[CANCER RESEARCH 64, 3376–3379, May 15, 2004]

Advances in Brief

Endogenous Galectin-3 Determines the Routing of CD95 Apoptotic Signaling Pathways

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Abstract

Studies of CD95 (APO-1/Fas), a member of the death receptor family, have revealed that it is involved in two primary CD95 apoptotic signaling pathways, one regulated by the large amount of active caspase-8 (type I) formed at the death-inducing signaling complex and the other by the apoptogenic activity of mitochondria (type II). To date, it is still unclear which pathway will be activated in response to an apoptotic insult. Here, we demonstrate that the antiapoptotic molecule galectin-3, which contains the four amino acid-anti-death-motif (NWGR) conserved in the BH1 domain of the Bcl-2 member proteins, is expressed only in type I cells. Transfection of galectin-3 cDNA into galectin-3 null cells (type II) resulted converting them to type I apoptotic phenotype. In addition, we show that galectin-3 is complexed with CD95 *in vivo* identifying galectin-3 as a novel CD95-binding partner that determines which of the CD95 apoptotic signaling pathways the cell will select.

Introduction

CD95 (APO-1/Fas) is one of the members of the death receptor family (1). The CD95 signaling pathway is characterized by a sequential activation of a number of caspases. Recent studies have described the existence of two types of CD95-mediated apoptotic signaling pathways that depend on the amount of active caspase-8 produced at the death-inducing signaling complex (DISC; Ref. 2). In type I cells, stimulation of CD95 results in a very efficient recruitment of both Fas-associating protein with death domain and caspase-8 to CD95, leading to DISC formation. This step is dependent on the availability of F-actin, which is followed by formation of large CD95 surface clusters and internalization of activated CD95 (3). The DISC in type II cells forms very inefficiently without the involvement of F-actin (3). However, it remains unknown which molecule(s) determine the CD95 dependent apoptotic pathway. Galectin-3 has been reported to protect epithelial cells from apoptosis induced by staurosporine, cisplatin, genistein, and anoikis (4–6). Introduction of galectin-3 into fibroblasts and breast cancer cells resulted in rearrangements of actin filaments (7, 8). The rearrangements may explain, in part, the antiapoptotic activity of galectin-3 (7). Moreover, recent studies have revealed that galectin-3 is associated with T-cell receptor complex and regulates its clustering (8).

We report herein that galectin-3 interacts with CD95 and determines which of the CD95 apoptotic signaling pathways the cell will select.

Materials and Methods

Cell Culture and Transfection. The human B-cell lymphoblastoid cell line SKW6.4 (TIB-215) and the human T-cell lines CEM (CCL-119) and Jurkat (TIB-152) were from American Type Culture Collection. All cells were maintained as described previously (2).

CEM cells were electrophorated with 10 μg of pcDNA3.1/Zeo(-), constructed by an insertion of a *Eco*RI-*Eco*RI fragment of pBK-CMV-Gal-3 (4), into the *Eco*RI site in the pcDNA3.1/Zeo(-) (Invitrogen, Carlsbad, CA). Cells were pulsed at 200V/960 μF in a Gene Pulser. Cells were cultured for 48 h in complete media and transferred to complete media containing 300 μg/ml zeocin. Limiting dilution isolated individual clones.

Apoptosis Assay. A total of 2×10^5 cells was treated with several concentrations of anti-CD95 or C_2-ceramide in a total volume of 200 μl for different periods of time at 37°C (2, 9). Apoptotic cells were measured by annexin V binding and propidium iodide permeability and analyzed using a Becton Dickinson FACScan and CellQuest software.

Caspase Activation Assay. To investigate the caspase activity, we measured DEVDase and IETDase activity in Jurkat cells treated with anti-Fas antibody (CH-11; Kamiya Biomedical Company) as described previously (6). Cells were lysed in cell extract buffer [20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT] containing 0.03% NP40. Lysates were centrifuged at 15,000 $\times$ *g* for 10 min, and 50 μl of the cytosolic fraction were incubated for 60 min at 37°C in a total volume of 200 μl of caspase buffer [10 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM DTT] containing 25 μM acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin or acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Bachem, King of Prussia, PA). Using a Spectra Maxi Germini fluorescence plate reader (Molecular Devices, Menlo Park, CA), 7-amino-4-methylcoumarin fluorescence, released by caspase activity, was measured at 460 nm using 360-nm excitation wavelength. Caspase activity was normalized per microgram of protein determined by the BCA protein assay kit.

