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Increased expression of interleukin-8 and aminopeptidase N by cell-cell contact: interleukin-8 is resistant to degradation by aminopeptidase N/CD13

Abstract

In rheumatic joints, high concentrations of interleukin-8 (IL-8) have been measured in synovial fluid and in pannus tissue. In both locations aminopeptidase N (APN)/CD13, an exopeptidase with reported activity towards IL-8 is also present. The surprising stability of IL-8 in the presence of an alleged

IL-8-degrading peptidase prompted us to undertake the present study. Cocultivation of fibroblast-like synoviocytes (SFC) with T cells or with T lymphocytic cell membranes, or of T cells with SFC cell membranes, all resulted in increased IL-8 mRNA expression and IL-8 secretion into the medium, and an increase of APN expression on lymphocytes. IL-8 degradation was monitored by Western blots and HPLC. IL-8(72), as a partially processed form, was used throughout this study since it is abundant in tissues and has increased biological activity in comparison to IL-8(77). Thus its degradation/inactivation is considered of high biological significance. Whereas trypsin as a positive control rapidly degraded IL-8, we did not see any IL-8 degradation, either by a variety of soluble APNs, by leucine aminopeptidase or by APN expressed on the surface of SFC, or on ECV304 cells transfected with an APN expression vector. The much more sensitive HPLC technique resulted in negative results as well.

Full text

INTRODUCTION

APN/CD13 (EC 3.4.11.2) is a membrane-bound enzyme of almost ubiquitous occurrence (for reviews see 1, 2, 3) that for a long time has been considered as a myeloid marker on haematopoietic cells. Our group became interested in aminopeptidase N (APN)/CD13 expression on lymphocytes when we observed that lymphocytes from pannus tissue or synovial fluid of rheumatoid arthritis (RA) joints are strongly APN/CD13 positive, quite in contrast to peripheral blood lymphocytes (PBL) which are not [4]. Further investigations revealed this as a phenomenon of more general importance insofar as we also found APN/CD13 positive lymphocytes in pericardial fluid [5] and in tumours [6, 7]. Therefore, the questions were obvious: how APN/CD13 expression on lymphocytes is regulated and what its functions on immune cells might be.

To find out the conditions and signals necessary for this transition from an APN/CD13 negative to a positive state, we performed co-culture experiments with SFC and lymphocytes of different origin (PBL, tonsillar lymphocytes, HUT78, Jurkat) and recorded a remarkably rapid increase of APN mRNA in the lymphocytes, and CD13 antigen and enzymatic activity on them, within less than one hour [8, 9].

In more detailed experiments in this field, we also observed a strong stimulation of interleukin-8 (IL-8) upon coincubating SFC with lymphocytes. The presence of IL-8 in rheumatic joints however, has been reported earlier and has been discussed in relation to the cellular infiltration in rheumatic joints [10-12]. IL-8 is a member of the large cytokine family of chemokines (CXC-group), having molecular weights close to 8 kD (for more recent reviews see [13-15]). IL-8 is a potent chemoattractant for neutrophils, but also for basophils, eosinophils and T cells. It has additional pro-inflammatory activities including degranulation of the neutrophil specific and azurophilic granules, expression of adhesion molecules and co-mitogenic action on keratinocytes and angiogenic effects.

The experiments reported here had a twofold purpose. Firstly, we wanted to differentiate the origin and kinetics of IL-8 induction in cocultivation experiments. To this end we used intact cells as well as membrane preparations of SFC and lymphocytes, the latter setting to avoid complications due to the presence of two viable cell types in the culture. Secondly, in view of the report by Kanayama *et al.* [16] on the degradation of IL-8 by APN, we sought an answer to the obvious contradiction of the close coexistence of a degradative enzyme and its alleged substrate. Indeed, we were rather sceptical as to whether APN could degrade IL-8. Despite having an amino-terminal sequence susceptible for attack by APN, never before has a substrate of this size been shown to be degraded by APN, only shorter N-terminal peptides (hexapeptides > duodeka peptides and no cleavage of larger ones) of a series of cytokines [17]. Additionally, the two disulfide bridges at the N-terminus of IL-8, involving Cys 7 and Cys 9, will very probably be a hindrance to aminopeptidase action under non-reducing conditions. To confirm or disprove the results of Kanayama *et al.*, soluble APNs of different commercial sources, a human recombinant APN produced in our laboratory [18], as well as cell-bound APN/CD13 expressed on SFC and on an APN/CD13 transfected cell line [18], were incubated with recombinant IL-8(72) or with IL-8 in cell culture supernatants obtained in our laboratory. We used the Western blotting technique as well as HPLC to detect IL-8 degradation which, however, was never observed regardless of the conditions.

MATERIALS AND METHODS

Enzymes

APN-PKM = leucine aminopeptidase (LAP) Sigma No. L5006, type IV-S from pig kidney microsomes. Despite being designated as LAP, this preparation in the Sigma catalogue has been given the EC-designation EC 3.4.11.2, which refers to APN. This was the preparation used by Kanayama *et al.* [16].

LAP: leucine aminopeptidase (microsomal), Sigma No. L9776.

APN-B: aminopeptidase M from pig kidney, EC 3.4.11.2, Boehringer/Germany, cat. No. 102768.

Soluble APN of ECV304 p8-28 cells (for details see below), secreted into the medium and used after concentration by ultrafiltration, is designated APN-ECV.

Trypsin, Sigma, No. T8003, was used as a positive control.

