Evidence for a crucial role of neutrophil-derived serine proteases in the inactivation of interleukin-6 at sites of inflammation

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Abstract The bioactivity of interleukin-6 (IL-6) was found to be dramatically reduced in fluids from sites of inflammation. Here, we provide evidence that the neutrophil-derived serine proteases elastase, proteinase 3 and cathepsin G are mainly involved in its degradation and subsequent inactivation. The initially hydrolyzed peptide bonds were detected to be Val[11]-Ala[12] and Leu[19]-Thr[20] (elastase), Phe[78]-Asn[79] (cathepsin G) and Ala[145]-Ser[146] (proteinase 3). The soluble IL-6 receptor elicits a protective effect against the IL-6 inactivation by cathepsin G only. The inactivation of IL-6 by neutrophil-derived serine proteases might act as a feedback mechanism terminating the IL-6-induced activation of neutrophils.

Key words: Interleukin-6; Inactivation; Elastase; Proteinase 3; Cathepsin G

1. Introduction

The pleiotropic cytokine interleukin-6 (IL-6) plays a major role in the regulation of acute inflammation by eliciting both, proinflammatory and anti-inflammatory effects [1,2]. IL-6 is involved in the activation of unspecific immune defense reactions and is necessary for the antigen specific immune response [1,2]. It is reported that IL-6 is also a key cytokine for the limitation and resolution of the inflammatory process: In contrast to the classic proinflammatory cytokines, IL-1 and TNF-α, IL-6 induces the release of several anti-inflammatory factors [2,3]. However, these wide spreading and apparently paradoxical effects of IL-6 are integrated in a complex network of cytokines and other inflammatory mediators. The IL-6 bioactivity is controlled by cell surface bound and soluble receptors, other binding proteins and probably by different tissue specific posttranslational modifications [4,5].

High IL-6 concentrations are detectable in the very early phase of inflammation, mainly released by endothelial cells, monocytes and neutrophils [6–10]. At local sites of inflammation we found excessively elevated IL-6 concentrations [8]. By comparative measurements of IL-6 concentrations and bioactivities, a discrepancy between high immunochemically detectable concentrations of IL-6 and its bioactivity became evident.

Since at sites of inflammation high amounts of active proteolytic enzymes are released by invading neutrophils in close temporal correlation to the elevated IL-6 concentrations [8], this study focused on the effect of these enzymes on the biological activity and molecular integrity of IL-6. The consequences of the proteolytical IL-6 inactivation for local inflammatory processes and the relevance of these findings with respect to the measurement of IL-6 concentrations in inflammatory exudates will be discussed.

2. Materials and methods

2.1. Patient samples

Inflammatory exudates were obtained from patients with acute inflammatory diseases as approved by the local Ethics Committee. Synovial fluids, obtained from patients with arthritis by arthrocentesis (knee) for diagnostic purposes, were provided from the Clinic of Rheumatology. Ascites and pleural effusions were obtained from pancreatitis patients during surgical treatments, CSF samples from neurotrauma patients via subdural drains established for monitoring and minimizing intracranial pressure. Cell-free supernatants of the exudates were prepared by a two-step centrifugation procedure (15 min, $700\times g$; 10 min, $900\times g$) within 30 min after collection and stored at −80°C until use.

2.2. Determination of IL-6 concentrations and bioactivities

IL-6 concentrations in exudates were detected by ELISA (R and D Systems), IL-6 bioactivities assayed by determining the proliferation rate of IL-6 sensitive B9 hybridoma cells (ATCC). In both assays purified human lymphocyte-derived IL-6 (Boehringer Mannheim), adjusted to the international IL-6 standard (NIBSC), was used as a standard.

2.3. Inactivation of IL-6 by neutrophils and purified neutrophil-derived proteases

Isolated neutrophil granulocytes (purity > 98%, viability 95–99%) as well as lysates and subcellular fractions of these cells were prepared as previously described [8,11]. Effects of vital neutrophils on the molecular integrity and bioactivity of IL-6 were proven by incubating 1 μg of carrier-free, rh IL-6 in the presence of 10^7 cells/ml serum-free DMEM. After various incubation periods, cells were removed by passing 100 μl-sample aliquots through 0.22 μm Filters (Millipore). Additionally, rhIL-6 was incubated with lysates and subcellular fractions of neutrophils or purified active elastase (NE), cathepsin G (Cat G) from ICN or proteinase 3 (PR 3) from IBL. Hydrolysis was stopped by adding a protease inhibitor mixture (IL-6 bioactivity measurements) or by immediate heating to 100°C in reducing sample buffer. IL-6 proteolysis products were analyzed by SDS-PAGE under reducing conditions (MiniProtean II, Bio-Rad), followed by silver staining or Western blotting using an affinity purified, polyclonal anti-human IL-6 rabbit IgG as first antibody and chromogenic or chemoluminescence detection systems.