Immunoprecipitation and Western Blot Analysis. Immunoprecipitation was performed as described previously (6). Western blot analyses were performed using the enhanced chemiluminescence detection system (Amersham) as described previously. Antibodies used were as follows: anti-galectin-3 monoclonal antibody (TIB166 ATCC); anti-tubulin; anti-caspase-3; anti-caspase-8 polyclonal antibody (Santa Cruz Biotechnology, Inc.); and anti-Bcl-2 (DAKO, Carpinteria, CA).

Confocal Immunofluorescence Microscopy. A total of 1×10^6 cells was fixed and permeabilized with 100% methanol for 15 min at −20°C blocked with 1% BSA in PBS and stained with anti-Bcl-2 antibody and anti-galectin-3 m antibody for 1 h. After three washes with PBS, the cells were incubated for 1 h with FITC-conjugated antimouse antibody and Texas Red-conjugated antirat antibody. After five washes, cells were dropped on glass microscope slides and mounted using Prolong Anti-fade mounting media (Molecular Probes). The stained cells were analyzed using Zeiss Laser Scanning Microscope 310 (Zeiss, Chester, VA). The cells were scanned by dual excitation of fluorescein (green) and Texas Red (red) fluorescence. Areas of green and red overlapping fluorescence were represented with a yellow signal.

Received 2/2/04; revised 3/16/04; accepted 3/29/04.

Grant support: NIH Grant CA-46120.

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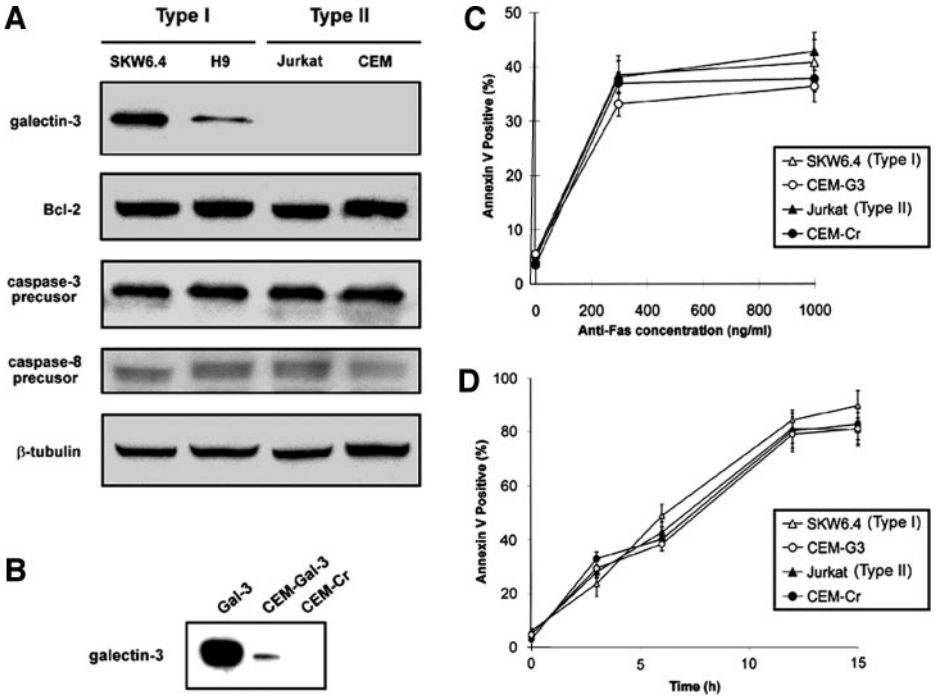