IL-8 was carrier free human recombinant IL-8(72) obtained from R & D Systems. In parallel, IL-8 from culture supernatants of SFC or glioblastoma cells LNZ308, after stimulation for 48 hours with 10 ng/ml IL-1beta and 20 ng/ml IL-17 was used. IL-8 concentrations in the supernatants were measured using the chemoluminescence enzyme immunoassay system, Immulite (BPC Biermann, Germany).

Cells

SFC were isolated from synovectomy tissues of patients suffering from juvenile chronic arthritis or RA as reported [19]. The cells were grown in RPMI 1640 containing 10% FCS and antibiotics, and were used between passages 3 and 7. ECV304 (ATCC), ECV304 1d-s (stable transfected with a full-length human APN/CD13 expression vector) and ECV304 p8-28 (stable transfected with a human APN/CD13 expression vector deleted for the APN/CD13 transmembrane domain; soluble enzymatically active APN/CD13 was found in the supernatant) were grown in RPMI 1640 containing 10% FCS and antibiotics [18]. The human lymphocytic cell lines Jurkat and HUT78 (ATCC) were cultured in RPMI with 10% FCS, antibiotics (1 mul/ml fungizone, 0.75 mul/ml Refobacin) and 50 mumol 2-mercaptoethanol. The human glioblastoma cell line LNZ308 (ATCC) was grown in DMEM (Cell Concepts, Umkirch, Germany) with 10% FCS supplemented with 1% penicillin/streptomycin (c.c.pro, Neustadt/W., Germany). Peripheral blood lymphocytes (PBL) were prepared from healthy donors by separating peripheral

blood mononuclear cells on Ficoll-Hypaque (Pharmacia, Freiburg, Germany), and depleting monocytes by adherence to a plastic surface. Flow cytometric control of the cellular composition of the PBL preparations revealed a composition of 96-98% lymphocytes and 2-4% monocytes.

Membrane preparation and co-culture experiments

SFC were detached from culture flasks using EDTA (0.2 mM) and subsequently trypsin (0.05 mg/ml), and washed three times with PBS at 4° C, resuspended at 3×10^7 cells/ml in fractionation buffer containing 0.25 M sucrose, 0.1 M $MgCl_2$, 10 mM Tris/HCl (pH 7.4) and 1 mM PMSF. Detached SFC or washed T cells were homogenized using a Potter type homogenizer. The homogenate was centrifuged at 1,000 x g for 15 min to remove unbroken cells and nuclei. The supernatant was then centrifuged at 100,000 x g for 30 min to separate the cytosol and membrane fractions. The membranes were washed twice with fractionation buffer, resuspended in AIMV medium (Gibco BRL, Karlsruhe, Germany) and stored at - 80° C. Protein was measured using a BCA Protein Assay Reagent Kit (KMF, Leipzig, Germany) with BSA as a standard, and Ala-pNA cleaving activity was estimated as described below. For investigation of the IL-8 induction after co-contact, SFC or T cells were incubated with the respective other cell or with membrane preparations of T cells or SFC for different times, as described [8]. Supernatants were collected for IL-8 protein quantification and cells were lysed for RNA preparations.

RNA isolation and cDNA synthesis

Total cellular RNA was isolated according to Chom-czynski and Sacchi [20]. The first strand of DNA was synthesized (after a 10 min incubation at 20° C) at 42° C for 50 min using 0.5 mug total RNA with six dilutions of the appropriate internal competitive standard RNA in 5.5 mul DEPC water, 2 mul 5 x first strand buffer (250 mM Tris/HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 0.5 mul dNTP mix (10 mM of each dNTP), 1 mul 0.1 M DTT, 0.5 mul (50 pmol) random primer and 0.5 mul (200 U/mul) SuperscriptTM II-RT (Gibco BRL, Karlsruhe, Germany).

Construction of the internal standard for RT-PCR

The construction of the IkappaB-alphainternal competitive standard RNA was performed as described earlier [21]. The IL-8 standard was constructed by a

procedure previously described [22].

PCR analysis

Two mul of the cDNA synthesized as described above were diluted in 50 mul of the following solution: 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mM of each dNTP, 1 U Taq polymerase (Qiagen, Hilden, Germany) and 50 pmol of each primer (IkappaB-alpha:5' GCTGAA GAA-GGAGCGGCTAC 3' and 5' ACATCAGCAC-CCAAGGACAC 3'; IL-8: 5' ATGACTTC AAG CTGGCCGTGGCT 3' and 5' TCTCAGCCCTCTTCAAAAACTTCTC 3'). PCR was conducted in a thermal cycler (Biometra, Göttingen, Germany) for 35 cycles of 60 s at 94° C, 60 s at 60° C and 60 s at 72° C. Ten mul of each reaction product were run on a 1.5% agarose gel containing 0.1 mug/ml ethidium bromide in a TAE buffer. The relative intensities of the bands corresponding to the target and standard PCR products were visualized in UV light. The relative amounts of target and standard products were calculated with densitometric analysis using the Scan Pack 2.0 software (Biometra).

