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Characterization of Galectin-9-Induced Death of Jurkat T Cells

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Galectin-9, a mammalian lectin with affinity for β-galactosides, is known as an apoptosis inducer of activated T lymphocytes. In the present study, we examined the properties of galectin-9-mediated cell death of Jurkat T cells. Galectin-9NC (wild-type), consisting of two CRDs (N-terminal and C-terminal carbohydrate recognition domains), and derivatives of it, galectins-9-NN and -9-CC, induced Jurkat T-cell apoptosis. However, a single CRD (galectin-9NT or -CT) had no effect, suggesting the stable dimeric structure of two CRDs is required for the activity. The apoptosis was inhibited by pretreatment with an *N*-glycan synthesis inhibitor, indicating that the expression of *N*-glycans in the cells is essential for galectin-9-induced apoptosis. We previously showed that the apoptosis of MOLT-4 cell is mediated by galectin-9 via a Ca^{2+}-calpain-caspase-1-dependent pathway. In Jurkat cells, the cell death by galectin-9, was insufficiently suppressed by caspase inhibitors, Ca^{2+}-chelator or calpain inhibitor. Furthermore, we observed the loss of mitochondrial membrane potential and significant AIF release in galectin-9-treated cells. These findings suggest that caspase-dependent and-independent death pathways exist in Jurkat cells, and the main pathway might vary with the T-cell type.

Key words: galectin-9, galectin, apoptosis, Jurkat, T cell.

Abbreviations: 7-AAD, 7-amino-actinomycin D; ActD, actinomycin D; AIF, apoptosis-inducing factor; BAPTA, *O*,*O*′-bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CRD, carbohydrate recognition domain; DiOC2(3), 3,3′-diethyloxacarbocyanine iodide; DMEM, Dulbecco's Modified Eagle's Medium; DMNJ, deoxymannojirimycin; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethyleneglycol-tetraacetic acid; EndoG, endonuclease G; Fluo 3, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Gal, galectin; $\Delta\psi m$, mitochondrial membrane potential; mAb, monoclonal antibody; PE, phycoerythin; PS, phosphatidylserine; RCA-120, *Ricinus communis* agglutinin; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling.

INTRODUCTION

Galectins are defined as a family of animal lectins that exhibit affinity for β-galactosides and share certain conserved amino acid sequences in the carbohydrate recognition domain (CRD) (*1, 2*). Fifteen members of the mammalian galectin family have been identified to date and are subdivided into three groups according to their structures: the proto type containing one CRD (galectins-1, -2, -5, -7, -10, -11, -13, -14 and -15); the chimera type consisting of an N-terminal Pro- and Gly-rich domain fused to a C-terminal CRD (galectin-3); and the tandem-repeat type containing two CRDs joined by a linker peptide (galectins-4, -6, -8, -9 and -12) (*3–10*). Since proto type galectins exist as dimers at high concentration (*3, 11*), galectin-3 forms pentamers upon binding to multivalent carbohydrates (*12*), and tandem-repeat galectins have two carbohydrate-binding sites, the galectin family can undergo bivalent or multivalent interactions with carbohydrates. Furthermore, galectins are distributed in a wide variety of tissues, and can interact with proteins and glycoconjugates extracellularly and intracellularly (*13, 14*), so the galectin family plays important roles in diverse biological processes such as cell adhesion, proliferation, death, differentiation and immunomodulation of inflammation (*14–17*).

In early works, galectin-9 was shown to possess eosinophil chemoattractant activity and to prolong eosinophil survival (*18–20*). In addition, galectin-9 is a prognostic factor with anti-metastatic potential in breast cancer and plays a role in acquired immunity by inducing human monocyte-derived dendritic cell maturation (*21, 22*). Furthermore, galectin-9 has been indicated to induce apoptosis in a variety of cells, including activated T lymphocytes and T-cell lines (*23–26*). In the galectin family, galectins-1, 2, 3, 7, 8, 9 and 12 have been reported to take part in the regulation of cell death. For example, galectin-1 induces the apoptosis of T cells, thymocytes, B cells, and even breast cancer and prostate cancer cells (*27–31*). Galectin-2 has been characterized