Fig. 1. CD95-mediated apoptosis. *A*, intracellular expression level of galectin-3 and apoptosis-related proteins such as Bcl-2, caspase-3 precursor, and caspase-8 precursor was measured in both CD95 type I and type II cell lines by Western blot analysis. A total of 30 μg of cell lysates was loaded in each lane. Tubulin was used as a loading control. *B*, galectin-3 expressing CEM (CEM-Gal-3) cells were transfected with an expression vector encoding a full-length galectin-3 and control cells only transfected with plasmid (CEM-Cr). *C*, dose response analysis of CD95-mediated apoptosis. Type I cell SKW6.4, type II cell Jurkat, CEM-Cr, and CEM-Gal-3 were incubated for 6 h at 37 °C with anti-Fas antibody. The percentage of apoptotic cells in each sample was measured by annexin V binding and propidium iodide permeability using flow cytometric analysis. *D*, kinetics of CD95-mediated apoptosis. Type I cell SKW6.4, type II cell Jurkat, CEM-Cr, and CEM-Gal-3 were treated with 1000 ng/ml anti-Fas antibody for different periods of time and analyzed as described in *C*. Data are mean ± SD of three independent experiments.

Results

Galectin-3 Is Expressed in Type II Cells but not in Type I Cells. In this study, we have used prototypic cells designated as type I cells (SKW6.4, H9) and type II cells (CEM, Jurkat), respectively (2, 9). First, we examined the expression levels of initiator caspase-caspase-8, effector caspase-caspase-3, anti-apoptotic protein-Bcl-2, and galectin-3 in these cells. Although expression levels of Bcl-2, procaspase-3, and procaspase-8 were almost identical in all cell lines, as previously reported (2), galectin-3 was found to be expressed only in type I cell lines (SKW6.4 and H9; Fig. 1*A*).

Next, we transfected galectin-3 cDNA into CD95 type II CEM cells and established a galectin-3 expression cell clone (CEM-Gal-3; Fig.

