IgA1 protease from *Neisseria gonorrhoeae* inhibits TNFα-mediated apoptosis of human monocytic cells

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Abstract The modulation of programmed cell death is a common theme in the patho-physiology of inflammation and infectious disease. The synthesis and secretion of an IgA1 protease is strictly associated with virulence of the *Neisseria* species. Here, we report on the inhibition of tumor necrosis factor α (TNFα)-mediated apoptosis of the human myelo-monocytic cell line U937 by highly purified IgA1 protease. Apoptosis was verified by the cell surface exposure of phosphatidyl serine and by terminal transferase mediated end-labeling of fragmented DNA. Interestingly, IgA1 protease specifically cleaved the TNF receptor II (TNF-RII) on the surface of intact cells whereas TNF-RI was not affected by the enzyme. Therefore, inhibition of TNFα-mediated apoptosis might be correlated to specific cleavage of the TNF-RII by neisserial IgA1 protease.

Key words: IgA1 protease; Tumor necrosis factor α; Apoptosis; Pathogenic *Neisseria* species

1. Introduction

The genus *Neisseria* includes a variety of harmless commensal species and two human specific pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, the causative agents of gonorrhea and bacterial meningitis, respectively. One outstanding characteristic of the pathogenic *Neisseria* species as well as the three clinically most important agents of bacterial meningitis, i.e. *N. meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, is their ability to produce extracellular IgA1 proteases [1,2]. Despite the obvious fact that IgA1 proteases strongly correlate with the virulence properties of bacterial species [2], the precise function of IgA1 proteases in pathogenesis is still elusive. It is believed that the primary function of this sequence specific endopeptidase in the infection process is to selectively cleave proline-rich sequences in the hinge region of human IgA1 [3]. Previous in vitro studies have shown that IgA1 protease is not required for gonococcal colonization and invasion of epithelial cells [4]. It is therefore possible that IgA1 protease functions in later stages of infection. IgA1 protease has been shown to cleave other substrates including synaptobrevin and the human lysosome-associated lamp-1 protein [3,5,6]. The biological significance of this cleavage, however, remains unclear.

Programmed cell death plays a key role in the regulation of the host immune response [7]; therefore modulation of apoptosis is a wide-spread mechanism exploited by various pathogens in order to facilitate tissue infiltration and/or to interfere with the host immune system [8]. In the immune system external stimuli by cytokines such as tumor necrosis factor α (TNFα) play a central role in initiating apoptotic events [9]. Furthermore, TNFα, originally identified by its ability to cause hemorrhagic necrosis of specific tumors [10], is now known to play a pivotal role in protection against parasitic, viral and bacterial infections [11]. Considering that the interference with TNFα-dependent processes would be an effective way to subvert the host immune response, we investigated the possible role of neisserial IgA1 proteases in TNFα-dependent signaling processes. Our studies revealed that purified IgA1 protease is capable of interfering with the TNFα response. In this respect, we demonstrate by annexin staining of translocated phosphatidylserines as well as by terminal transferase mediated end-labeling of fragmented DNA that TNFα-dependent apoptosis is prevented by neisserial IgA1 protease. Moreover, we present evidence suggesting that the inhibition of TNFα-dependent signaling is due to the enzymatic cleavage of the receptor type II for TNFα by the neisserial IgA1 protease.

2. Materials and methods

2.1. Cell line and culture

The TNF-sensitive human myelo-monocytic cell line U937 was obtained from the American Type Culture Collection (CRL 1593). Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FCS and 50 μM 2-mercaptoethanol.

2.2. IgGa1 protease purification

The enzyme was purified from recombinant *Escherichia coli* H2053 (UT5600/pC1857/pIP12) according to previously published procedures [3]. The purified IgA1 migrated as a single band of 106 kDa in a Coomassie-stained gel, indicating that this enzyme has been purified to homogeneity $>95\%$ (data not shown). Analysis of the lipopolysaccharides (LPS) content of purified IgA1 protease were done using the LAL-test according to the manufacturer's protocol (Haemochrom Diagnostika, Germany). The endotoxin concentrations were estimated using a series of Control Standard Endotoxin dilutions. Analysis of the LPS content of the IgA1 protease preparations using the Limulus test showed that only negligible amounts of ≤ 10 pg/ml were present.