2.4. Cleavage site analysis by N-terminal peptide sequencing and MALDI-TOF

The N-terminal sequencing of IL-6 cleavage products was performed by Edman-degradation (ABI Procise system). Additionally, cleavage sites were identified by MALDI-TOF mass spectrometry. Proteolytical degradation was performed with 0.2 μg/μl carrier-free, unglycosylated rh IL-6 in 40 mM Tris-buffer plus a final protease

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concentration of 0.01 μg/μl and stopped by adding 2′,5′dihydroxy acetophenon (Matrix, pH = 3.5). Mass spectra were obtained by using a Hewlett-Packard G2025A LD-TOF system, calibrated externally by Hewlett-Packard mass standards. The sequences of IL-6 fragments were predicted using the General Protein Mass Analysis for Windows 2.13 software.

3. Results

3.1. Discrepancy between biological activity and immunochemically detectable concentrations of IL-6 in inflammatory exudates

In various biological fluids derived from sites of acute inflammation, such as CSF, ascites, pleural effusions and synovial fluids, excessively elevated IL-6 concentrations (up to 60129 IU/ml) are detectable by ELISA. Since the high proteolytic potential at sites of inflammation might have considerable influence on the bioactivity of cytokines, we measured the bioactivity of IL-6 in comparison to the immunochemically detectable values.

For standardization and direct comparability of the results obtained by bioassay and ELISA, aliquots of an identical IL-6 standard preparation, adjusted to the international IL-6 standard, were used. Comparing the corresponding IL-6 values obtained by ELISA or bioassay, a significant discrepancy in the vast majority of inflammatory exudates became evident: Taken together, in about 76% of all examined inflammatory exudates ($n = 115$) the IL-6 bioactivity was considerably below the predicted value. In Table 1, the results of the comparing IL-6 measurements for each kind of the inflammatory exudates, are presented separately. In Fig. 1 the individual values of the immunochemically detected IL-6 concentrations versus the hybridoma growth promoting activities are given.

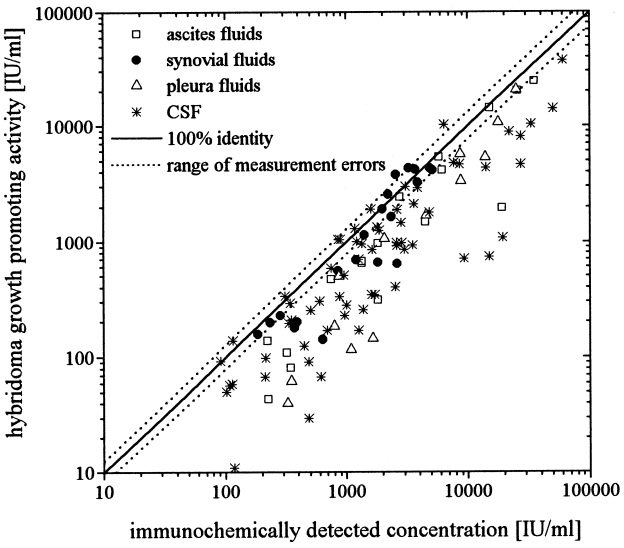

Fig. 1. Immunochemically detected IL-6 concentrations versus the hybridoma growth promoting activities in inflammatory exudates from local sites of inflammation. The concentrations of IL-6 in CSF, ascites, pleural effusions and synovial fluids were measured by ELISA, the bioactivities determined using the B9 hybridoma growth assay. The data calculated on the basis of the international IL-6 standard are given in IU/ml.

