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Acid sphingomyelinase mediated release of ceramide is essential to trigger the mitochondrial pathway of apoptosis by galectin-1

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Abstract

The mechanism of apoptosis induced by human galectin-1, a mammalian β-galactoside-binding protein with a remarkable cytotoxic effect on activated peripheral T cells and tumor T cell lines has been extensively investigated in this study. Here we first show that galectin-1 initiate the acid sphingomyelinase mediated release of ceramide and this event is critical in the further steps. Elevation of ceramide level coincides with exposure of phosphatidylserine on the outer cell membrane. The downstream events, decrease of Bcl-2 protein amount, depolarization of the mitochondria and activation of the caspase 9 and caspase 3 depend on production of ceramide. All downstream steps, including production of ceramide, require the generation of membrane rafts and the presence of two tyrosine kinases, $p56^{lck}$ and ZAP70. Based on our findings we suggest a model of the mechanism of galectin-1 triggered cell death.

Keywords: Galectin-1; $p56^{lck}$; ZAP70; Acid sphingomyelinase; Ceramide; Apoptosis

1. Introduction

Regulation of cell survival during tissue differentiation, immune development, immune response and tumor growth is a vital factor in maintaining homeostasis. Galectin-1 (Gal-1), a member of the galectin superfamily plays roles in regulation of cell growth and apoptosis [1] of activated peripheral T lymphocytes and tumor T cell lines [2], hence it contributes to the immunological balance in physiological and pathological situations. During the past several years, scores of data have been published about the mechanism of the Gal-1 induced apoptosis, however, these data remain largely controversial. It is still not clear which receptor transmits the apoptotic signal into T cells. It appears to be distinct from Fas/FasL pathway as it has been shown in a Fas resistant T cell line [3] and Fas deficient *lpr* mice [4]. Nevertheless a recent paper has shown that Gal-1 cooperates with Fas induced apoptosis in resting and activated peripheral T cells [5]. The Gal-1-binding surface glycoproteins (CD2, CD3, CD7, CD43 and CD45) [6–8] have been presumed as signal transmitters. However, only CD7 seems to fulfill the requirements for a transmitting receptor, as the absence of CD7 correlates with the failure of Gal-1 induced apoptosis, and complementation of CD7 restores cell death [6,9]. The receptor tyrosine phosphatase CD45, initially described as apoptotic receptor for Gal-1 [3,7] has recently been shown to be dispensable [10]. Caspases have been implicated by many authors [11,12], as death effectors in the intracellular death pathway, though, in contrast to the others, a group have recently reported that caspase cascade is not involved

Abbreviations: Ac-IETD, Ac-Ile-Glu-Thr-Asp-CHO; Ann V, Annexin V; aSMase, acid sphingomyelinase; β-CD, β-cyclodextrin; Desi, desipramine; FB1, fumonisin B1; Gal-1, galectin-1; PARP, poly (ADP-ribose) polymerase; PDBu, phorbol dibutyrate; PS, phosphatidylserine; PTK, protein tyrosine kinase; S1P, sphingosine 1 phosphate; zVAD-fmk, *N*-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone.

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in this process [13]. Bcl-2 downregulation and activation of AP-1 transcription factor [11] and translocation of endonuclease G from the mitochondria to the nucleus [13] have also been indicated. Gal-1 treatment induces partial TCRζ chain phosphorylation, generating pp21ζ and limited receptor clustering at the TCR contact site [14] and hence it antagonizes with the TCR signal transduction and promotes apoptosis. Accordingly we have recently described the indispensable function of $p56^{lck}$ and ZAP70 in Gal-1 induced T cell apoptosis [15]. The induction of classical [5] and non-classical [13] mitochondrial pathway of apoptosis was published generating a more confusing picture in the mechanism of Gal-1 induced T cell death.

Ceramide plays role in cell survival and homeostasis on diverse manners. It has been shown to be a general factor of apoptosis triggered by different ways, such as TNFR [16] or Fas [17] stimulation or UV irradiation [18], however in some cases this event is not essential for execution of apoptosis [19]. Early and transient release of ceramide essentially contributes to the formation of functional membrane rafts [20] as it occurs upon Fas ligation in T cell lines [21]. Nevertheless, the "second messenger concept" [22,20] cannot be ruled out since ceramide has several well-defined signaling targets. Ceramides act on enzymes participating in the transduction of death signals, for instance by activating the protein phosphatase A2 which rapidly dephosphorylates and inactivates Bcl-2 [23]. Role of ceramide was also indicated in Gal-1 induced apoptotic pathway [5].

Our aim in this work has been to carry out an extensive study on the mechanism of Gal-1 induced apoptosis. We prove that acid sphingomyelinase (aSMase) dependent ceramide production is essential to downstream apoptotic events induced by Gal-1. Inhibiting the activity of aSMase results in the failure of decrease of Bcl-2 protein amount and depolarization of mitochondrial membrane as well as activation of caspase 9 and 3. Based on our results we suggest the most complete model, available at this time, for Gal-1 induced apoptosis.

2. Materials and methods

2.1. Cell lines

Jurkat cells and I 9.2 (Jurkat lacking caspase 8) (ATCC) were maintained in RPMI-1640 (GIBCO-BRL) with 5% FCS (Plaz Med Kft.) in an incubator with 5% CO_2 at 37 °C. JCaM1.6 ($p56^{lck}$ deficient), JCaM1/Lck (JCaM1.6 re-transfected with $p56^{lck}$) [24], P116 (ZAP70 deficient) and P116WT (P116 re-transfected with ZAP70) [25] Jurkat variants were cultured in RPMI 1640 with 10% FCS under the same conditions. JCaM1/Lck cells were maintained in the presence of 250 μg/ml hygromycin. The cell lines, I 9.2 was a kind gift from Dr. V. Chitu (Albert Einstein College of Medicine of Yeshiva University, New York), P116 and P116WT were donated by Dr. R.T. Abraham (Mayo Clinic, Rochester) and JCaM/Lck was generously provided by Dr. A. Weiss (Howard Hughes Medical Institute, San Francisco). Peripheral blood mononuclear cells from healthy donor were isolated through Ficoll gradient and activated with 5 μg/ml PHA for 72 h in RPMI containing 10% FCS then cultured with 20 ng/ml IL-2. IL-2 was removed 24 h before apoptosis assays.