2.3. Enzyme activity assay

For demonstration of enzyme activity 1 μg of immunoglobulin A1 isolated from human sputum (Sigma, St. Louis, MO, USA) was incubated with purified recombinant IgA1 protease (1 μg/μl) for 4 h at 37°C. The proteins were separated by SDS electrophoresis on 10% polyacrylamide gels and protein bands were visualized by silver stain-

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ing. According to this procedure, the enzymatic activity of heat denatured (boiling for 20 min) IgA1 protease was estimated and revealed to be completely destroyed. To determine the amount of antibody and specific inhibitor required for maximal inhibition of IgA1 protease activity, either serial dilution of different anti-IgA1 protease antibodies (AK130, mouse monoclonal), AK24 and AK25 (polyclonal rabbit), or peptide prolyl-boronic acid (a generous gift from A.G. Plaut, New England Medical Center, MA, USA) were added to 0.1 μg IgA1 protease and pre-incubated for at least 1 h at room temperature. Following incubation of these mixtures with 1 μg human IgA1 for 4 h at 37°C, cleavage activity was analyzed in silver-stained SDS–PAGE. The concentration which achieved maximal inhibition was subsequently used in the TNFα cytotoxicity assays.

2.4. Assessment of TNFα-dependent cytotoxicity and apoptosis

Generally, U937 cells were seeded into 96-well culture plates at 2×10^5 in 100 μl of culture medium. 20 ng/ml of recombinant human TNFα (10^8 U/mg, Pharma Biotechnologie Hannover, Germany) with or without the indicated amount of IgA1 protease were applied to each well and incubated at 37°C and 5% CO_2. After over night incubation analysis of cell viability was carried out using the addition of 3-(4,5-dimethylthiazol-2yl-)-2,5 diphenyl tetrazolium bromide (MTT) as described earlier [12]. Optical density was measured with a test wave length of 550 nm and a reference wave length of 620 nm using a 96-well multiscanner autoreader (DigiScan, Asys Hitech GmbH, Eugendorf, Austria). The cytotoxic activity of TNFα was estimated by reduced absorbance compared to untreated control cells. Percentage of dead and viable target cells, respectively, was determined as follows: Relative cell counts (%) = 100 × (OD in treated wells/OD in untreated control wells) − 100. Each experiment was carried out at least four times. Results are presented as mean ± S.D. of $n = 12$ of one representative experiment. Detections of apoptotic cells were done by annexin V-FITC staining of translocated phosphatidylserines according to the manufacturer's protocol (ApoAlert™ Annexin V Apoptosis kit, Clontech, Palo Alto, CA, USA). DNA fragmentation was assessed by catalytically incorporating fluorescein-12-dUTP at the 3′-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT) (Promega, Mannheim, Germany), which forms a polymeric tail using the principle of the TUNEL assay [13] according to the manufacturer's protocol. Negative controls were treated identically but in the absence of the TdT enzyme. Positive controls were treated with DNase I prior to TdT staining. Stained cells were subsequently analyzed with a FACScan flow cytometer (Beckton Dickinson, Heidelberg, Germany). The data collected with FACScan were examined using the software CELLQUEST (Beckton Dickinson). Dead cells were excluded on the basis of forward and side scatter.

2.5. Immunoprecipitation of TNF-RII

U937 cells were adjusted to 10^6/ml and either labeled with ^{35}S methionine or not. The cells were lysed in 500 μl 50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 10 mM EDTA, pH 8.0, 2% NP-40, 0.1 mM Pefabloc, 2 μg/ml leupeptin, 10 μg/ml pepstatin on ice for 1 h. Following incubation of the cell lysate with either 2.5 μl of the rat monoclonal anti-TNF-RII antibody (clone 2B7/97, Biotrend, Köln, Germany) or 2.5 μl of an isotype matched control antibody, the immunocomplexes were precipitated using 20 μl protein A/G-Sepharose. After extensive washing with lysis buffer, the samples were solubilized in loading buffer and separated on a 10% SDS–PAGE. ^{35}S methionine-labeled immunoprecipitates were detected by autoradiography. Unlabeled immunocomplexes were blotted onto a PVDF-membrane and probed with a goat polyclonal antibody specific for the extracellular domain of TNF-RII (Santa Cruz Biotech., Santa Cruz, CA, USA). After incubation with the secondary antibody, a monoclonal anti-goat alkaline phosphatase conjugate (Sigma), TNF-RII molecules were visualized by NBT/BCIP reaction.