Assays for measuring IL-8 degradation by soluble or cell-bound APN

Assays for Western blot analysis using soluble APN preparations: 10 ng IL-8 (recombinant or in cell culture supernatants) were incubated at 37° C, plus 100 mul dilutions of the above mentioned aminopep-tidase preparations in Sörensen buffer pH 7.4, containing 0.1% bovine serum albumin (BSA) as carrier. Control experiments proved that addition of BSA neither influenced the Ala-pNA-cleaving activity of APN nor did it interfere with IL-8 degradation insofar as assays without BSA also did not show any IL-8 degradation by APN or any of the other aminopeptidases in Western or HPLC assays (data not shown). Enzyme activity of the aminopeptidases investigated was tested with Ala-pNA and was, in all cases, 1,000-1,500 pKat/ml. Trypsin as a positive control was used as a 5 mug/ml assay mixture. Assay using cell-bound APN: the APN transfected ECV304 1d-s cell line was used. Ten ng rh IL-8/100 mul, containing 3×10^5 cells (viability > 90% [trypan blue]) with an APN activity of 110 pKat (380 pKat/10^6 cells). After the respective incubation time (37° C) the supernatant was collected and used for Western blot as described above. In alternative assays, adherent SFC, having approximately 70 pKat per well (1,450 pKat/10^6 cells), were incubated for 2-24 hours with 20 ng rh IL-8/200 mul RPMI (without FCS). Western blot with the supernatants were done as follows: one tenth of

digestions were separated on 10% Bis-Tris gels with MES running buffer (NOVEX, Frankfurt-Hoechst, Germany). Proteins were electroblotted from NuPAGE gels onto Hybond ECL membranes (Amersham, Freiburg, Germany). The membrane was blocked with 5% dried milk in TBST for 1 hour at room temperature. Blots were incubated with the primary antibody (anti-IL-8 C19 sc-1269; Santa Cruz, Heidelberg, Germany; 1:1,000 dilution) in TBST with 5% dried milk at room temperature for 2 hours. Blots were washed three times and then incubated for 1 hour with the secondary antibody (1:1,000 dilution, Dianova, Hamburg, Germany) coupled with horseradish peroxidase. Immunodetection was accomplished using the ECL Western blotting detection reagents (Amersham) for chemiluminescent detection. Immunoreactivity was quantified by scanning densitometry using the Scan Pack 2.0 software (Biometra).

HPLC assays for measuring IL-8 degradation

Assays of IL-8 with APN for HPLC measurement contained, in a 10 mul mixture, 50-150 pKat APN and 100-2,000 pMol (0.8-16 mug) of IL-8 in Dulbecco's buffer pH 7.4 (incubations for 24 hours were done with 50 mul assays). Two mul assay aliquots were used for F-Moc derivation, resulting in 320 mul, of which 10 mul were used for HPLC (60RP-8 column, Hewlett-Packard system HP1090 with fluorescence detector 1046A and ^{3D}Chem-Station). In the settings choosen, 1.5 pMol amino acid resulted in 30-45% fluorescence and 1.5 pMol amino acid corresponded to a release of 12% of the N-terminal serine or glutamic acid or alanine, so the beginning of a degradation should have been detectable.

Measurement of IL-8

T cell lines HUT78 or Jurkat or PBL were incubated with membrane preparations of SFC for 48 hours. SFC were cultured in the presence of intact cells or membrane preparations of HUT78 cells, Jurkat cells or PBL for 48 hours. Supernatants were collected and assayed for IL-8 using the Chemiluminescence-Enzyme Immunoassay System, Immulite (DPC Biermann, Bad Nauheim, Germany).

Enzyme assay for aminopeptidase activity

Enzyme activity was assayed using 1.5 mM alanine p-nitroanilide (Ala-pNA) as a substrate for APN. Tests for aminopeptidase activity were incubated at 37° C in

67 mM Na_2HPO_4/KH_2PO_4 buffer at pH 7.2, with the substrate for 30-60 min. The amount of p-nitro-aniline formed was measured in the supernatant by reading the OD at 390 nm. Tests were run in quadruplicate; enzyme-free and substrate-free blanks were run in parallel and the results were calculated as pKat/ml or pKat/10^6 cells.

Statistical analysis

Data were expressed as mean ± SD. The Mann-Whitney rank sum test was used to determine whether two experimental values were significantly different, $p < 0.05$ indicated with *, $p < 0.01$ with **.

RESULTS

Cell-cell contact induces expression of IL-8 mRNA and IL-8 protein in SFC

Using competitive RT-PCR, we quantified the IL-8 mRNA expression in SFC stimulated by T cell membrane preparations (Figure 1). We used membrane preparations of T cells instead of intact T cells to prevent cross-contamination of SFC RNA preparation with T cell RNA [8]. We detected an up-regulated IL-8 mRNA expression in SFC, which was time-dependent, after contact with T cell membrane preparations. We found a 7-fold increase in IL-8 mRNA expression after 24 hour stimulation (Figure 1). Furthermore, supernatants obtained from T cell membrane-stimulated SFC contained significantly higher amounts of IL-8. We found 65.3 ng/ml of IL-8 after 48 hours stimulation with Jurkat membrane preparations, and 110.3 ng/ml of IL-8 after stimulation with HUT78 membrane preparations, compared to 20.2 ng/ml in the supernatant of untreated SFC. Similar results were obtained in coculture experiments with intact cells. Direct cell-cell contact of SFC with Jurkat cells increased the amount of IL-8 to 26.4 ng/ml, with HUT78 cells to 63.5 ng/ml and with PBL to 139.6 ng/ml, compared to 20.2 ng/ml of IL-8 in the medium of untreated SFC (Figure 2A).