3.2. Proteolytical degradation of IL-6 in the presence of vital neutrophils, neutrophil lysates and supernatants of activated neutrophils

Since in inflammatory exudates high amounts of activated neutrophils (up to 98×10^6 cells/ml) as well as high concentrations and activities of neutrophil proteases (up to 40 μg/ml) were detectable [8], we focused our interest on the effects of activated neutrophils on the molecular integrity and biological activity of IL-6 in vitro. Purified, glycosylated rh IL-6 was exposed to 10^7 vital, activated neutrophils, which release active proteases by degranulation and bear several endoproteolytic enzymes on the plasma membrane. Western blot analysis using a polyclonal affinity purified anti-IL-6 antibody revealed a rapid cleavage of IL-6 into immunoreactive fragments with an molecular weight below 14 kDa (Fig. 2, lane 2). Incubation for 120 min led to a further hydrolysis of these fragments (Fig. 2, lane 3). Among several group specific protease inhibitors only the serine protease inhibitor diisopropyl fluorophosphate (DFP) prevented the IL-6 cleavage almost completely (Fig. 2, lanes 4 and 5). In contrast, the IL-6 degradation was not blocked in the presence of metalloproteinase inhibitors (Fig. 2, lane 5).

To get further information, on which proteases are involved in the IL-6 cleavage, rh IL-6 was incubated with lysates and subcellular fractions of activated neutrophils. The exposure of IL-6 to neutrophil lysates resulted in a rapid cleavage of IL-6 too (Fig. 3, lane 5). This was inhibited by DFP (Fig. 3, lane 6), whereas the metalloproteinase inhibitors EDTA plus ortho-phenanthroline (lane 7) or the cysteine protease inhibitor E-64 elicited only slight effects (lane 8). Analyzing the IL-6 cleavage by various subcellular fractions, a remarkable IL-6 cleavage was observable in the presence of the granule and membrane fraction (Fig. 3, lanes 2 and 3) only.

3.3. Cleavage of IL-6 by purified neutrophil-derived serine proteases

The fact that most of the IL-6-degrading activity is inhibitable by DFP and is localized in the granule and membrane

Table 1
IL-6 bioactivity versus immunochemically detected IL-6 concentrations, summarized for each kind of inflammatory exudate, separately

	n	IL-6 (IU/ml) mean ± S.E.M.	relative amount of samples with IL-6 bioactivity		
			lower than predicted	as predicted	higher than predicted
Ascites	17	7167 ± 2500	76.5%	23.5%	0%
Synovial fluids	20	1987 ± 345	40%	50%	10%
Pleural effusions	13	6568 ± 2184	92.3%	7.6%	0%
Drained CSF	65	5665 ± 1406	84.6%	13.9%	1.5%
Summary	115	5349 ± 917	76.5%	20.9%	2.6%

The intra- and interassay deviations of IL-6 measurements by ELISA or bioassay (approved with citrated plasma, CSF and medium spiked with natural IL-6) did not exceed values of 9.8% and 22%, respectively. Based on these results, bioactivity values with more than 25% deviation from the predictions based on ELISA data were considered to be significantly reduced (or elevated).

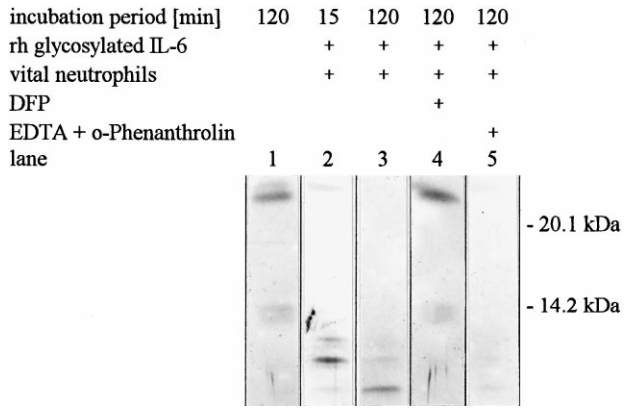

Fig. 2. Western blot analysis after exposure of IL-6 to vital activated neutrophils. 1 μg rh glycosylated IL-6 was incubated in the presence of 10^7 vital activated neutrophils under serum-free conditions. After the indicated time points, sample aliquots were applied to a 15% SDS-PAGE gel, run under reducing conditions and blotted on nitrocellulose membrane. IL-6 and fragments were detected using an affinity-purified, polyclonal anti-IL-6 IgG as primary antibody. The positions of molecular weight standards are given at the edge of the panel.

fraction of neutrophils, suggested a crucial role of serine proteases derived from the granules of neutrophils in the proteolysis of IL-6. Because of their enzymatic features the serine proteases NE, PR 3 and Cat G from the azurophilic granules are main candidates for an involvement in the IL-6 fragmentation. Therefore, the capacity of each of the proteases to catalyze the cleavage and inactivation of IL-6 was analyzed. The incubation of rh IL-6 with purified NE, PR 3 or Cat G under serum-free conditions resulted in a time-dependent loss of its bioactivity. No significant difference between the inactivation kinetics of unglycosylated or glycosylated rh IL-6 was observable (Fig. 4).