3. Results

3.1. Neisserial IgA1 protease prevents U937 cells from TNFα-mediated cytotoxicity

Human myelo-monocytic U937 cells have been reported to respond to TNFα treatment with a cytopathic outcome [14,15]. Initially, various concentrations of TNFα were used to determine the dose-dependent response of U937 cells for cell death. Because the cells were not synchronized apoptosis occurred only in a fraction of the cell population. Therefore, also the percentage of cell loss of TNFα-treated cells and the percentage of proliferation of untreated cells varied. In our hands, TNFα-treatment induced cell loss of about 20% to a maximum of about 40%. After over night incubation, the TNFα-mediated cell loss was monitored by MTT-treatment. At a treatment of cells with 20 ng/ml TNFα, about 40% cell loss was observed compared to untreated control cells (Fig. 1A). Therefore, in order to assess the effect of IgA1 protease of TNFα-dependent signaling processes, U937 cells were treated with 20 ng/ml TNFα and different amounts of purified IgA1 protease. As illustrated in Fig. 1B IgA1 protease-treatment protected the cells from TNFα-mediated cytotoxicity in a concentration dependent manner. When IgA1 protease was applied at concentrations of 0.4–2 μg/ml cell proliferation could be observed as in TNFα-untreated control cells. This effect was strictly TNFα-dependent since treatment of the cells with various amounts of IgA1 protease alone did not influence cellular viability in any detectable way (data not shown). Furthermore, to exclude any effect of the LPS contaminants in IgA1 protease preparations, TNFα-treated U937 cells were incubated with various amounts of *E. coli* LPS. Even an excess amount of LPS did not influence the cellular TNFα response (data not shown), indicating that the minor LPS contamination of the purified IgA1 protease does not account for the observed effect.

3.2. IgA1 protease inhibits TNFα-induced phosphatidylserine translocation

As an early event of apoptosis, most cell types translocate the membrane phospholipid phosphatidylserine (PS) from its normal location at the inner face of the plasma membrane in non-apoptotic cells to the cell surface. Surface exposed PS can be easily detected by a FITC conjugate of annexin V, which

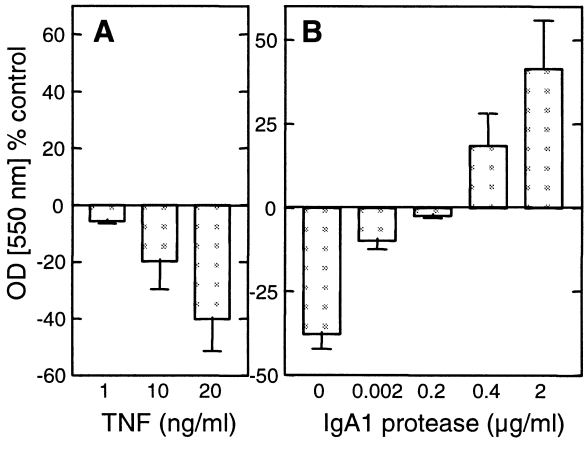

Fig. 1. Effect of IgA1 protease on TNFα-mediated cytotoxicity. A: Dose response effect of TNFα on U937 cells. 2×10^5 cells were treated over night with different concentrations of TNFα. B: Concentration dependent inhibition of TNFα-mediated cytotoxicity by IgA1 protease. Cells were treated with 20 ng/ml TNFα and various amounts of IgA1 protease. Cell viability was measured by the MTT assay. All experiments were repeated for at least four times with $n = 12$ each. The data shown give the mean ± S.D. of $n = 12$ of one representative experiment. The data are presented as relative cell counts based on untreated control cells which were set at 100%.