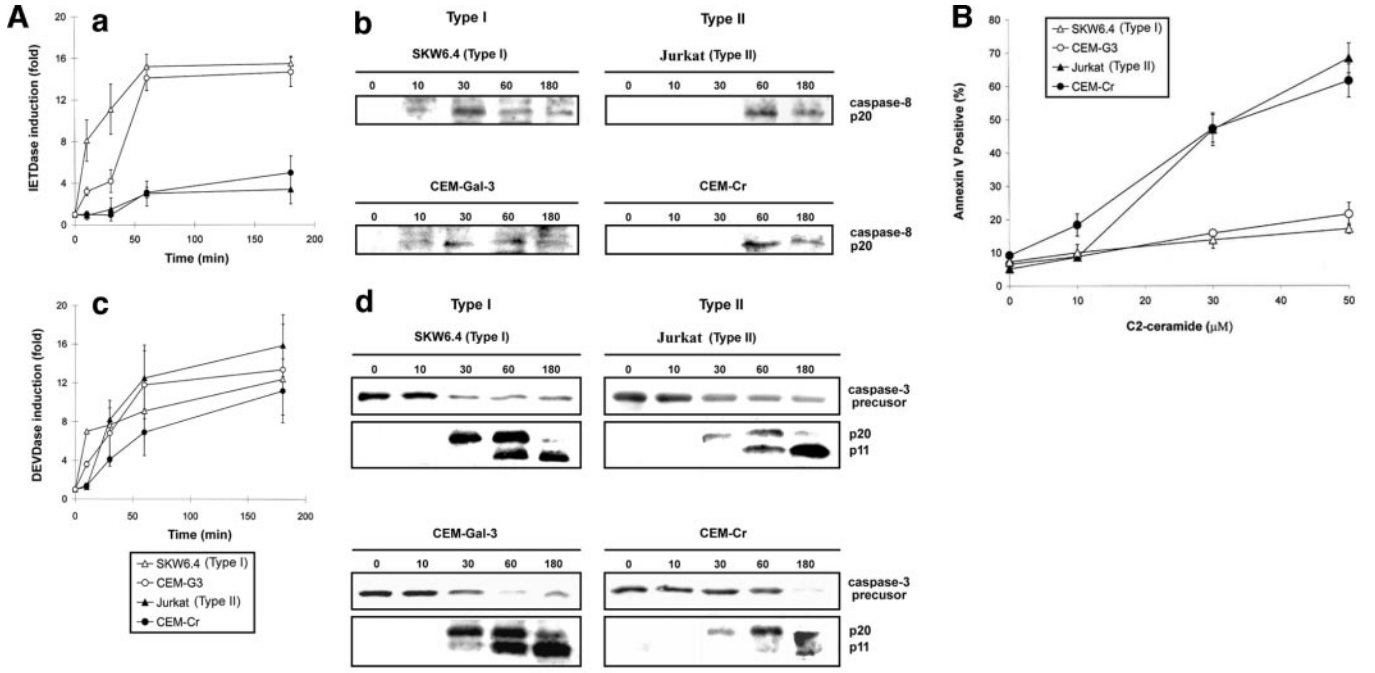

Fig. 2. *A*, caspase-8 and caspase-3 were activated with different kinetics in type I and type II cells. *a*, time course of caspase-8 activation in type I cell SKW6.4 and type II cell Jurkat and CEM-Cr and CEM-Gal-3 cells. Caspase-8 activities were determined by IETDase activities. *b*, time course of caspase-8 cleavage. Type I cell SKW6.4 and type II cell Jurkat and CEM-Cr and CEM-Gal-3 cells were treated with 1000 ng/ml anti-Fas antibody for indicated periods of time as described in *a*. Lysates were subjected to 10% SDS-PAGE and immunoblotted with polyclonal anti-caspase-8 antibodies. *c*, time course of caspase-3 activation in type I cell SKW6.4 and type II cell Jurkat and CEM-Cr and CEM-Gal-3 cells. All samples were the same as in *a*. Caspase-3 activities were determined by DEVDase activities. *d*, time course of caspase-3 cleavage. All samples were the same as in *b*. Lysates were subjected to 10% SDS-PAGE and immunoblotted with polyclonal anti-caspase-3 antibodies. *B*, sensitivity to C_2-ceramide-induced apoptosis. Type I cell SKW6.4 and type II cell Jurkat and CEM-Cr and CEM-Gal-3 cells were incubated for 6 h with different concentration of C_2-ceramide. Annexin V binding using flow cytometric analysis measured the percentage of apoptotic cells in each sample. Data are mean ± SD of three independent experiments.