*Cell-cell contact induces expression of IL-8 mRNA and IL-8 protein as well as I*kappa*B*-alpha *mRNA in T cells*

Considering the coculturing system for the T cells, we wondered whether the contact between T cells and SFC membranes is sufficient for increased IL-8 secretion from T cells. After 2 days of coculture of Jurkat cells with SFC

membrane preparations, the amount of IL-8 secreted increased from 7.9 ± 2.9 pg/ml to 63.4 ± 21.2 pg/ml (Figure 2B). We found also an increase in IL-8 protein secretion with PBL, to 2,120 pg/ml after contact stimulation with SFC membrane preparations as compared to unstimulated cells secreting 303 pg/ml (Figure 2C). Additionally, we investigated the ability of cell-cell interactions to activate nuclear factor NF-kappaB. Activation of NF-kappaB involves the degradation of its cytoplasmatic inhibitor IkappaB-alpha, which allows the nuclear translocation of NF-kappaB, and ensures transcriptional activation of genes including IL-8 and IkappaB-alphaitself. Using a competitive RT-PCR, we detected a time-dependent, up-regulated lymphocytic IkappaB-alpha mRNA expression following contact with SFC membrane preparations. Already after 30 min cocultivation of T cells with SFC membrane preparations, we measured a 2.5-fold increase of IkappaB-alpha mRNA expression compared to unstimulated T cells, and after 24 hours, we detected a 3.3-fold increase in lymphocytic IL-8 mRNA expression (Figure 3).

IL-8 is resistant to degradation by APN/CD13

The effects of cell-cell contact: increased expression of IL-8 protein on the one hand and upregulated expression of APN/CD13 on the other prompted us to investigate the enzymatic activity of APN/CD13 against the substrate IL-8 in light of the report by Kanayama *et al.* [16], who reported on IL-8 degradation by APN. The reasoning of these authors was as follows: IL-8 in its aminoterminus has proline at residue 16. Since peptide bonds between the proline imidogroup and the carboxy groups of other amino acids cannot be cleaved by APN, a degradation of IL-8 with APN would stop here, and the size of the molecule of IL-8 would be reduced by about 2 kD. They followed the degradation of IL-8 by APN using SDS gel electrophoresis, and demonstrated a 6 kD IL-8 fragment that by amino acid sequencing showed Lys or Pro N-terminally. This confirmed the conclusion of APN degradation of IL-8 up to aa 15 or 16. Our experimental approach was detection of IL-8 *via* Western blotting, using an antibody reacting at the C-terminus of IL-8. The degradation of human recombinant IL-8 by the following enzyme preparations was tested: soluble APN secreted from ECV304p8-28 (APN-ECV), APN-PKM, APN-B, LAP and trypsin. As shown in Figure 4, neither of the aminopeptidase preparations used was able to degrade human recombinant IL-8(72) (result with LAP is not documented). Only with the control enzyme trypsin, could degradation of IL-8 be obtained as shown in Figure 5. Any influence of BSA content on APN catalytic activity (*e.g. via* substrate

competition) could be excluded as described in the Materials and Methods section, and below. In the next experiments, we investigated the ability of the different aminopeptidase preparations to degrade IL-8 contained in culture medium obtained from IL-1beta- and IL-17-stimulated SFC and LNZ308. This was done to exclude the possibility that the negative results with recombinant IL-8 could have been caused by some structural derivation at the N-terminus of the human recombinant IL-8 obtained from R&D systems. Again, none of the aminopeptidases apart from trypsin was able to degrade this human biosynthetic IL-8 (data not shown). Furthermore, we investigated the ability of membrane-bound aminopeptidase on intact cells to degrade human recombinant IL-8. ECV304 cells (APN/CD13 negative), ECV304 1d-s cells (APN/CD13 positive) and SFC (APN/CD13 positive) were cultured with human recombinant IL-8 for different time intervals. We were not able to detect any IL-8 degradation with the membrane bound aminopeptidase in Western blot either (Figure 5 shows the example with adherent growing SFC and hr-IL-8).

The Western blot method may not be sensitive enough to detect the very first stage in IL-8 degradation perhaps involving only a few of the N-terminal amino acids. To avoid this criticism, we used as an additional detection method HPLC with F-Moc derivation for detection of even the faintest amino acid release from IL-8. In Figure 6, one example is given showing a diagram of APN-ECV action on hr-IL-8. The positions of serine, glutamine acid (pos. 1 and 2) and alanine (pos. 4 of IL-8) are indicated. Even after a 24 hours incubation at 37° C, there is no release of amino acids from the N-terminus and this is in agreement with the Western blotting results. In conclusion, all efforts made to demonstrate a degradation of the cytokine IL-8 by aminopeptidases were unsucessful.

DISCUSSION

Cocultivation experiments of isolated cells as models of the situation in organs and tissues under normal and pathological situations, may give results which could prove to be helpful in understanding and explaining findings of *in vivo* or *ex vivo* analyses. In our case with APN/CD13 expression detected on lymphocytes from RA joints, coculturing of lymphocytes with SFC resulted in stimulation of lymphocytic APN/CD13 expression by the SFC. The parallel stimulation of the chemokine IL-8 synthesis and secretion presented the paradox of producing a situation in which both, a substrate, and an enzyme which is allegedly able to degrade that substrate, are stimulated by cocultivation and are readily

detectable *in vitro*. Therefore, we wanted to determine the direction of signaling under cocultivation conditions of SFC and lymphocytes as measured by the IL-8 response and to explain the above-mentioned paradox.

As to the first part, the direction of signaling could easily be followed with the experimental approach of a simplified cocultivation assay, in which cell membranes of the one cell type are used to study effects on the intact "responding" cell, taking for granted that the response observed (IL-8 secretion) comes only from the intact cells and not from the plasma membranes used for stimulation.