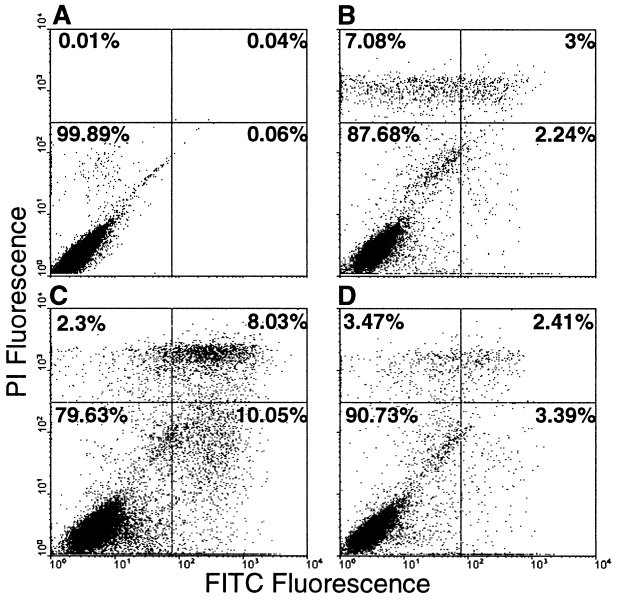

Fig. 2. Inhibition of TNFα-induced annexin V staining by IgA1 protease. A: Unstained cells. B–D: U937 cells were stained with Propidium iodide (PI) and Annexin V-FITC. Controls: A and B: Untreated cells. C and D: U937 cells were exposed to 20 ng/ml TNFα with (D) or without 2 μg/ml IgA1 protease (C) for 6 h after which cell labeling and subsequent FACS analysis were performed. Description of quadrants: upper right: late apoptotic or necrotic, lower left: viable cells, lower right: early apoptotic.

has a strong, natural affinity of PS [16]. Hence, apoptotic cells can be readily identified by their specific staining with annexin V. For this purpose, TNFα cytotoxicity assays with U937 cells were performed and after staining with annexin V, the cells were analyzed by FACS. The data obtained demonstrated that treatment of U937 cells with TNFα resulted in a cell fraction with strong annexin V staining (Fig. 2C). In the presence of IgA1 protease, however, the annexin V positive cell population was markedly decreased (Fig. 3D) indicating that IgA1 protease protects U937 cells from TNFα-mediated apoptosis.

3.3. IgA1 protease inhibits TNFα-induced DNA fragmentation

Endonucleolysis is a further key biochemical event of apoptosis, resulting in the cleavage of nuclear DNA into oligonucleosome-sized fragments. U937 cells treated with 20 ng/ml TNFα exhibited a positive signal for fragmented DNA as assessed by the TUNEL assay and subsequent FACS analysis, however, IgA1 protease also diminished DNA fragmentation in TNFα-mediated apoptosis (Fig. 3B).

3.4. Inhibition of TNFα-mediated apoptosis requires enzymatically active IgA1 protease

To clarify whether the enzymatic activity is responsible for, or at least involved in the activity exerted by IgA1 protease on TNFα-treated cells, different methods were applied. First, the IgA1 protease was denatured by extensive boiling and then analyzed for enzymatic activity using its natural substrate IgA1. Neither cleavage of IgA1 nor inhibition of TNFα-dependent cytotoxicity was observed with the denatured enzyme (data not shown).

In *Neisseria*-infected hosts [17] or in animals immunized with IgA1 proteases [18], antibodies which specifically neutralize the enzymatic activity are produced. To confirm the requirement of active IgA1 protease for the inhibition of cytotoxicity, several antibody preparations were used in subsequent tests. The specific monoclonal antibody AK130 was found to most effectively inhibit the cleavage of human IgA1. IgA1 protease was thus pre-incubated with serial dilutions of AK130 for at least 1 h at 37°C. The immune complexes were then added to TNFα-treated cells. After overnight incubation, the cytotoxic activity of TNFα was determined by the number of remaining cells. As a result, TNFα-dependent cytotoxicity was no longer inhibited indicating that inactivation of IgA1 protease by the antibody abolished its inhibitory effect in the cytotoxicity assay (Fig. 4).

Since IgA1 protease is a relatively large enzyme and may therefore harbor additional functions, we intended to inhibit the proteolytic activity of this enzyme using peptide prolyl boronic acid as specific inhibitor [19]. First, the amounts required for inhibition of enzymatic activity were determined. As illustrated in Fig. 5A 10–100 μM of the peptide prolyl boronic acid preparation almost completely inhibited the enzymatic degradation of human IgA1. Subsequently, TNFα-treated and untreated cells were incubated with peptide prolyl boronic acid to demonstrate that even high amounts of peptide prolyl boronic acid did not influence cell viability or the ability of the cells to respond to TNFα. These experiments clearly indicated that peptide prolyl boronic acid interfered with neither cellular viability (data not shown) nor TNFα-dependent cytotoxicity (Fig. 5B). Based on these results, IgA1 protease was pre-incubated with the specific inhibitors prior to incubation with TNFα-treated cells. Consistent with

