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Short Communication

Identification of ADAM10 as a major TNF sheddase in ADAM17-deficient fibroblasts

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ABSTRACT

ADAM17 (a disintegrin and metalloprotease)-deficient murine fibroblasts stably transfected with proTNF cDNA release significant amounts of biologically active soluble TNF. The enzyme responsible for this activity is a membrane protein that hydrolyzes the peptide bond Ala^{76}:Val^{77} within proTNF. Its activity is inhibited by 1,10-phenantroline and GM6001, insusceptible to TIMP-2 (tissue inhibitor of metalloproteinases-2), and stimulated by ionomycin. These characteristics match ADAM10. The moderate silencing of ADAM10 by shRNA resulted in a significant inhibition of TNF shedding. There was no correlation between the level of ADAM10 expression and the presence of active ADAM17. Our results indicate that ADAM10 may function as the TNF sheddase in cells which lack ADAM17 activity.

1. Introduction

TNF is a major pro-inflammatory cytokine with broad immune effects [1]. This pleiotropic cytokine is expressed mainly by macrophages as a 26 kDa transmembrane protein (proTNF) that is converted to a 17 kDa soluble molecule (sTNF). Membrane TNF, alike its soluble counterpart, is able to induce apoptosis in various cell types [2,3], and to activate immune and endothelial cells [1,2,4]. However, the overall biological consequences of the two forms' action are not identical, primarily because only sTNF may exert systemic effects [2] and only proTNF is involved in the process of reverse signaling [5]. Thus, the overall effects of TNF depend on the ratio of the soluble and the membrane forms of the cytokine.

ADAM17 was identified as a major TNF convertase [6,7]. Apart from TNF, ADAM17 is able to shed numerous cytokines, chemokines, growth factors, and their receptors, which implicates its involvement in the regulation of immune system functioning and in processes of cell growth and differentiation [8]. ADAM10 shares most structure similarity and some substrate specificity with ADAM17. Both enzymes play the role of secretase for numerous membrane proteins, including fractalkine, IL6-R, CD44, HB-EGF, APP, Prp [8]. Early publications indicated that TNF too is susceptible to ADAM10 proteolysis [9,10] but later experiments challenged the previous reports [11]. Other metalloproteases, such as transmembrane ADAM19 [12] and soluble MMP7 [13], may also be involved in TNF processing.

We present here the results of our study that identified ADAM10 as the enzyme that is, at least partially, responsible for TNF shedding from ADAM17-deficient fibroblasts.

2. Materials and methods

2.1. Murine cell lines

$ADAM17^{-/-}$, Ras-Myc-immortalized murine fibroblasts isolated from $ADAM17^{\Delta Zn/\Delta Zn}$ mice, a gift from Dr. R.A. Black (Amgen Inc., Seattle, WA, USA); $ADAM17^{-/-}TNF^{+}$, $ADAM17^{-/-}$ stably transfected with human proTNF cDNA [14]; L929 (fibroblast), NIH3T3 (fibroblast) and P388D1 (murine monocyte/macrophage-like), all from American Type Culture Collection; MBE-SV, murine brain microvascular endothelial cells (a gift from Dr. R. Auerbach, Madison, WI, USA) immortalized with SV40 large T antigen.

2.2. Cell cultures

All cell lines were cultured in DMEM/Glutamax-1 supplemented with 10% FBS at 37 °C in 5% CO_2. Culture medium for $ADAM17^{-/-}TNF^{+}$ cells was enriched in geneticin (1.5 mg/ml) and in zeocin (500 μg/ml) every few passages.

2.3. Generation of $ADAM17^{-/-}TNF^{+}$ cells stably transfected with ADAM17 cDNA

$ADAM17^{-/-}TNF^{+}ADAM17^{+}$ were generated by $ADAM17^{-/-}TNF^{+}$ cells transfection with pcDNA3.1(Hygro)/murine ADAM17 and a

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selection of hygromycin-resistant clones. The level of ADAM17 expression was analyzed in individual clones by RT-PCR and was confirmed by Western blot analysis of cell lysates.

2.4. Enzyme-linked immunosorbent assay

The TNF level in culture media of ADAM17$^{-/-}$TNF^{+} cells was measured using ELISA. Polyclonal anti-huTNF IgG (Sigma Chemicals Co., USA) was used as coating antibody and murine mAb anti-huTNF (Biocentrum Ltd., Poland) followed by HRP-conjugated-anti-murine IgG (Becton-Dickinson) were used as detecting antibodies. The enzymatic reaction was performed using TMB Substrate Reagent Set (PharMingen, USA).

2.5. Immunoprecipitation and Western blot analysis of TNF

Sample of medium (200 μl), collected from ADAM17$^{-/-}$TNF^{+} culture was immunoprecipitated according to a standard protocol [15] using Protein L-Sepharose beads (Santa Cruz Biotechnology Inc., USA; 10 μl) and murine anti-TNF IgG (2 μg). For Western blot analysis rabbit Ig anti-human TNF (2 μg/ml) and HRP-conjugated anti-rabbit IgG (0.05 μg/ml) were used. Antibody-reactive proteins were detected using enhanced chemiluminescence reagents (ECL kit, Amersham-Pharmacia Biotech., Sweden).

2.6. Western blot analysis of ADAM17

Cell lysates were prepared using RIPA buffer containing the protease inhibitors cocktail (Roche Applied Science). Fractions enriched in glycoproteins were obtained by ConA-Sepharose chromatography [16] and protein concentration was determined in each sample using BCA assay (Sigma). For Western blot analysis of ADAM17 rabbit IgG generated in our laboratory (4 μg/ml) [17] and HRP-conjugated anti-rabbit IgG (0.05 μg/ml, Sigma) were used. The signals were analyzed by using Quantity One software (Bio-Rad).