The results presented here unequivocally show that both the cell types studied, SFC and lymphocytes (PBL, HUT78, Jurkat), can be stimulated to synthesize IL-8 mRNA as well as protein secretion by cell membranes of the other cell type. Marsh *et al.* [23] studied the role of Fc receptors in the stimulation of monocyte IL-8 secretion, and they postulated the involvement of a soluble lymphocyte factor. In our experiments, neither SFC nor lymphocytes seemed to produce IL-8 under the influence of Fc receptors or soluble factors, since stimulation by plasma membranes exclude both as necessary components. Other authors showed that in cocultures of PBMC with HUVEC or fibroblasts, IL-1alpha stimulates IL-8 secretion [24]; during monocyte-HUVEC interactions Lukacs *et al.* [25], however, did not see an influence of antibodies to IL-1 (nor to TNF-alpha), but they found a significantly decreased IL-8 expression due to soluble matrix proteins, collagen type I or fibronectin. In cocultivation systems including monocytes [26], platelets and endothelial cells [27], the CD40/CD40L pair was shown to strongly influencing MCP-1 and IL-8 expression. Wechsler *et al.* [28] invoke the CD28 costimulatory pathway for T cell PMA-stimulated IL-8 expression. The triggering interactions thus seem to vary in depending on the interacting cells.

When interpreting the results of cell-cell cocultivation, together with the results of membrane-cell cocultivation assays, the conclusion to us seems justified that activation of NF-kappaB is involved, as shown by activation of IkappaB-resynthesis (1-3 hours, Figure 3) preceding the peak of IL-8 mRNA (24 hours, Figure 1) and protein secretion (3 days, Figure 2). The kinetics of the response to stimulation *via* plasma membrane preparations of both SFC as well as of lymphocytes, is not much different to the stimulation by intact cells. Lukacs *et al.* [25], reported the mRNA peak at 8 hours, the IL-8 protein peak at 24 hours for

IFN-gamma-activated HUVECs stimulated by monocytes. Kolios *et al.* [29] studied the chemokine expression on the human colonic epithelial cell line HT-29 under TNF-alpha plus IFN-gamma stimulation and found IL-8 mRNA peaking at 6 hours (RANTES mRNA only at 30 hours), and IL-8 protein at 48 hours. The amounts of IL-8 in our SFC membrane-stimulated Jurkat cultures remain lower than those of PBL or SFC, but the latter indeed are of the same order of magnitude as in those experiments using cocultures of viable cells, not plasma membranes for stimulating a response, or cytokines as stimulators [25-29].

The other part of the results presented here concerns the degradation of IL-8(72) by APN. We deliberately used this form of IL-8 throughout since this is the form used by Kanayama *et al.* [16]. In our experiments, we could not confirm the results of Kanayama *et al.* [16]. To be on the safe side, we extended this study to also using related aminopeptidases (leucine aminopeptidase), and we took advantage of the availability in our laboratory of a human APN/CD13, produced by transfection of the APN negative cell line ECV304 with an expression vector containing the APN cDNA lacking the transmembrane anchor. This cell line thus produces large quantities of APN released into the culture medium which after concentration could be used directly in the present experiments. In our opinion, this preparation to date is the only APN of clearly defined human origin. The other commercial APN preparations leave some doubt as to their biochemical characterization (see Materials & Methods section) and identification as APN/CD13. All have been shown, however, with Ala-pNA to be active enzymes, and we took pains to use roughly identical activities in the assays, as indicated. The results obtained here agree so strongly - and are also in line with those of Hoffmann *et al.* [17] that in our opinion it is no longer justified to regard IL-8 as a substrate of APN/CD13. Our controls, without addition of BSA (cf. Materials & Methods), make sure that this is true not only in assays free of added "carrier proteins" but also in the presence of other proteins; the latter situation is shown in experiments with cell-bound APN (cf. Figure 5), as well as when using a conditioned medium as a source of IL-8 - and of course also *in vivo*. The conflicting results of Kanayama *et al.* [16] deserve more comment. In the one part of their study, they used as a natural source of APN, a plasma membrane preparation of human neutrophil leukocytes, and show IL-8 inactivation in a chemotaxis assay. They did not prove, however, the absence of endopeptidases in the plasma membrane preparations used, except for lysosomal elastase. In the literature, the presence of various endopeptidases (proteinase 3 [30]; neprilysin/CD10 [31]) in neutrophil plasma membranes has been reported,

enzymes that may well be responsible for or at least participating in the IL-8 inactivation seen by Kanayama *et al.* The inhibition of IL-8 inactivation by bestatin, which has only low specificity for APN, does not prove beyond doubt the action of APN alone in IL-8 degradation. Additionally, Kanayama *et al.* show degradation of IL-8 with APN-PKM (Sigma), which we did not find to be active in this respect. At present, we do not have an explanation for the results obtained by Kanayama *et al.* [16]. There is, however, a strong doubt from an enzymatic point of view, concerning the susceptibility of IL-8 in non-reduced form to degradation by aminopeptidases. The two disulfide bridges Cys7 - Cys34 and Cys9 - Cys50 will form most probably an unsurmountable hurdle for aminopeptidase attack. Unfortunately, for APN, the influence of disulfide bonds on degradation of cystine peptides does not seem to have been studied thoroughly (A. J. Turner, personal communication). Even endopeptidases (cathepsin G, elastase, proteinase-3, thrombin, plasmin) degrade native, nonreduced IL-8 rather slowly as compared to IL-8 after reduction [32-34].