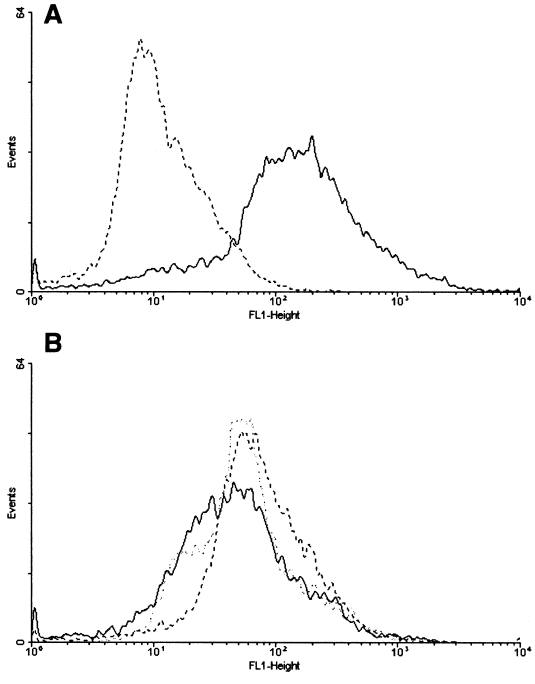

Fig. 3. Inhibition of TNFα-induced DNA fragmentation by IgA1 protease. A: Controls: (solid) Positive control, cells were treated with DNase I, (dashed) negative control, untreated cells without TdT enzyme. B: U937 cells treated as described in Fig. 2 were incubated over night and fragmented DNA was subsequently detected by TUNEL staining and FACS analysis. (dotted) Untreated cells, (dashed) TNFα-treated cells, (solid) cells treated with TNFα and IgA1 protease.

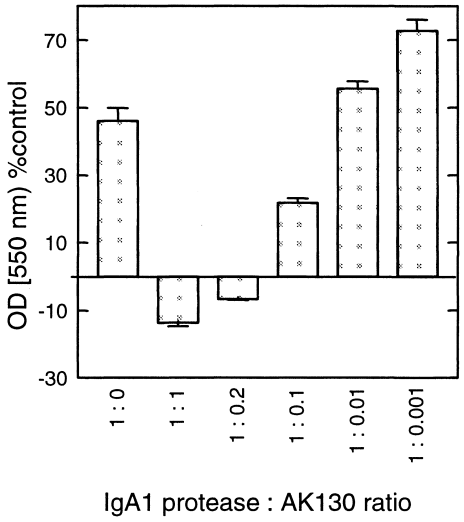

Fig. 4. Inhibition of IgA1 protease activity by monoclonal antibody. AK130 (2 μg/ml) was used in TNFα-dependent cytotoxicity assays. For this purpose, cells were treated with 20 ng/ml TNFα, 2 μg/ml IgA1 protease were pre-incubated with the indicated dilutions of AK130 prior to adding to TNFα-treated cells. Cytotoxicity analysis revealed a clear inhibition of the protease effect on TNFα-treated cells. Controls were TNFα-untreated cells, TNFα-treated cells for TNFα-mediated cytotoxicity, effect of antibody alone on TNFα-treated cells, activity of IgA1 protease alone on TNFα-treated cells. Cell loss as well as cell proliferation were determined as described in Fig. 1.