Western blot analysis revealed that the hydrolysis of rh IL-6 catalyzed by NE initially led to the formation of two intermediate, immunochemically detectable fragments (Fig. 5A, lane 4 and 5). By mass spectrometry, the molecular weights of the fragments of unglycosylated rh IL-6 were determined to be 19 770 kDa and 18 906 kDa. The N-terminal sequences

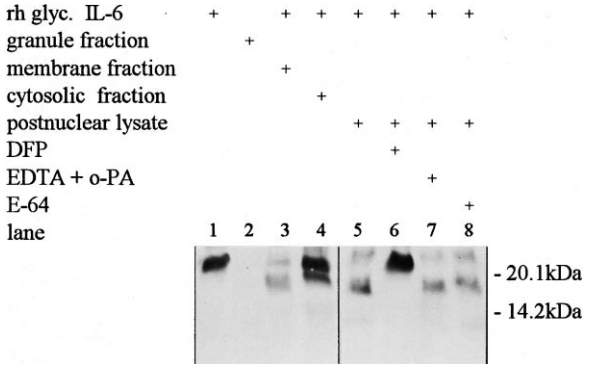

Fig. 3. Hydrolysis of IL-6 by subcellular fractions and lysates of neutrophils. Glycosylated rh IL-6 (0.25 μg per lane) was incubated for 30 min with 10 μl of subcellular fraction extracts or a lysate of 5×10^8 freshly isolated neutrophils. Group specific inhibitors were added as indicated. The degradation of IL-6 was analyzed by SDS-PAGE and Western blotting. The positions of molecular weight standards are given at the edge of the panel.

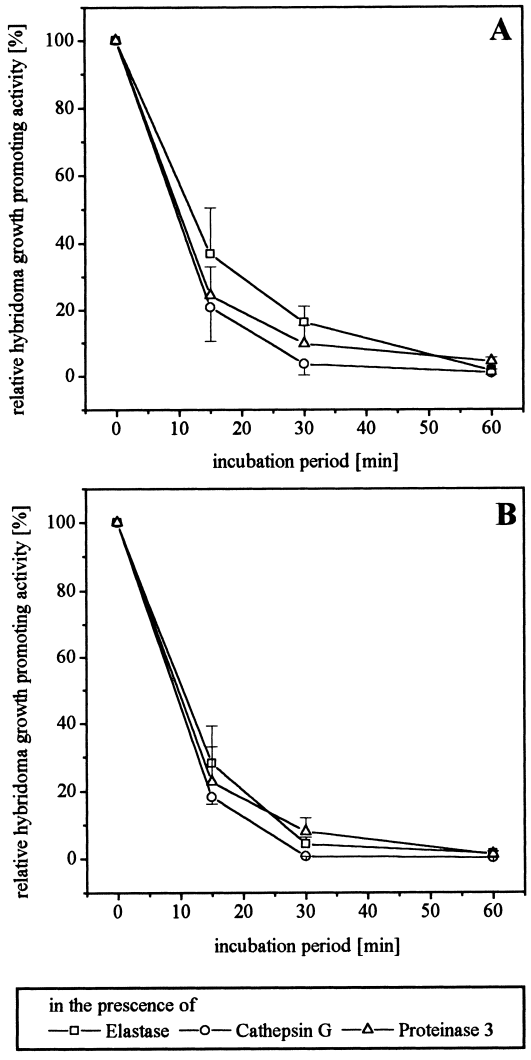

Fig. 4. Influence of NE, Cat G or PR 3 on the bioactivity of unglycosylated IL-6 (A) or glycosylated IL-6 (B). Both forms of rh IL-6 were incubated with purified NE, Cat G or PR 3 for 15, 30 and 60 min. The hybridoma growth promoting activity of IL-6 after the protease treatment was determined in triplicates. The results are given as percentage of the initial bioactivity of the untreated control IL-6.