As a substrate we did not only use the human recombinant IL-8 from R&D Systems but also IL-8 freshly secreted from cultured human cells. The idea was again - as with the enzymes - not to test only one product. Our control enzyme, trypsin, readily degraded both the IL-8s, rhIL-8 (R&D systems) as well as that contained in our conditioned media. This, in our opinion, also excludes a binding or masking of IL-8 by heparin or heparansulfate as the reason for the resistance of IL-8 to APN/CD13 [35, 36]. Looking at the coculturing assays as well as at the findings in RA joints, the coexistence of IL-8 and APN/CD13 to us no longer presents a paradox, but is the simple consequence of the inability of APN/CD13 to degrade the chemokine IL-8.

CONCLUSION

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Figures

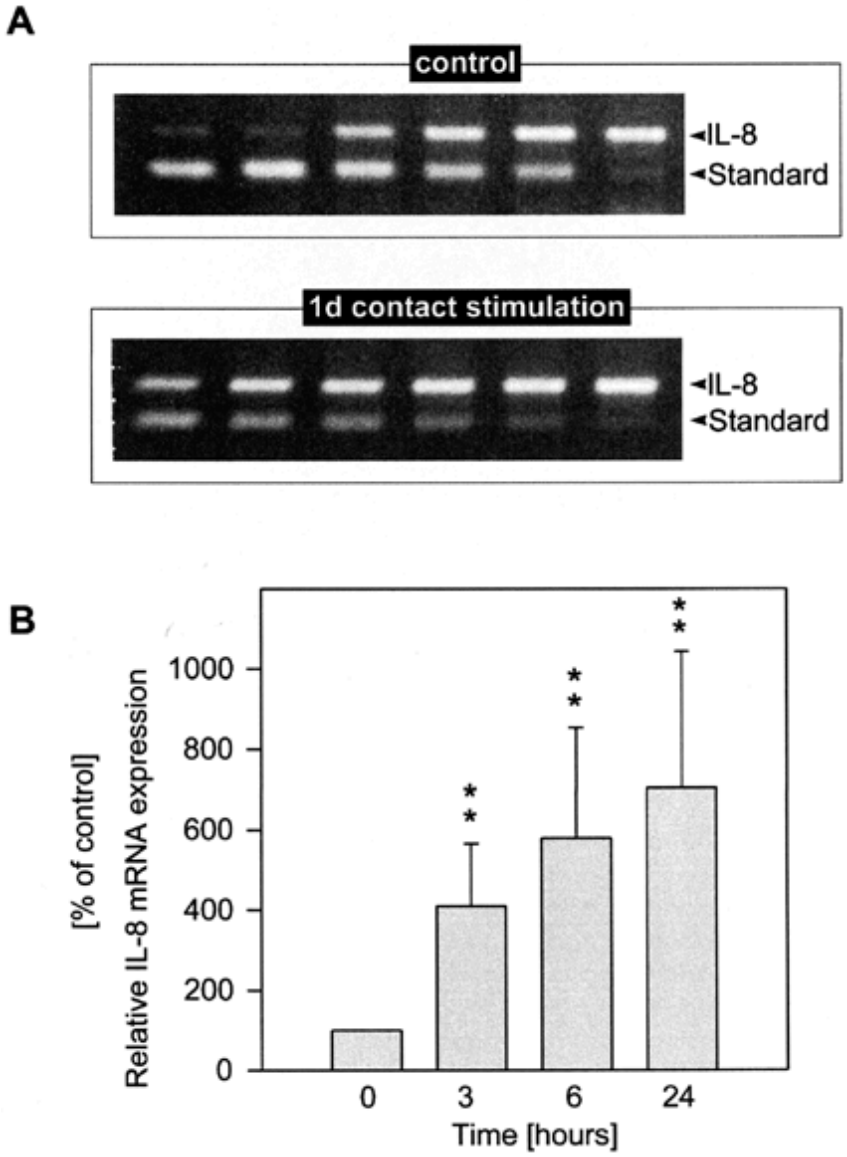


Figure 1

Contact-induced expression of IL-8 mRNA in SFC, stimulated with a membrane preparation of HUT78 cells.

A: Results of competitive RT-PCR. 0.5 mug of total RNA was used for cDNA synthesis in the presence of six dilutions of IL-8 internal competitive standard RNA and random primer in a volume of 10 mul. 1.5 mul of the synthesized cDNA was amplified in the PCR by means of the appropriate primers as described in Materials and Methods. Ten mul of PCR reaction product was separated on an agarose gel containing ethidium bromide. The relative intensities of the bands of IL-8 product and

standard product were visualized in UV light. **B:** Results of competitive RT-PCR are given as a percentage of the basal control (culture without membrane preparation = 100%, n = 6, ** at the p < 0.01 level).

