]-γ100 U/ml, lipopolysaccharide (LPS) 1 μg/ml, phorbol myristate acetate (PMA) 50 ng/ml) as well as time intervals (1–18 h) are indicated. hrIL-1α was obtained from Hoffmann-La Roche, Basel, Switzerland, hrTNFα from Knoll/BASF, Ludwigshafen, Germany, HrIFNγ from Genentech Inc., San Francisco, CA, LPS (S. Minnesota) from Calbiochem, Marburg, Germany, and PMA from Sigma, Heidelberg, Germany. Culture supernatants as well as coverslips were recovered for sandwich ELISA and immunocytochemistry, respectively.

Monoclonal NAP-1/IL-8 Antibodies Monoclonal antibodies (MoAbs) were produced and characterized as described earlier [11]. Mice had been immunized with natural NAP-1/IL-8 purified from supernatants of stimu-

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Abbreviations: CTAP, connective tissue activating peptide; hr, human recombinant; NAP-1/IL-8, neutrophil activating peptide 1/interleukin 8.

* Schröder J-M, Christophers E: Identification of a novel family of highly potent neutrophil chemotactic peptides in psoriatic scales (abstr). *J Invest Dermatol* 91:395, 1988

† Schröder J-M, Sticherling M, Henneicke-von-Zepelin HH, Smid P, Herlyn M, Christophers E: Keratinocyte-derived neutrophil-activating proteins (KENAPs) (abstr). *Arch Dermatol Res* 283:57, 1991

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lated human peripheral blood monocytes. MoAbs were purified from spent culture medium of respective hybridoma clones by ammonium sulfate precipitation and subsequent chromatography on fast protein liquid chromatography (FPLC) protein A columns (Pharmacia-LKB, Bromma, Sweden). Before being used in the sandwich ELISA, aliquots of monoclonal antibodies were coupled to N-hydroxy-succinimidobiotin according to manufacturer's instructions (Sigma, Heidelberg, Germany).

Immunocytochemical Detection of NAP-1/IL-8 in Cultured Human Fibroblasts and Endothelial Cells Cells grown and stimulated on coverslips were fixed in 4% paraformaldehyde (v/v) in water for 10 min at room temperature. After washing they were incubated with anti-NAP-1/IL-8 MoAb 52E8 contained in spent culture supernatant (RPMI 1640, 7% FCS). Appropriate dilutions had been tested in advance. Immunoreactivity was detected using the avidin-biotin-peroxidase technique (ABC-kit, Vector Laboratories, Burlingame, CA) and diaminobenzidine as substrate. Two hundred cells were microscopically counted and the percentage of intracellularly stained cells was determined.

NAP-1/IL-8 Sandwich ELISA Polystyrene plates were coated overnight at 4°C with purified antibody (10 μg/ml) in sodium carbonate buffer, pH 8.9, and blocked thereafter with 1% BSA (w/v) in phosphate-buffered saline (PBS) for 1 h at room temperature. Samples were incubated in duplicate at 100-μl volumes for 1 h at room temperature. After washing, biotinylated MoAbs were added at appropriate dilutions tested in advance. The enzymatic color reaction was performed using the avidin-biotin-peroxidase method (ABC-kit, Vector Laboratories) and o-phenylendiamin as substrate. Optical density was measured at 492 nm in a Behring ELISA processor II.

Peptides from the β-Thromboglobulin Supergene Family Tested in the Sandwich ELISA Peptides from the β-thromboglobulin (β-TG) supergene family were either commercially available (platelet factor 4 [PF 4]; UCB, Belgium) or kindly provided by Dr. E. Brandt, Forschungsinstitut Borstel, Germany (β-TG, NAP-2).

NAP-1/IL-8 (72-residue form) was purified in our laboratory from supernatants of stimulated human monocytes as described earlier [5]. After HPLC analysis, the peptide was quantified by measuring optical density at 215 nm. In addition, the number of 50% effective doses (ED_{50}) of chemotaxis was evaluated [5]. One ED_{50} in this test system represents an IL-8 concentration of about 1 ng/ml.

RESULTS

Immunocytochemical Detection of NAP-1/IL-8 in Cultured Human Fibroblasts and Endothelial Cells Incubation of both endothelial cells and fibroblasts with IL-1α and TNF-α resulted in the appearance of intracellular IL-8 immunoreactivity as early as after 1 h (Figs 1*a,b*, 2). Thirty to fifty percent of cells were stained in a coarse focal pattern located near the nucleus. The percentage of positive cells rose to 60–70% after 4 h and to more than 90% after 8 h (Fig 2*b,d*). IL-8 immunoreactivity remained at this level upon further incubation for up to 18 h.