2.2. Reagents

The following reagents were used in this study: nitrocellulose membrane (Schleicher and Schuell), anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology Inc), anti-lactosyl-ceramide (Biomeda Corp.), rabbit anti-mouse IgG conjugated to HRP, biotinylated rabbit anti-mouse IgM and streptavidin-FITC (DAKO), ECL plus detection system (Amersham Bioscience), X-ray film (Medifort SFB), Annexin V-FITC (Pharmingen), Alexa647-cholera toxin B and MitoTrackerRed CMX-Ros (Molecular Probes), prestained molecular weight marker (GIBCO-BRL), mouse anti-PARP mAb (Serotec), rabbit anti-ZAP70 (produced in our laboratory), mouse anti-ZAP70 mAb (BD Transduction Laboratory), rabbit-anti-Lck (Santa Cruz Biotechnology), mouse anti-Lck mAb (produced in our laboratory), mouse-anti-Bcl-2 (Upstate), phytohemagglutinin-M (Calbiochem), IL-2 (Proleukin, Chiron Corporation), rabbit anti-actin (Abcam), Ficoll-Paque™ Plus (Amersham), Protein G Sepharose (Pharmacia), caspase inhibitor I (zVAD-fmk), caspase 8 inhibitor (Ac-IETD-CHO), herbimycin A and bongkrekic acid (Calbiochem), anti-ceramide mAb, MID 15B4 (Alexis Biochemicals), Caspases-Glo™ 9 Assay and Caspases-Glo™ 3 Assay (Promega), ^{32}P-γATP (Isotope Inst. Kft, Budapest, Hungary). TNFα was a kind gift of Dr. Erno Duda (Biological Research Center, Szeged, Hungary). Other reagents were purchased from Sigma.

Recombinant Gal-1 was cloned and purified by lactose affinity chromatography as previously described [26]. The purity of the recombinant Gal-1 was stringently analyzed by SDS-PAGE, reverse-phase HPLC in a Vydac C4 column and mass spectrometry (data not shown). The empty vector was cloned and the bacterial lysate was processed on the same way as for Gal-1 purification and used as control in all experiments.

2.3. Western blotting

2.3.1. Tyrosine phosphorylation

The cells were stimulated at a concentration of 5×10^7 cells/ml in RPMI without FCS by adding 1.8 μM of Gal-1 for the indicated time at 37 °C. Activation was stopped by addition of equal volume of 2× concentrated ice-cold lysis buffer (1× lysis buffer: 50 mM HEPES pH 7.4, 1% Triton X-100, 150 mM NaCl, 20 mM NaF, 200 μM Na_3VO_4, 2 mM EDTA, 1 mM PMSF and 10 μg/ml leupeptin). The cells were lysed for 30 min on ice and cleared off the nuclear/cytoskeletal components by centrifugation at $12{,}000\times g$ for 15 min. The proteins were separated on a 7.5–15% gradient of SDS polyacrylamide gel and transferred to nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membranes were blocked using Tris-buffered saline (TBS) containing 0.05% Tween 20 and 3% cold fish gelatin and subsequently probed with anti-phosphotyrosine mAb, 4G10 and rabbit anti-mouse IgG-HRP. Immunoreactive proteins were visualized by ECL plus detection system.

2.3.2. PARP and Bcl-2 immunoblotting

The cells were lysed in SDS-PAGE sample buffer (40 mM Tris–HCl pH 6.8, 5% glycerol, 0.5% SDS, 0.5% 2-ME) (10^7 cells/ml) and boiled for 5 min. The lysate was then centrifuged with $12{,}000\times g$ for 15 min at room temperature. Twenty microliters of the lysate was loaded onto a 6–12% SDS polyacrylamide gel for separation of PARP and its degraded forms or to a 10% SDS polyacrylamide gel for analysis of Bcl-2 and then transferred onto nitrocellulose membrane. The membrane was blocked using TBS containing 0.05% Tween 20 and 5% nonfat milk and subsequently probed with mouse anti-PARP or anti-Bcl-2 and rabbit anti-mouse IgG-HRP. For loading control the membranes were hybridized with rabbit anti-actin followed by goat anti-rabbit IgG-HRPO. Immunoreactive proteins were visualized by ECL plus detection system.

Images of all immunoblots were captured with Hewlett Packard scanner, exported to Adobe Photoshop 7.0, and then Tiff images were placed for final presentation in CorelDRAW 10. For estimation and quantitation of Bcl-2/actin ratio the X-ray film were analyzed with UVP's System 5000 gel documentation system using UVP GelBase software (UVP Inc.).

2.4. Annexin V labeling

To detect phosphatidylserine exposure on the outer cell membrane, the Jurkat cells were treated as indicated, then washed twice with PBS and resuspended in binding buffer (0.01 M HEPES, 0.14 M NaCl and 2.5 mM $CaCl_2$). Annexin V-FITC and propidium iodide (10 μg/ml) were added to the cells for 15 min in dark, at room temperature. After washing, the cells were analyzed on FACSCalibur cytofluorimeter (Becton Dickinson).