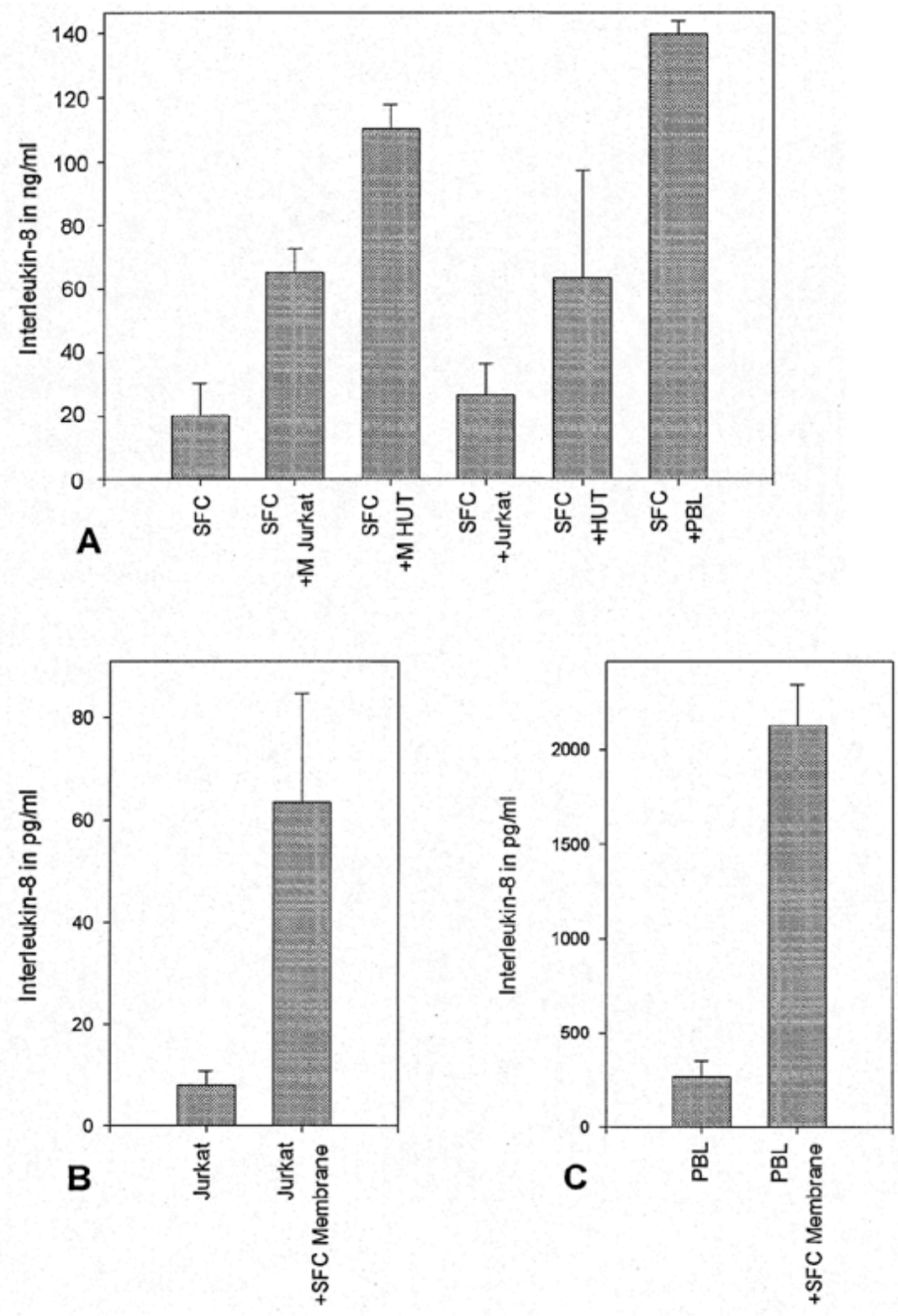


Figure 2

Contact-induced secretion of IL-8 in cell cultures (48 hours) of SFC, or lymphocytes stimulated by membrane preparations of the respective other cell.

A: Stimulation of SFC with membrane preparations (M) of different lymphocytes or with intact cells. **B:** Stimulation of Jurkat cells with SFC-membrane preparations. **C:** Stimulation of PBL with SFC-membrane preparations.

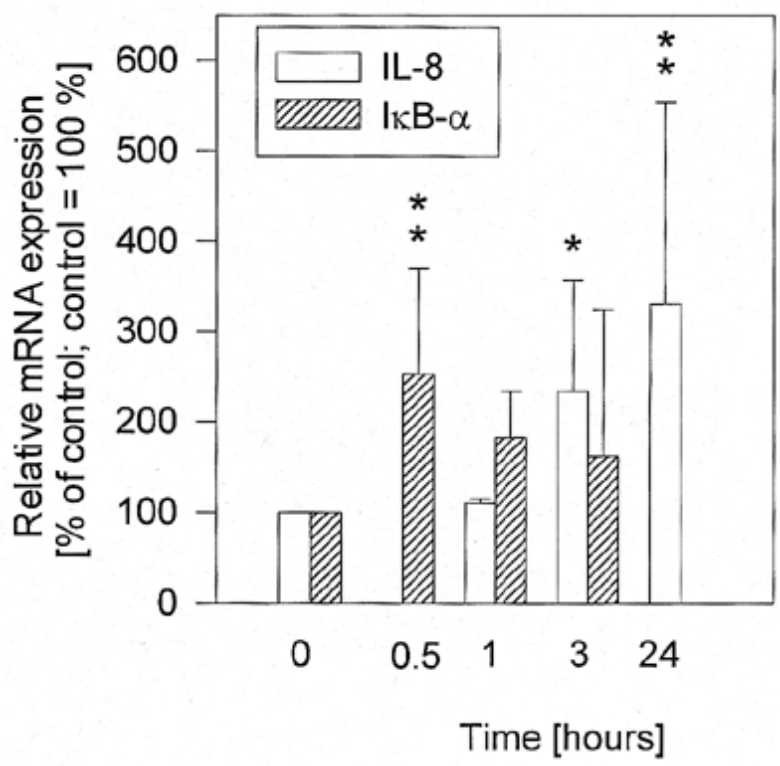


Figure 3

Contact of SFC with T cells induces expression of IkappaB-alpha mRNA and IL-8 mRNA in T cells.