Similar results of a distinct increase of intracellular IL-8 immunoreactivity were seen in endothelial cells upon incubation with PMA or LPS, whereas IFN-γ did not induce detectable effects (Fig 1*b*). In contrast, fibroblasts did not respond to any of these three stimuli (PMA, LPS, IFN-γ) at any time interval tested (Fig 1*a*).

NAP-1/IL-8 Sandwich ELISA Different combinations of monoclonal NAP-1/IL-8 antibodies (8C4, 14E4, 46E5, 52E8) were tested for sensitivity and specificity. As shown in Fig 3, the combination of MoAb 52E8 as coating and 8C4 as detecting antibody is able to detect NAP-1/IL-8 down to 0.2 ng/ml. With CTAP III, NAP-2, β-TG, and PF 4, in any of the possible antibody combinations, only minimal extinction could be found even at very high concentrations (100 ng/ml). This finding excludes the possibility that these peptides act as potential cross-reactants causing false-positive results. For subsequent experiments, the combination of MoAb 52E8 as coating and 8C4 as detecting antibody was used.

Testing supernatants of stimulated human fibroblasts and endothelial cells revealed a dose- and time-dependent increase of NAP-1/IL-8 concentrations that was parallel to the increasing intracellular staining of both cell types (Fig 4*a,b*). After stimulation with IL-1α (100 ng/ml), 10–20 ng/ml NAP-1/IL-8 can be detected in the culture supernatant of both cell types during the first 2 h of incubation. With prolonged incubation time, IL-8 rose up to 100 ng/ml after 18 h. With TNF-α, similar results were obtained in both cell types. In contrast to PMA, LPS, and, to some extent, IFN-γ, which are able to induce IL-8 secretion in endothelial cells (Fig 4*b*), no response to these stimuli was seen in fibroblasts (Fig 4*a*).

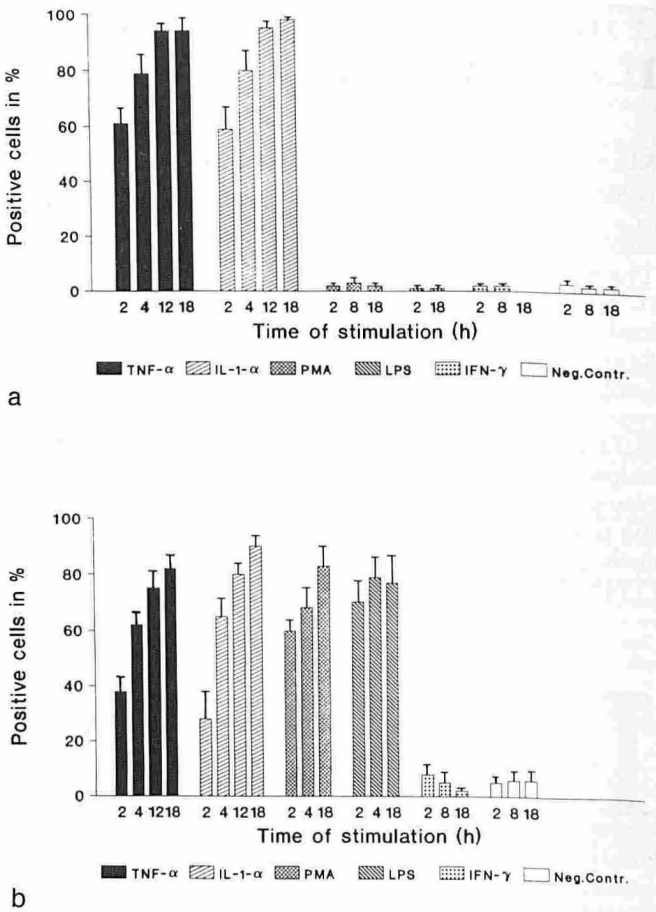

Figure 1. Percentage of immunocytochemically stained cells in *(a)* dermal fibroblasts, *(b)* endothelial cells demonstrating time- and stimulus-dependent intracellular production of NAP-1/IL-8 (hrIL-1α 100 U/ml, hrTNF-α 100 ng/ml, hrIFN-γ 100 U/ml, LPS 1 μg/ml, PMA 50 ng/ml). After staining with monoclonal anti-NAP-1/IL-8 antibody 46E5, 200 cells were counted and the percentage of intracellularly stained cells was determined. *Error bars,* standard deviation.

DISCUSSION

A number of different cell types have in the past been shown to produce NAP-1/IL-8 either constitutively or after stimulation with various stimuli [1,2]. Apart from monocytes [1,2,5], various sessile cutaneous cell populations like endothelial cells [8], fibroblasts [6,7], and keratinocytes [7,12]† were shown to produce the peptide or variants thereof. Furthermore, alveolar macrophages [14], mesothelial cells [15], chondrocytes [16], and synovial macrophages [17] are able to produce IL-8.

