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Research paper

FasL on the surface of Tag7 (PGRP-S)-activated lymphocytes induces necroptosis in HLA-negative tumor cells with the involvement of lysosomes and mitochondria

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ABSTRACT

Recently we have found that cytokine IL-2 and innate immunity protein Tag7 activate cytotoxic lymphocytes that kill HLA-negative tumor cells, inducing both apoptosis and necroptosis. Here we decrypt the processes, taking part in necroptosis execution after FasL-Fas interaction. Necroptosis begins with RIPK1 activation and necrosome formation. Subsequent activation of MLKL results in the increase of Ca2+ level in the cell and activation of Ca2+-dependent enzymes causing lysosomal membrane permeabilization and the release of cathepsins to the cytosol. STAT3 translocation to the mitochondria and binding to a component of the respiratory chain complex I causes ROS accumulation. We have shown that transduction of necroptotic signal via TNFR1 and Fas has many common points. It is known that apoptosis plays a major role in physiological cell death; however, under pathological conditions necroptosis is very common. That is why the detailed mechanisms of FasL-Fas necroptosis can help in understanding the processes of elimination of tumor cells that have blocked apoptosis signal transduction.

1. Introduction

The search for new protective agents in immunotherapy is inextricably associated with studies on the processes of cell death induced by effector lymphocytes and an insight into the mechanisms helping tumor cells to survive. A cytotoxic effect against virus-infected and tumor cells may be exerted by soluble factors as well as by specialized lymphocyte populations, but in both cases the mechanism of cell killing involves the interaction of specific ligands with corresponding receptors on their surface.

These receptors are combined into a subclass of cell death receptors that have long been considered to be capable of inducing only apoptosis [1,2]. Today, however, it is known that they can induce both cell proliferation and cell death signals, with the central role in signal transduction being played by RIP1 protein kinase (RIPK1), which interacts with the cytoplasmic part of the receptor[3]. The same ligand bound to the receptor can induce opposite signals depending on the degree of RIPK1 ubiquitination [3,4]. Non-ubiquitinated RIPK1 dissociated from the receptor-bound complex to the cytoplasm and binds to the death-inducing DISC complex. Following mechanism of cell death depends on procaspase-8 activation, which results in the formation of caspase-8 and consequent activation of the caspase cascade and eventual cell death by apoptosis [5–7]. In the absence of caspase-8, RIPK1 is autophosphorylated and forms a stable complex with RIP3 kinase (RIPK3), named necrosome, which, in turn, activates the protein kinase cascade and thereby induces programmed cell necrosis, or necroptosis [8–10]. It is noteworthy that one of the ways for tumor cells to evade the immune defense is to block apoptotic signals, but in this case necroptotic pathway is activated, and the cells die anyway.

Receptors TNFR1, TRAIL-R, and Fas belong to the family of tumor necrosis factor (TNF) death receptors. The mechanisms of apoptotic and necroptotic signal transduction during the interaction of TNFα with TNFR1 have been studied in most detail. We have recently shown that the Tag7–Hsp70 cytotoxic complex is a ligand for TNFR1 and described the mechanisms of apoptosis and necroptosis induced by their interaction [11,12]. It has long been considered that Fas is a classic apoptosis receptor, regardless of evidence that its soluble ligand (FasL) can induce caspase-independent cell death by a mechanism where RIP1 kinase is the effector molecule [13]. We have shown that the well-known cytokine IL-2 and innate immunity protein Tag7 activate cytotoxic T lymphocytes that kill HLA-

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positive tumor cells by inducing apoptosis or necroptosis through the FasL–Fas interaction [14,15].

The Tag7 protein, also referred to as PGRP-S or PGLYRPL, plays a role in innate immunity and has both antibacterial and antitumor activity [16,17]. This protein in insects interacts with the Toll receptor to activate a protective pathway leading to antimicrobial peptide secretion [18]; in mammals, it induces secretion of anti-inflammatory cytokines activating antimicrobial defense [15,19]. We have described several new functions of Tag7 in antitumor defense. In particular, Tag7 in complex with the main heat shock protein Hsp70 can have a lethal effect on certain cell lines and retard tumor growth in mice [20,21]. In complex with Ca^{2+}-binding protein Mts1 (S-100A4), Tag7 has chemotactic activity and can attract lymphocytes (mainly NK cells) to the focus of lesion [22]. Our recent experiments have shown that long-term incubation of healthy donor leukocytes with Tag7 results in activation of cytotoxic lymphocyte populations [15], with $FasL^+$ T lymphocytes killing tumor cells by triggering both apoptotic and necroptotic pathways, as in the case of activation by IL-2. However, the specific mechanisms of necroptosis caused by $FasL^+$ T lymphocytes in tumor cells have not yet been studied sufficiently.

This study was performed to elucidate molecular mechanisms involved in Fas-dependent necroptosis in target cells following their exposure to Tag7-activated lymphocytes.

2. Material and methods

2.1. Cell culture

K562 cells were cultured in RPMI-1640 with 2 mM L-glutamine and 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA). Human peripheral blood mononuclear cells (PBMCs) were isolated from the total leukocyte pool of healthy donors by Ficoll-Hypaque density gradient centrifugation, as described [23], and cultured at a density of 4×10^6 cells/mL in RPMI-1640 with 10^{-9} M Tag7 protein for 6 days. Cell sorting was performed using standard magnetic bead kits (Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's protocol. Specific antibodies to MicA (Sigma, St. Louis, MO, USA) were coupled to magnetic beads coated with anti-rabbit antibodies. All PBMC experiments and procedures were performed as approved by the Ethics Committee of the Institute of Gene Biology.