AAPHR and TSSERI of these fragments indicate that peptides of 11 or 19 amino acids are released from the N-terminus of the IL-6 molecule, respectively. Both, the Val^{11}-Ala^{12} and the Leu^{19}-Thr^{20} cleavage sites, are in excellent agreement with the results of mass analysis and sequence based calculations. During longer incubation periods, both fragments disappeared progressively. In silver stained gels as well as by mass spectrometry a further stepwise degradation of IL-6 into small peptides via 16.2 kDa, 15.1 kDa and 12.8 kDa fragments was observed.

PR 3 initially releases an immunoreactive 16.2 kDa fragment from unglycosylated rh IL-6 (Fig. 5A, lanes 2 and 3), but was not as potent as NE or Cat G. The N-terminal sequence of this fragment corresponds to the N-terminus of intact IL-6. By mass spectrometry the Ala^{145}-Ser^{146} peptide bond was identified as initial cleavage site. Cat G initially catalyzes the hydrolysis of unglycosylated IL-6 into two fragments with apparent molecular weights of 9 and 12 kDa. Only

the 12 kDa fragment was capable of binding the polyclonal anti-IL-6 antibody (Fig. 5A, lanes 6 and 7). Mass spectrometry and the N-terminal sequencing revealed that these fragments were released by hydrolyzing the Phe^{78}-Asn^{79} bond.

In vivo, IL-6 was found to form complexes with the soluble form of the ligand-binding IL-6 receptor subunit gp 80 (sIL-6R) and the soluble form of the signal-transducing receptor subunit gp 130 (sgp130). The aim of further investigations was to evaluate the influence of sIL-6R on the degradation of its ligand IL-6 by NE, PR 3 and Cat G. (Fig. 5, part B). The Western blot shows that only the cleavage of IL-6 by Cat G was almost completely abolished in the presence of the sIL-6R (lane 9, control in lane 8).

3.4. Inactivation of IL-6 by protease-rich inflammatory exudates

By Western blotting of 11 inflammatory CSF-samples with excessive high IL-6 concentrations (> 100 ng/ml), very weak immunoreactive protein bands with an apparent molecular weight of approximately 10 kDa were detectable. Since these results allowed no clear conclusion about the formation of IL-6 fragments in vivo, the capacity of selected inflammatory exudates to inactivate exogenously added rh IL-6 and the influence of various group specific protease inhibitors was investigated. As demonstrated in Fig. 6, glycosylated IL-6 is degraded by a protease-rich CSF or a pleural effusion sample. In both cases, IL-6 fragments (molecular weight > 21.5 kDa) are detectable by Western blotting after 2 or 6 h of incubation. The IL-6 degradation by proteases in these samples was abolished by DFP only (lanes 4 and 8). By comparing the fragment pattern in this experiments with those generated by each of the purified enzymes (Fig. 5, part A), fragments comparable with the NE or PR 3 generated proteolysis products were found.