Recently, we have extensively characterized monoclonal antibodies that have been raised against natural NAP-1/IL-8 purified from supernatants of human peripheral monocytes [11,13]. The four MoAbs used in this study (8C4, 14E4, 46E5, 52E8) do react with fibroblast-derived IL-8 (FINAP) [6,13], a 77-amino acid extended version of the 72-amino acid genuine peptide, and with endothelial-cell-derived NAP (ENAP) [8,13], a processed 69-amino acid form [8]. Consequently, the ELISA immunoreactivity in supernatants as well as the intracellular staining of stimulated human fibroblasts and endothelial cells as demonstrated in this study can be attributed to NAP-1/IL-8 or variants thereof.

In earlier studies on cultured human fibroblasts [18] and periph-

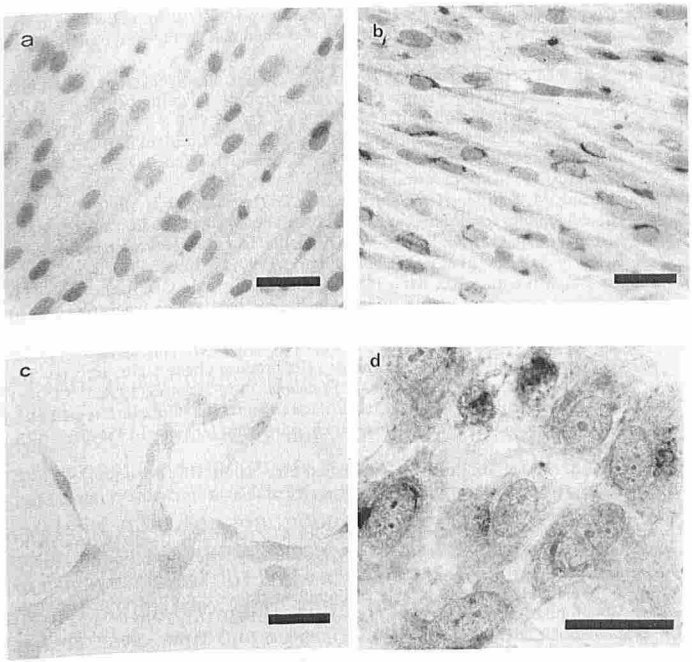

Figure 2. Immunocytochemical staining with monoclonal anti-NAP-1/IL-8 antibody 46E5 demonstrating intracellular focal staining of fibroblasts [*(b) bars,* 50 μm] and endothelial cells *(d)* bars 50 μm) after stimulation with IL-1α (100 U/ml) for 8 h. *a)* fibroblasts prior to stimulation; *c)* endothelial cells prior to stimulation (*bars,* 50 μm).

eral monocytes [19] using mRNA *in situ* hybridization as well as IL-8 immunoassays, we have been able to demonstrate the sequence of events from transcription of IL-8 message, expression of intracellular IL-8, to release of IL-8.

Using Northern blot in combination with RIA techniques, Kristensen *et al* [20] recently were able to demonstrate IL-8 message and peptide in endothelial cells, fibroblasts, and keratinocytes stimulated with IL-1α. IL-8 was, however, not localized within the cells. In the present study, our previous observations have now been extended using additional stimuli as well as different cell types that demonstrate ready and distinct production of IL-8. Furthermore, the consecutive steps of IL-8 expression were pursued by comparing intracellular IL-8 expression and IL-8 secretion. Accordingly, upon stimulation IL-8 is not only readily expressed, but also stored intracellularly as well as secreted. The number of pro-inflammatory stimuli capable of inducing IL-8 in both endothelial cells (LPS, PMA, IL1-α, TNF-α) and fibroblasts (TNF-α, IL1-α) emphasizes the important role of these stimuli as well as of IL-8 under inflammatory conditions.

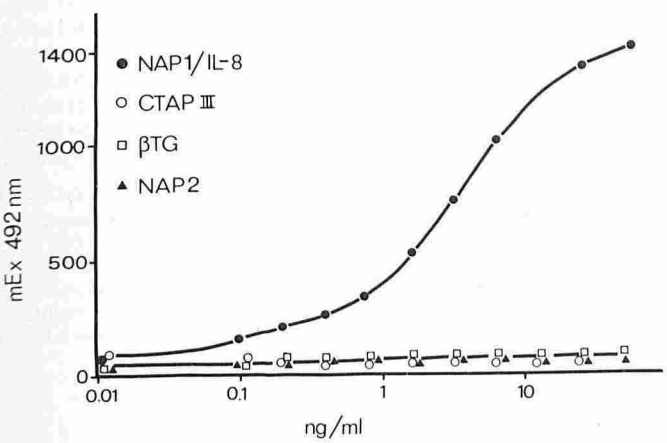

Figure 3. Sandwich-ELISA results with MoAb 52E8 as coating and MoAb 8C4 as detecting antibody. NAP-1/IL-8 and related platelet-derived peptides were tested in the concentrations indicated.