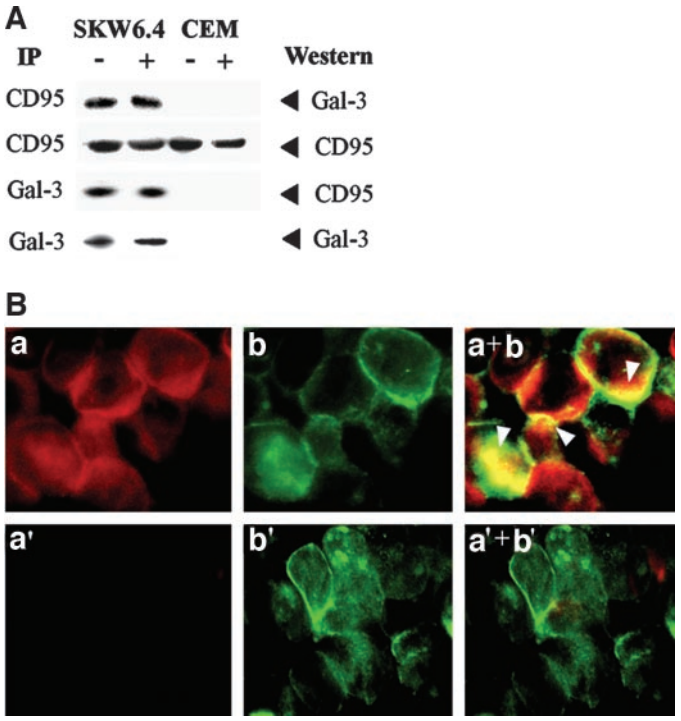

Fig. 3. The association of galectin-3 and CD95. *A*, the lysates of SKW6.4 and CEM cells without (−) or with (+) 1000 ng/ml anti-CD95 were immunoprecipitated with either anti-CD95 antibody or anti-galectin-3 antibody. The samples were subjected to SDS-PAGE and immunoblotting with either anti-galectin-antibody or anti-CD95 antibody. *Left*, immunoprecipitated with the designated antibodies. *Right*, Western blot analysis with the designated antibodies. *B*, SKW 6. 4 cells (*a*, *b*) and CEM cells (*a′*, *b′*, *a′* + *b′*) were stained with anti-galectin-3 antibody (*a*, *a′*) and anti-CD95 antibody (*b*, *b′*). *a* + *b* and *a′* + *b′* are the merge images of *a* and *b* and *a′* and *b′*, respectively.

1*B*). The sensitivity to CD95-induced apoptosis was tested in CD95 type I SKW6.4 cells, type II Jurkat cells, CEM control (CEM-Cr) cells and CEM-Gal-3 cells for both dose dependence and kinetic analyses (Fig. 1, *C* and *D*). In SKW6.4, Jurkat, CEM-Cr, and CEM-Gal-3 cells, there was no difference in Fas-induced apoptosis. All four cells expressed CD95 on the cell surface (data not shown). Previously, it was reported that the most significant difference between CD95 type I and type II cells is the time lag of caspase-8 activation (2, 9). To test the kinetics of caspase-8 and caspase-3 activation, we measured caspase-8 and caspase-3 cleavage products for the indicated periods of time [Fig. 2*A* (*b* and *d*)]. IETDase and DEVDase activity in SKW6.4, Jurkat, CEM-Cr, and CEM-Gal-3 cells were then analyzed after treatment with anti-CD95 antibody [Fig. 2*A* (*a* and *c*)]. Caspase-8 was rapidly cleaved in CD95 type I SKW6.4 cells after anti-CD95 treatment, whereas caspase-8 cleavage was delayed in type II Jurkat cells as reported previously (2). In Jurkat cells, the first cleavage products of caspase-8 did not appear until after 60 min of stimulation [Fig. 2*A* (*b*)]. Interestingly, in CEM-Gal-3 cells, caspase-8 was cleaved as rapidly as in type I cells relative to CEM-Cr cells. Furthermore, caspase-8 activity of type I SKW6.4 cells, after treatment with anti-CD95 antibody, was higher than that of type II Jurkat cells after 30 min of stimulation [Fig. 2*A* (*a*)]. Caspase-8 activity of CEM-Gal-3 was also higher than that of type II CEM-Cr, suggesting that galectin-3 changes the level and kinetics of caspase-8 activation, thereby affecting the CD95-mediated apoptosis signaling pathway.

Although caspase-3 was also rapidly cleaved within 30 min in CD95 type I SKW6.4 cells and CEM-Gal-3 cells, its activation in type II Jurkat cells and CEM-Cr cells was delayed. The cleavage products of caspase-3 in type I SKW6.4 cells and CEM-Gal-3 cells were clearly observed after 30 min of stimulation, whereas the cleavage products of caspase-3 in type II Jurkat cells and CEM-Cr cells were not readily apparent at this time point [Fig. 2*A* (*d*)]. Caspase-3 activity, measured by DEVDase, type I SKW6.4 and CEM-Gal-3, was markedly higher than that of type II Jurkat and CEM-Cr 10 min after CD95 treatment. There was no difference in caspase-3 activity after 30 min of stimulation between CD95 type I and CD95 type II cells [Fig. 2*A* (*d*)]. Thus, in the early phase (10 min) after CD95 treatment, both caspase-3 and caspase-8 were rapidly activated but in only type I and CEM-Gal-3 cells.

Recently, it has been shown that C_2-ceramide induces apoptosis only in CD95 type II cell lines such as CEM and Jurkat cells because C_2-ceramide represents a CD95-independent apoptotic signal pathway and affects mitochondrial components directly (10). Therefore, we tested whether C_2-ceramide-induced apoptosis was diminished in CEM-Gal-3 cells, similarly to type I cells (Fig. 2*B*). As previously reported (9), type II Jurkat cells were sensitive to C_2-ceramide-induced apoptosis, whereas type I cells were resistant. Interestingly, CEM-Gal-3 cells acquired resistance to C_2-ceramide-induced apoptosis similarly to type I SKW6.4 cells, whereas CEM-Cr cells were sensitive to C_2-ceramide (Fig. 2*B*). Thus, intracellular galectin-3 inhibited C_2-ceramide-induced apoptotic signals in type I cells.