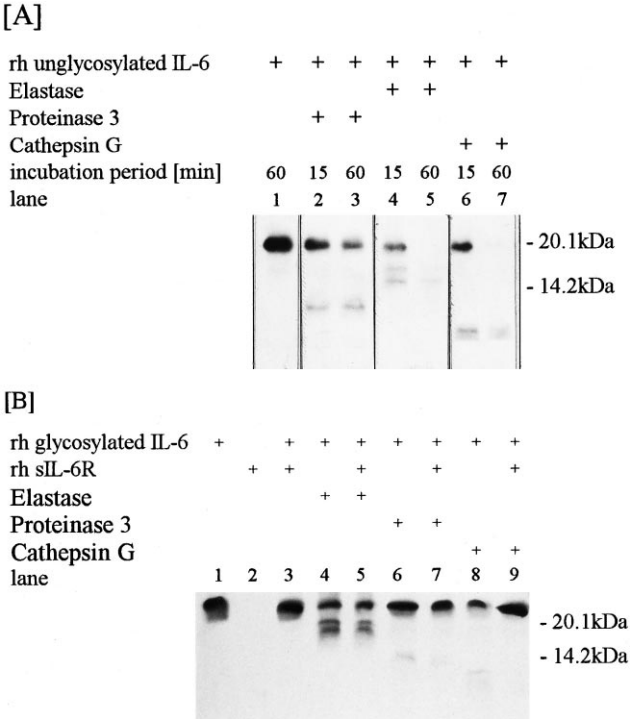

Fig. 5. Hydrolysis of unglycosylated IL-6 by purified NE, Cat G or PR 3 (A) and influence of sIL-6R on the IL-6 degradation (B). A: Unglycosylated rh IL-6 (0.25μg per lane) was incubated with 0.05 μg PR 3, NE and Cat G for 15 or 60 min, and subjected to Western blot analysis using the polyclonal anti-IL-6 IgG as primary antibody (control: lane 1). B: rh IL-6 preincubated in the presence or absence of a five-fold molar surplus of rh sIL-6R, was exposed to NE, PR 3 or Cat G for 30 min. Western blot analysis was performed as described in (A).

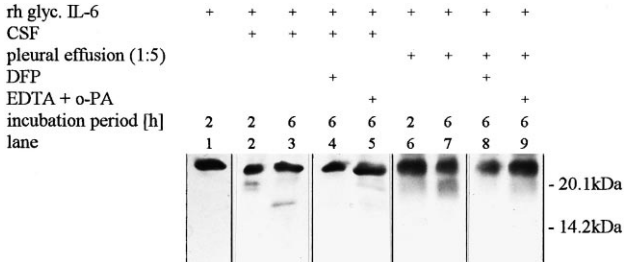

Fig. 6. Degradation of exogenously added IL-6 by proteases in inflammatory exudates. Glycosylated rh IL-6 (0.25 μg per lane, untreated control in lane 1) added to a CSF sample (lanes 2–5) or to a prediluted pleural effusion sample (lanes 6–9) was incubated in the absence or presence of serine or metalloproteinase inhibitors.

4. Discussion

In this report, we show that in inflammatory exudates the bioactivity of IL-6 is often considerably below the predicted level based on the immunochemically detected concentration. Since at sites of inflammation high amounts of active proteases are released, especially by invading neutrophils in temporal correlation to elevated IL-6 concentrations, an influence of these enzymes on the molecular integrity and bioactivity of IL-6 is highly probable. Other conceivable causes for a reduced IL-6 bioactivity in these samples, as e.g. the presence of immunosuppressive drugs or anti-IL-6 auto-antibodies, which may affect the accuracy of IL-6 detection systems [12,13], could be virtually excluded.

Indeed, our results provide evidence that NE, PR 3 and Cat G play a crucial role in the inactivation of IL-6 at sites of inflammation. Each of these proteases is capable of inactivating IL-6. Although the primary amino acid sequence of IL-6 contains numerous potential cleavage sites [14,15], each of the proteases was found to catalyze preferentially the hydrolysis of distinct peptide bonds within the intact IL-6 molecule, independently of its glycosylation status. Examinations of the tertiary structure of the human IL-6 molecule (Brookhaven-code 1IL6, RasMol-software) showed that the initially cleaved peptide bonds are located outside the main four helical bundle [16]. The peptide bonds, initially cleaved by NE, are located within the free accessible N-terminal loop sequence. Since the importance of this sequence region for the IL-6 bioactivity is controversially discussed [17,18], we assume that the rapid loss of bioactivity is a result of structural changes in other parts of the molecule following the removal of the N-terminal loop sequence.

PR 3 attacks initially the Ala^{145}-Ser^{146} bond of the IL-6 molecule, which is located in a small helical region outside the main four helical bundle and is thought to play a pivotal role in the structural stabilization of the helix bundle [16]. Thus, it is probable that proteolysis within this region results in drastic structural changes. Especially with respect to the similar substrate specificities of PR 3 and NE [19], it is interesting that PR3 and NE cleave preferentially different peptide

bonds of the IL-6 molecule. This supports the idea that both enzymes prefer different molecular interactions in extended substrate binding sites.