Results of competitive RT-PCR for IkappaB-alpha mRNA and IL-8 mRNA expression in HUT78 and Jurkat cells are given as a percentage of the basal control (culture without contact = 100%, n = 6, * denotes statistically significant differences from control at $p < 0.05$, ** at the $p < 0.01$ level).

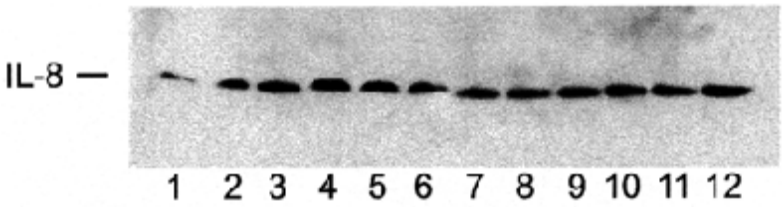


Figure 4

Results of incubation of rh IL-8 with different aminopeptidases.

After 2, 4, 6 hours at 37° C, reactions with 1 ng IL-8 per lane were separated, blotted and probed with an antibody specific for IL-8 as described in Materials and Methods. Results of one representative of three experiments is shown. Lane 1-3: rh IL-8 without APN; lane 4-6: incubation with APN-ECV; lane 7-9: incubation with APN-PKM; lane 10-12: incubation with APN-B.

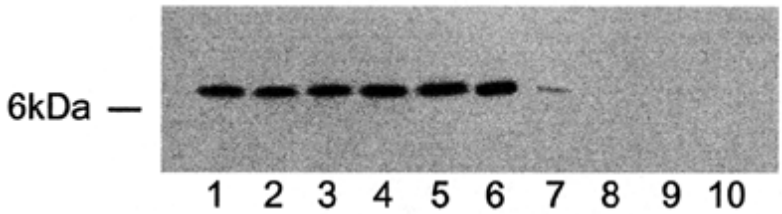


Figure 5

Results of incubation of rh IL-8 with adherent growing SFC.

After different times at 37° C, reactions with 1 ng IL-8 per lane were separated, blotted and probed with an antibody specific for IL-8 as described in Materials and Methods. Results of one representative of three experiments is shown. Lane 1-5: incubation of rh IL-8 with SFC for 0, 2, 4, 6, 24 hours; lane 6: rh IL-8 without SFC (control); lane 7-10: incubation of rh IL-8 with trypsin for 10 min, 1, 2, 4 hours.

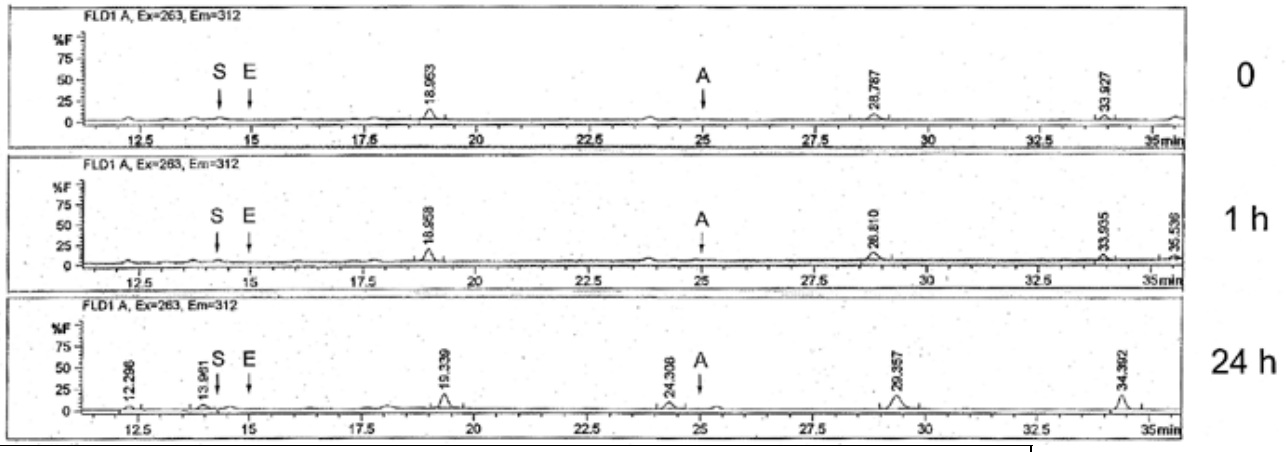


Figure 6

HPLC diagram of an assay of 50 pKat APN-ECV + 8 mug = 1 nMol IL-8.

At the indicated times, 2 mul assay were Fmoc derivatized to 320 mul, and 10 mul of this mixture were injected in the HPLC column. In this example, 1.5 pMol standards of serine (S), glutamic acid (E) and alanine (A), three of the four amino acids at the N-terminus of IL-8 (lysine at position 3 is not detectable in this run), resulted in peaks of 45, 30 and 35% fluorescence. Hundred per cent

degradation of IL-8 under the conditions of this HPLC run would have resulted in 12.5 pMol of each amino acid, this 1.5 pMol corresponded to 12% degradation and this would have given 45% fluorescence. We interpret the diagram as full confirmation of the negative results of the Western blot assays.

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