2.2. Proteins and antibodies

The cDNAs encoding recombinant human Tag7 (GenBank accession no. NM_005091) was subcloned in pQE-31 and expressed in *E. coli* M15 (pREP4) (Qiagen, Germany). Tag7 was purified on nickel nitrilotriacetic acid-agarose (Qiagen) as recommended by the manufacturer. Limulus amebocyte lysate (LAL) chromogenic endpoint assays (Cambrex, East Rutherford, NJ, USA) of recombinant Tag7 did not detect LPS above the detection limit of 1 endotoxin unit (EU) per microgram protein. Soluble FasL was from (Sigma, St. Louis, MO, USA). Antibodies used in the study were as follows: rabbit monoclonal anti-pRIPK1 (Cell Signaling Technology, USA), mouse monoclonal anti-beta-actin (Sigma, St. Louis, MO, USA), mouse monoclonal anti-pSTAT3-Ser727 (Santa Cruz Biotechnology, USA), mouse monoclonal anti-VDAC1/Porin (Abcam, Cambridge, UK), rabbit anti-MicA (Sigma, St. Louis, MO, USA).

2.3. Cytotoxicity assays

K562 cells cultured in 96-well plates (6×10^4 cells per well) were mixed with Tag7-activated $FasL^+$ lymphocytes added at a 20:1 ratio or with soluble FasL (250 ng/mL) and incubated at 37 °C for 20 h.

Enzyme inhibition tests were conducted with specific inhibitors: RIP1 kinase inhibitor Nec1, lysosomal enzyme inhibitor chloroquine, MKLK inhibitor NSA (5 μM each), Ca^{2+} chelator EGTA (2 μM), antioxidants ionol (1 μM) and, cathepsin B inhibitor Ca-074Me (10 μM), cathepsin D inhibitor pepstatin A (P5318, 10 μM), and calpain inhibitor peptide (C9181, 10 μM) were from Sigma-Aldrich (St. Louis, MO, USA); Necrox-2 (1 μM), STAT3 inhibitor Stattic V (10 μM), and cPLA2 inhibitor U-73122) (1 μM) were from Santa-Cruz Biotechnology (Dallas, TX, USA). The cells were initially treated with an inhibitor for 1 h, and then $FasL^+$ lymphocytes or soluble FasL were added. As a control, the lymphocytes were pre-incubated with Nec1 for 1 h and washed before addition to the K562 cells. Dead cells were detected with a Cytotox 96 Assay kit (Promega, Madison, WI, USA). The death rate of control cells (lymphocytes or target cancer cells) did not exceed 3%.

2.4. Isolation of mitochondrial and cytosolic fractions

K562 cells incubated with $FasL^+$ lymphocytes (for 60 min) were purified on MicA conjugated beads according to the manufacturer's protocol. K562 cells were incubated with soluble FasL (for 30 or 60 min or for 30 min in the presence of Nec1). Untreated K562 cells were used as control. Approximately 5×10^6 cells were ground with a glass homogenizer in an ice bath (22 strokes) and extracted with 20 mM HEPES buffer, pH 7.4, containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM DTT, 10 μM PMSF, and 250 mM sucrose. Cytoplasmic and mitochondrial fractions were separated by differential centrifugation (700 g for 10 min, 1500 g for 10 min, and 12 000 g for 30 min at 4 °C). The supernatant (cytosolic fraction) and pellet (mitochondrial fraction) were collected and lysed in SDS sample buffer.

2.5. Immunoprecipitation of RIPK1, RIP3K, MLKL and STAT3

RIPK1 was immunoprecipitated with anti-pRIPK1 antibody (Cell Signaling Technology, Beverly, MA, USA) from the cytosolic fraction of K562 cells treated with soluble FasL and of K562 cells incubated with Tag7-activated $FasL^+$ lymphocytes and purified on magnetic beads according to the manufacturer's protocol. RIPK3 was immunoprecipitated with anti-pRIPK3 antibody (Abcam, Cambridge, MA, USA) MLKL was immunoprecipitated with anti-pMLKL antibody (Abcam, Cambridge, MA, USA). STAT3 was immunoprecipitated with anti-pSTAT3-Ser727.5 antibody (Santa Cruz Biotechnology) from the mitochondrial fraction of K562 cells treated with soluble FasL. The immunoprecipitates were resolved by 10% SDS-PAGE and blotted onto a PVDF membrane (GE Healthcare Ltd., Little Chalfont, UK). The membrane was blocked with 1% nonfat dry milk and incubated with primary antibodies: rabbit monoclonal anti-pRIPK1 (1:1000, Cell Signaling Technology), anti-pRIPK3 (1:1000, Abcam), anti-pMLKL (1:1000, Abcam), and mouse monoclonal anti-pSTAT3-Ser727 (1:1000, Santa Cruz Biotechnology). As control were used mouse monoclonal anti-beta-actin (1:10 000, Sigma-Aldrich) or mouse monoclonal anti-VDAC1/Porin (1:10 000; Abcam, Cambridge, UK). Horseradish peroxidase-conjugated secondary antibodies were goat anti-rabbit (1:15 000) or rabbit anti-mouse (1:15 000), both from GE Healthcare. The immunoreactive bands were visualized with an ECL Plus kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's protocol.