2.7. Cytotoxicity assay

The cytotoxic activity of TNF was determined by the MTT test [15]. L929 cells cultured in a 96-well plate (10^4 cells/well) were incubated in DMEM containing 5% FBS and actinomycin D (125 ng/ml) or in DMEM enriched in 5% FBS and actinomycin D containing: (*i*) the conditioned medium aspirated after 50-min incubation of ADAM17$^{-/-}$ or (*ii*) ADAM17$^{-/-}$TNF^{+} (containing 10 ng/ml TNF); (*iii*) fresh medium containing TNF (10 ng/ml) purified from ADAM17$^{-/-}$TNF^{+} medium (as described below); (*iv*) rhu TNF (10 ng/ml) or (*v*) ADAM17$^{-/-}$TNF^{+} medium deprived of TNF by immunoprecipitation with Protein A/rabbit anti-huTNF. The negative control (100% viability) consisted of L929 incubated with actinomycin D only, and the positive control (0% viability) consisted of L929 incubated with actinomycin D + rhuTNF (10 ng/ml). MTT assay was performed after 16-h incubation. The absorbance of solubilized formazan was measured at 562 nm. Cell viability was calculated as follows:

$$\text{Cell viability}\,(\%) = \frac{A_{562}\,(\text{sample}) - A_{562}\,(\text{positive control})}{A_{562}\,(\text{negative control}) - A_{562}\,(\text{positive control})} \times 100\% \quad (1)$$

2.8. Nitrite assay

The media collected after 50 min-incubation of ADAM17$^{-/-}$ or ADAM17$^{-/-}$TNF^{+} (final concentration of TNF – 10 ng/ml) were transferred to MBE-SV cells cultured in a 96-well plate. To some wells fresh medium containing TNF (10 ng/ml) purified from ADAM17$^{-/-}$TNF^{+} medium, rhuTNF (10 ng/ml) or ADAM17$^{-/-}$TNF^{+} medium deprived of TNF by immunoprecipitation was added. MBE-SV cells were then stimulated with IL-1 (10 ng/ml) and IFNγ (10 ng/ml) for 24 h. Nitrite concentration in the medium was determined using Griess reagent [18].

2.9. Amino-terminal sequence analysis

TNF was purified from medium collected from ADAM17$^{-/-}$TNF^{+} cells cultured in the absence of FBS by means of affinity chromatography on mAb anti-huTNF (Biocentrum Ltd.) coupled to Sepharose. The sample of purified protein (2 μg) was resolved in SDS–PAGE and electroblotted onto PVDF membranes. After staining with Coomassie Blue-G250 the protein band was cut out and subjected to amino-terminal sequence analysis with an Applied Biosystems 491 Protein Sequencer according to the program designed by the manufacturer.

2.10. Analysis of TNF-shedding activity in the ADAM17$^{-/-}$ culture medium

P388D1 cells were incubated for 16 h with LPS (100 ng/ml) in order to stimulate TNF synthesis. Next, the medium was replaced with serum-free DMEM, or with serum-free DMEM collected after 24-h incubation of ADAM17$^{-/-}$ or ADAM17$^{-/-}$TNF^{+}. After 1-hour incubation the levels of TNF released to the medium were measured using ELISA (BenderMedSystem, Austria).

2.11. Analysis of the effect of metalloprotease inhibitors on TNF-shedding activity in ADAM17$^{-/-}$TNF^{+} and ADAM17$^{-/-}$TNF^{+}ADAM17^{+} cells

The cells were incubated with 10 mM 1,10-phenantroline or with 10 μM GM6001 (galardin) (Biomol Int.), both dissolved in DMSO or with the adequate volume of the solvent. In order to determine whether TNF-shedding activity is susceptible to TIMP-2, cells (1×10^4) were incubated for 30 min in 50 μl of serum-free DMEM containing 100 nM TIMP-2 (R&D Systems) dissolved in TCNB buffer (50 mM Tris, 5 mM $CaCl_2$, 100 mM NaCl, 0.05% Brij-35, pH 7.5) or containing the appropriate volume of TCNB buffer. The activity of TIMP-2 was confirmed by the inhibition of azocoll hydrolysis catalyzed by collagenase from *C. histolyticum* (Sigma). The levels of TNF released to the media were determined by ELISA. The lack of cytotoxic effects of the inhibitors was verified by MTT assay performed 15 h after removing the inhibitor from the medium.

2.12. Analysis of the effect of ionomycin or PMA on TNF shedding in ADAM17$^{-/-}$TNF^{+} or in ADAM17$^{-/-}$TNF^{+}ADAM17^{+} cells

The cells (1×10^4/100 μl) were incubated in serum-free medium for 30 min in the presence of ionomycin (5 μg/ml) or PMA (250 ng/ml) or with the adequate volume of ethanol (the solvent; the concentration of ethanol did not exceed 0.5%). The levels of TNF released to the media were determined by ELISA.

2.13. The effect of ADAM10 gene silencing by specific shRNA on TNF shedding in ADAM17$^{-/-}$TNF^{+}

ADAM17$^{-/-}$TNF^{+} were transfected with pGeneClip™Vector (Promega) coding for ADAM10 shRNA or for non-interfering control shRNA or with a plasmid coding for green fluorescent protein, pEGFP-C1 (Clontech) using lipofectamine-2000 (Invitrogen Life Technologies) according to the manufacturer's protocol. Based on preliminary results two vectors out of four were chosen for further