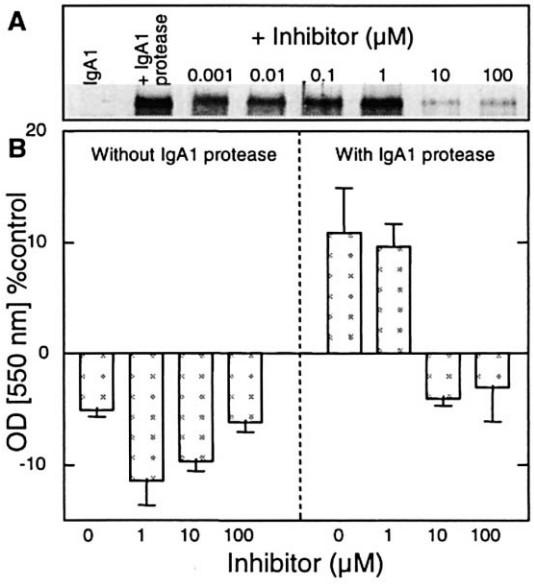

Fig. 5. Peptide prolyl boronic acid as specific inhibitor of IgA1 protease activity. A: 1 μg human IgA1 was treated with 20 ng IgA1 protease pre-incubated with the indicated amounts of inhibitor. Cleavage activity could be inhibited almost completely using 10 μM or 100 μM inhibitor. B: Effect of peptide prolyl boronic acid on the inhibition of IgA1 protease activity in TNFα-dependent cytotoxicity assays. Peptide prolyl boronic acid alone did not interfere with the cellular TNF-response. In accordance with the inhibition profile in A, the effect of IgA1 protease in the TNFα assay was abolished at 10–100 μM concentrations. Controls: TNFα-untreated cells, cells treated with 20 ng/ml TNFα alone. Cell loss as well as cell proliferation were determined as described in Fig. 1.

the inhibition of IgA1 cleavage, the enzyme did not prevent the TNFα-dependent cytotoxicity at prolyl boronic acid concentrations of 10–100 μM (Fig. 5B). Altogether, these results provide evidence for a function of the enzyme's proteolytic activity in the inhibition of TNFα activity.

3.5. IgA1 protease cleaves TNF-RII

Since TNFα functions by binding to either or both TNF receptors I and II (TNF-RI, TNF-RII), we speculated that IgA1 protease might cleave one of the TNF receptors or TNFα itself. IgA1 proteases represent highly specific endopeptidases cleaving distinct proline-rich sequence motifs, particularly in the hinge region of human IgA1 [3]. We therefore used the known cleavage motifs in a computer-based search for putative cleavage sites in TNFα as well as in TNF-RI and TNF-RII. The search revealed that the extracellular domain of TNF-RII contains several proline-rich motifs similar to the IgA1 protease consensus cleavage sequence. Thus, we assessed the susceptibility of the TNF receptor type II to cleavage by IgA1 protease. Initially, TNF-RII was immunoprecipitated from cellular lysates with a monoclonal antibody from rat specific for the extracellular domain and incubated with recombinant IgA1 protease. After resolving the immunoprecipitates by SDS–PAGE, TNF-RII molecules were detected by autoradiography. As illustrated in Fig. 6, lanes 3 and 4, the amount of ~75 kDa receptor molecules decreased substantially and an additional band appeared at ~62 kDa (*lane 4*), indicating cleavage by IgA1 protease. Next, we analyzed the capability of IgA1 protease to cleave the native membrane bound receptors on viable U937 cells. For this purpose, U937 cells were treated over night with IgA1 protease. Cells were then lysed and type II receptors were immunoprecipitated using the specific antibody. As a control, the type I receptors were precipitated. Immunocomplexes were subsequently analyzed by Western blotting. Consistent with the lack of putative IgA1 protease cleavage sites in TNF-RI, no cleavage of this receptor was detected (Fig. 6, lanes 7 and 8). In contrast, a cleavage product of the TNF-RII at ~62 kDa

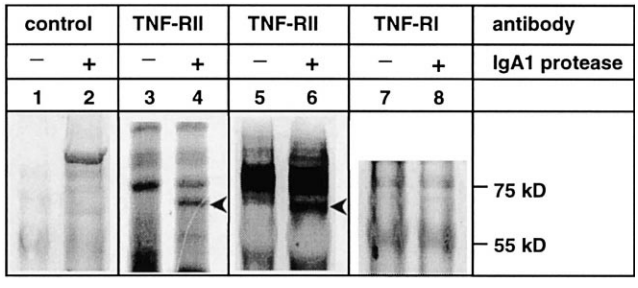

Fig. 6. Cleavage of TNF-RII by IgA1 protease. Following immunoprecipitation from cellular lysates with a monoclonal antibody specific for the extracellular domain of TNF-RII the immunocomplexes were incubated with (lane 4) or without (lane 3) IgA1 protease. As it is shown in lane 4, a cleavage product of ~62 kDa (arrowhead) could be clearly detected. To evaluate the cleavage activity of IgA1 protease on native cells, U937 cells were treated with (lane 6 and 8) or without (lane 5 and 7) IgA1 protease prior to cellular lysis and immunoprecipitation with either a monoclonal antibody specific for TNF-RI (lane 7 and 8) or TNF-RII (lane 5 and 6). As shown in lane 6 IgA1 protease is also active on viable cells thereby cleaving TNF-RII (arrowhead), however as it was suggested – due to the lack or proline-rich motifs – TNF-RI was not cleaved by IgA1 protease (lane 8). Lanes 1 and 2, immunoprecipitation with an isotype matched control antibody.