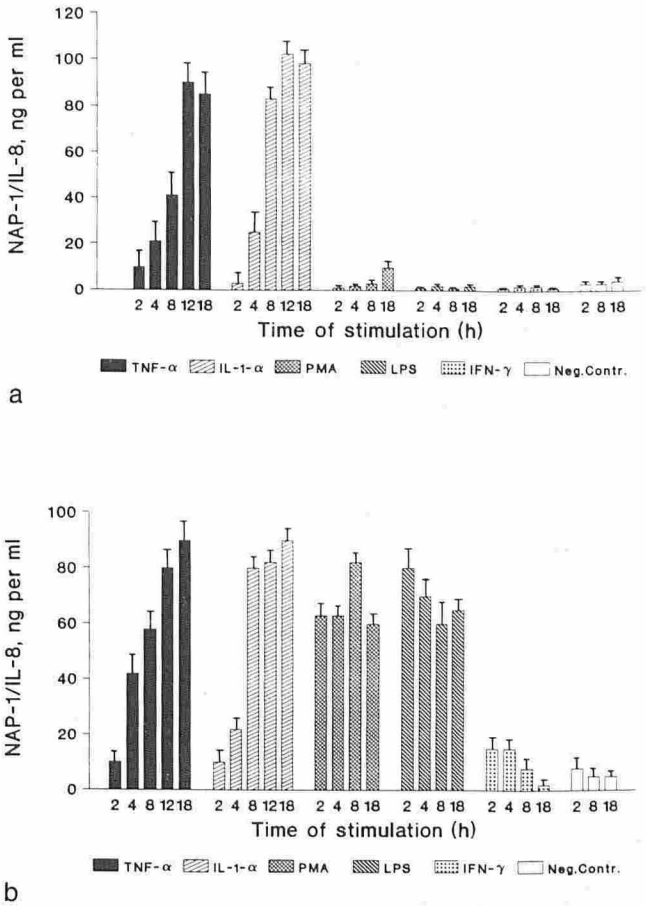

Figure 4. ELISA detection of NAP-1/IL-8 demonstrating time- and stimulus-dependent secretion (hrIL-1α 100 U/ml, hrTNF-α 100 ng/ml, hrIFN-γ 100 U/ml, LPS 1 μg/ml, PMA 50 ng/ml). *a)* dermal fibroblasts; *b)*, endothelial cells). *Error bars,* standard deviation.

From the clinical point of view, testing biologic fluids for NAP-1/IL-8 content would be of great interest. Any biologic assay may, however, be influenced by the presence of additional factors that could either stimulate or inhibit functional activities of the responder cells. This could cause major problems when analyzing complex biologic fluids for the presence of biologically important factors.

In recent studies, a family of structurally related peptides has emerged called the β-thromboglobulin supergene family [21]. Among them are platelet-derived peptides like platelet factor 4 [22], β-thromboglobulin [23], its precursor molecule CTAP III [24], or processed forms like NAP-2 [25].

An ELISA as described here for detecting the IL-8 molecule helps circumvent these problems. Our assay proved to be both sensitive and specific for the measurement of the peptide content in biologic fluids. Indeed, in our ELISA format no related peptide could be detected (data for PF 4 not shown) and therefore these can be excluded as a major source of immunoreactivity.

A further aspect of immunoreactivity noted under these conditions relates to precursor molecules of IL-8 or processed forms. Preliminary data from our laboratory reveal that several peptides with higher molecular weight seem to exist that lack biologic activity although they can be detected by the antibodies raised against IL-8. At present this immunoreactive material has not been examined biochemically.

In our assay the standard curve was established by the use of genuine NAP-1/IL-8 purified from human monocyte supernatants [5]. Quantification was performed by measuring optical density and determining the number of ED_{50s} present in the preparation. The equivalent of one ED_{50} was established at 1 ng/ml [5].

These data may serve as a baseline for further standardization of this molecule in biologic fluids. Unfortunately, up to now use of different immunoassays has hampered careful quantification of NAP-1/IL-8, as pointed out previously [26].

It will be interesting to study NAP-1/IL-8 contents in different pathologic conditions as well as in relation to disease activity to find out whether the peptide can be detected within the circulation or is locally restricted to the site of inflammation.

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