The Phe^{78}-Asn^{79} bond hydrolyzed initially in the presence of Cat G, is located within the loop sequence connecting helix A and B. The Phe in P_1-position is in agreement with the previously reported preference of Cat G for the catalyzation of the hydrolysis of peptide bonds containing aromatic amino acids [20]. The side chains of Phe^{78} and Asn^{79} are located right next to the Met side chain of the C-terminus of IL-6. Interactions between these side chains seem to be essential for a stable C-terminal structure, which was described to play a critical role for the IL-6 bioactivity [21]. This might explain the loss of bioactivity following the cleavage of the Phe^{78}-Asn^{79} bond. Moreover, the Phe^{78} residue was described to be involved in the IL-6 receptor binding [22]. This is in agreement with our findings, that in the presence of sIL-6R the cleavage of IL-6 by Cat G is almost completely prevented. It is likely that the Phe^{78}-Asn^{79} peptide bond is not accessible in the IL-6/sIL-6R complex, supporting that sIL-6R functions as a carrier for bioactive IL-6 by protecting it against proteolytical degradation [23,24]. Possible effects of the soluble gp130 molecule, which efficiently binds to IL-6/sIL-6R complexes, on the proteolytical inactivation of IL-6 remain to be elucidated.

The direct comparison of the immunoreactive IL-6 fragment pattern found after the exposure of IL-6 to vital activated neutrophils with the pattern generated in the presence of the single purified enzymes, suggests that in vivo IL-6 is inactivated by synergistic action of different surface-bound and soluble proteases. Unfortunately, we were not able to provide a convincing detection of naturally occurring IL-6 fragments, which allows comparisons to in vitro formed IL-6 fragments. The concentrations of IL-6, and potentially formed fragments, in the CSF samples are near the detection limit of the Western blotting method and all engagements to concentrate or isolate IL-6 fragment(s) were accompanied with a substantial loss of immunoreactive protein. By proving the effects of protease-rich inflammatory exudates on exogenously added rh IL-6, we found fragments which correspond to those released in the presence of purified NE and PR 3. However, this and the results of the inhibitor studies supports that these proteases play a pivotal role in the initiation of the IL-6 inactivation at sites of inflammation.

As mentioned above, there are hints that in vivo other proteases from different cell types may be involved in the further degradation of IL-6. Interestingly, it is likely that besides host proteinases also bacterial proteases may process cytokines in infected tissues: Recently, it has been shown that proteases derived from *Porphyromonas gingivalis* in periodontal plaques are potent to inactivate IL-6 rapidly [25].

In the circulation IL-6 seems to be protected against the rapid degradation by such enzymes, because protease inhibitors as well as protective carrier proteins are available in much higher concentrations than at sites of inflammation (data not shown).

Our data rise the question of the putative biological consequences of the proteolytical inactivation of IL-6 at sites of inflammation. A number of reports demonstrated that especially neutrophil-derived proteases are important tools for the regulation of the biological activity of inflammation-related cytokines [26–33]. Since IL-6 has been described to elicit both, pro-inflammatory and anti-inflammatory effects [1,2], it is difficult to assess whether the inactivation of IL-6 is rather limiting or promoting the local inflammatory process. We and others could previously demonstrate that IL-6 induces the release of serine proteases from azurophilic granules of neutrophils [8,34,35]. Therefore, the inactivation of IL-6 by the same enzymes might represent a direct negative feedback mechanism with respect to IL-6 effects on neutrophils. This is in analogy to conclusions drawn in reports describing the proteolytical inactivation of the neutrophil stimulating cytokine TNF-α by neutrophil-derived enzymes [30,31]. A combined inactivation of IL-6 and TNF-α was recently shown to result in the prevention of the acute phase response [36]. Beyond that, neutrophil-derived serine proteases are reported to be involved in the release of active IL-1β or TNF-α from the membrane-bound precursors as well as to enhance the bioactivity of IL-8 [32,37]. Interestingly, some fragments of cytokines released by proteolysis were found to elicit new biological activities [33,38,39]. A comparable relevance of intermediate IL-6 fragments is conceivable, but in view of the rapid further cleavage, doubtful.

Furthermore, the observed discrepancy between the immunoreactive levels and bioactivities of IL-6 in inflammatory exudates shows that it is impossible to draw direct conclusions to the bioactivity of a cytokine from ELISA data. Partially-degraded cytokines may be not detectable by bioassays or may cause variable results of various ELISAs using different antibodies for the cytokine detection. Consequently, our results emphasize that the handling of samples with a putative high proteolytic potential requires improved, standardized protocols.

In summary, the present study demonstrates that neutrophil-derived serine proteases play a crucial role in the regulation of the biological potency of IL-6 at local sites of inflammation. The proteolytical inactivation of stimulating cytokines may act as feedback mechanism important for the limitation and termination of immunostimulating signals.

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