was clearly observed (lane 6). However, only partial cleavage of the type II receptor molecules was seen, possibly due to the high turn over rate of TNF-RII [20]. Thus, results from these experiments indicated that inhibition of TNFα-dependent apoptosis by IgA1 protease is associated with the specific proteolytic degradation of receptor type II for TNFα.

4. Discussion

Despite the fact that IgA1 proteases strongly correlate with the virulence properties of bacterial species [2], the precise function of IgA1 proteases in pathogenesis is still elusive. The results shown here provide first evidence that neisserial IgA1 protease is a defined bacterial factor capable of preventing human monocytic cells from TNFα-induced apoptosis. Furthermore, our data indicate that the enzymatic activity of the IgA1 protease is directly involved in the inhibition of TNFα-dependent apoptosis. We also demonstrate the cleavage of TNF-RII, but not TNF-RI, by purified IgA1 protease. This strongly suggests that the observed effect could be due to specific cleavage of TNF-RII although a causal link between these two intriguing observations is still missing.

TNF-RI and TNF-RII, together with other similar type I integral membrane proteins, constitute a distinct receptor super-family which is characterized by multiple cysteine-rich repeats in the extracellular domain [21]. The activation of these receptors is thought to occur by aggregating two or three receptor molecules on the cell surface upon binding of the homotrimeric ligands. This extracellular complex leads to an activation of the intracellular domains and finally to TNFα-mediated signal transduction via a complex network of adapter proteins. Cleavage of TNF-RII by IgA1 protease might prevent this activation process.

The extracellular portions of TNF receptor type I and II exist also as soluble fragments [22,23]. This mechanism occurs naturally in vivo by proteolytic cleavage of the complete extracellular part of the receptor from its transmembrane domain. The soluble forms specifically bind TNFα, competing for its binding to the cell surface TNF-R's thus interfering with the biological function of TNFα in an agonist–antagonist pattern. Moreover, shedding of TNF-R's is used by the cells to modulate TNF receptor expression [24]. In addition to naturally occurring soluble TNF-R's, some viruses utilize this cellular mechanism to interfere with TNFα-activity [25,26]. However, concerning the cleavage of TNF-RII by IgA1 protease, a similar shedding mechanism is very unlikely. Soluble TNF-R's as well as similar viral proteins comprise all four extracellular cysteine-rich domains which are necessary for TNFα binding [27]. However, due to the cleavage product of ~62 kDa shown in Fig. 6 and on the basis of the known amino acid sequence of TNF-RII [22] the cleavage site of IgA1 protease appears to be located near the binding site for TNFα in the first N-terminal cysteine-rich domain thus leading to a receptor fragment which comprises only the TNFα binding site and part of the first cysteine-rich domain. This very small fragment might therefore not be able to bind TNFα. So, we conclude that inhibition of TNFα-dependent apoptosis is directly mediated by cleavage of the ligand-binding domain from cellular TNF-RII rather than by a shedding mechanism.

Although the extracellular domains of the TNF receptors exhibit similar structures, they share no homology between the cytosolic domains [21]. This has led to the suggestion that the two TNF receptor transduce distinct signals [28]. For example, the TNF-RI contains a death domain, whereas the TNF-RII does not. It has also been shown that the intracellular domain of TNF-RI recruits several signaling proteins distinct from that of the TNF-RII [9]. However, due to the co-expression of both types of receptors on most cells, a dissection of their distinct signaling properties has been difficult. It is generally accepted that most of the TNFα signals are mediated through the receptor type I, particularly the signals necessary for NF-κB activation and apoptosis [9]. However, it has been shown recently that TNF receptor type II activates NF-κB and induces apoptosis [29]. In this respect, our data demonstrating inhibition of TNFα-dependent apoptosis putatively by cleaving TNF-RII support and extend these observations.

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