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as a proapoptotic factor for activated T cells via the caspase pathway (*32*). Although extracellular galectin-3 can induce T-cell apoptosis, intracellular galectin-3 protects T cells and some types of carcinoma cells such as human bladder carcinoma cells from death (*33–36*). Galectin-7 exerts a proapoptotic function through JNK activation and mitochondrial cytochrome *c* release (*37*). Galectin-8 is thought to be associated with cell adhesion, but recently was also reported to govern the choice between growth arrest and apoptosis through cyclin-dependent kinase inhibitors and the JNK pathway (*38*). It has been reported that galectin-12 may participate in the apoptosis of adipocytes (*39*). Cell death is a very complex process in which a lot of multiple death signals participate and which plays a role in the control of the proper development of multicellular organisms, in the regulation of immune system homeostasis and in the prevention of neoplastic disease. Although we previously showed that galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway in MOLT-4 cells (*25*), we still found a lot of new features of galectin-9-induced T-cell death. In the present study, we compared the cell death activities of galectin-9 and derivatives of it with those of other galectins, plant lectins and strong apoptosis inducers such as anti-Fas antibodies and actinomycin D in Jurkat T cells. The data revealed that the dimeric structure of two-CRDs of galectin-9 and *N*-glycans in target cells were essential for galectin-9-mediated Jurkat T-cell apoptosis. Furthermore, we demonstrated that both caspase-dependent and -independent pathways must be involved in this cell death.

MATERIALS AND METHODS

Reagents and Antibodies—RPMI-1640 medium (Nissui, Tokyo, Japan); Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA); a pET System (Novagen, Darmstadt, Germany); lactosyl-agarose and *Ricinus communis* agglutinin (RCA-120) (Seikagaku, Tokyo, Japan); actinomycin D (Wako, Osaka, Japan); caspase inhibitors: caspase-1 inhibitor (zYVAD-fmk), caspase-3 inhibitor (zDEVD-fmk), caspase-8 inhibitor (zIETD-fmk), caspase-9 inhibitor (zLEHD-fmk), and a broad-specific pan-caspase inhibitor (zVAD-fmk), a caspase-3 substrate (Ac-DEVD-AFC), A23187, benyl-2-acetamide-2-deoxy-D-galactopyranoside (BG), deoxymannojirimycin (DMNJ), and calpain inhibitor III (Calbiochem, San Diego, CA, USA); annexin V-PE, 7-amino-actinomycin D-PE (7-AAD-PE) and anti-CD7 (M-T701) mAb (BD Biosciences, Franklin Lakes, NJ, USA); anti-CD29 (TDM29) mAb (Southern Biotechnology Associates, Birmingham, AL, USA); anti-CD45 (T29/33) mAb (DakoCytomation, Denmark); anti-CD45 (135-4C5) antibodies (Cymbus Biotechnology, Hants, UK); anti-CD7 (H-126) antibodies, anti-CD29 (M-106) antibodies, anti-AIF antibodies and anti-cytochrome *c* antibodies (Santa Cruz Biotechnology, Santa Crus, CA, USA); anti-Smac/DIABLO antibodies (ProSci, Poway, CA, USA); anti-Fas (CH-11) mAb (Medical & Biological Laboratories, Nagoya, Japan); a Mebstain Apoptosis Kit Direct (Immunotech, Fullerton, CA, USA); a Mitoprobe $DiOC_2(3)$ Assay Kit (Molecular Probes, Eugene, OR, USA); and a Cell Counting Kit-8, Fluo 3-AM and BAPTA-AM (Dojindo Laboratories, Kumamoto, Japan).

Cell lines—Jurkat and MOLT-4 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 mU/ml penicillin and 100 μg/ml streptomycin at 37°C under a 5% CO_2–95% atmosphere.