Galectin-3-CD95 Interaction. Next, we examined the association of galectin-3 and CD95 (Fig. 3*A*). When the cell lysates of SKW6.4 cells (type I) were immunoprecipitated with anti-CD95 antibody, galectin-3 was pulled down, and when the lysates were immunoprecipitated with anti-galectin-3 antibody, CD95 was co-precipitated with galectin-3. The galectin-3-CD95 binding did not change after CD95 treatment. Next, we examined whether galectin-3 colocalized with CD95 *in vivo*. Confocal microscopic analysis revealed that both galectin-3 [Fig. 3*B* (*a*)] and CD95 [Fig. 3*B* (*b*)] were predominant at the cell surfaces and colocalized *in vivo* [Fig. 3*B* (*a* and *b*)].

Discussion

The data presented here indicate that CD95-induced apoptotic signaling pathway in types I and type II cells is determined, at least in part, by endogenous galectin-3. In type I cells, galectin-3 interacts with CD95 and promotes caspase-8 activation. On the basis of the data, we revised the original model for CD95-mediated apoptosis

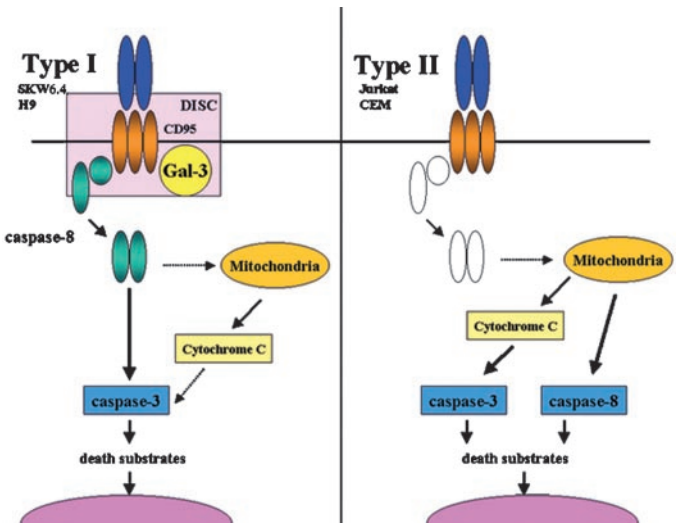

Fig. 4. Models of the galectin-3 involvement with CD95-induced apoptosis signaling pathway. Intracellular galectin-3 associates with CD95 and stimulates caspase-8 activation and interferes with apoptotic signaling pathway from caspase-8 to mitochondria and also inhibits C_2-ceramide-induced apoptosis.

(Ref. 2; Fig. 4). In type I cells, galectin-3 interacts with CD95 and promotes DISC formation, resulting in the generation of large amounts of active caspase-8 at DISC, followed by the direct cleavage of caspase-3. By contrast, in type II cells, few DISC and active caspase-8 molecules are insufficient for caspase-3 cleavage, although sufficient to induce the apoptogenic activity at the mitochondria (2).

Clinically, thymocytes and activated T cells have a type I phenotype because they are not protected from CD95-induced apoptosis by a Bcl-2 transgene (11). We suggest that for therapeutic strategies using CD95-mediated and C_2-ceramide-mediated apoptosis, cell typing of cancer cells should include galectin-3. Treatment with C_2-ceramide and soluble CD95 ligand may be useful for type II cancer cells (12, 13). The determination of endogenous galectin-3 expression is important for other cancer treatments such as immunotherapy and chemotherapy because both induce apoptosis of cancer cells, at least in part, through CD95.

In summary, galectin-3 was found to modulate CD95-mediated apoptotic signaling pathway and to determine cell phenotype to be either type I or type II.

Acknowledgments

We thank Vivian Powell for editing this manuscript and Drs. Gen Wu and Jerome Horwitz for reviewing this manuscript.

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