Construction of Expression Vectors for Galectins—The cDNAs for a human galectin-9 isoform with the shortest linker peptide (G9S), galectin-1 (G1), and galectin-3 (G3) were amplified from first-strand cDNA prepared from a poly $(A)^+$RNA fraction of the Jurkat T-lymphocytic cell line by polymerase chain reaction (PCR) using forward primers tagged with an extra 5′ *Nde*I sequence (G9-F1, G1-F1 and G3-F1), and reverse primers tagged with an extra *Bgl*II (G9-R1) or *Bam*HI (G1-R1 and G3-R1) sequence. The amplified cDNAs were digested with *Nde*I and *Bgl*II (G9S) or *Nde*I and *Bam*HI (G1 and G3), and then inserted into the *Nde*I-*Bam*HI sites of pET-11a (Stratagene, La Jolla, CA). Expression vectors for G9NT and G9CT (N- and C-terminal carbohydrate recognition domains of galectin-9), and G9NN and G9CC (mutant forms of galectin-9 consisting of two N-terminal CRDs and C-terminal CRDs), and galectin-8 were prepared as described previously (*40–42*). The DNA sequences of all the expression vectors were confirmed to be as follows by automated sequencing.

G9-F1, 5′-CGTCCTCATATGGCCTTCAGCGGTTC CCAG-3′;

G9-R1, 5′-CGACCGAGATCTCTATGTCTGCACATGGG TCAG-3′;

G1-F1, 5′-CGTCCTCATATGGCTTGTGGTCTGGTC GCC-3′;

G1-R1, 5′-CGACCGGGATCCTCAGTCAAAGGCCACAC ATTT-3′;

G3-F1, 5′-CGTCCTCATATGGCAGACAATTTTTCG CTC-3′;

G3-R1, 5′-CGACCGGGATCCTTATATCATGGTATATG AAGC-3′;

Expression and Purification of Recombinant Proteins—Expression of recombinant human galectin-9 (galectin-9NC), its derivatives (galectins-9NN, -9CC, -9NT and -9CT), galectin-1, galectin-3 and galecin-8 in *Escherichia coli* BL21 cells was carried out as described previously (*41*). Recombinant proteins were obtained using a pET-expression vector, and purified by affinity chromatography on lactosyl-agarose columns, the bound proteins being eluted with elution buffer (TBS/0.2 M lactose). The recombinant proteins were dialyzed against PBS, then examined by SDS-PAGE and Coomassie brilliant blue R-250 staining, and finally stored at −80°C before use. The protein concentrations were determined using BCA protein assay reagent (Pierce) and bovine serum albumin as a standard.

Proapoptosis or Apoptosis Assay—Annexin V-PE/7-AAD-PE staining

Cells (2×10^5 cells/well) treated with various stimulants and/or inhibitors were washed with cold PBS and then resuspended in annexin V-binding buffer. Annexin V-PE

and 7-AAD-PE were added to each cell suspension, and then the mixture was incubated for 20 min in the dark at room temperature. After that the suspension was analysed by flow cytometry (FACSCalibur, BD Bioscience).

DNA fragmentation by agarose gel electrophoresis

Cells lyzed in 50 mM tris-HCl (pH 8.0)/10 mM EDTA/0.5% sodium lauroyl-sarconate after culture (2×10^5 cells/well) for 2–12 h were treated with RNase A (3 mg/ml) at 50°C for 30 min and Protease K (3 mg/ml) at 50°C for 1 h. After being dissolved in gel loading buffer, the resulting cell digests were separated by 2% agarose gel electrophoresis and the DNA fragments were stained with ethidum bromide.

TUNEL assay

After treatment with galectins or other stimulants, Jurkat T cells (2×10^5 cells/well) were washed with PBS containing 0.2% BSA, then fixed in 4% paraformaldehyde buffered with PBS at 4°C for 30 min, and finally permeabilized with 70% ethanol at −20°C for 30 min. Then 30 μl of TdT reaction reagent was added to gently resuspend cell pellets, followed by incubation at 37°C for 1 h. After washing the cells with PBS, DNA fragments generated in the cells were analysed by flow cytometry (FACSCalibur, BD Bioscience).

Cell proliferation assay—Cells (5×10^3 cells/well) were plated on 96-well plates and then incubated for 48 h. After the addition of various concentrated reagents, the culture was continued for 24 h. Then, the WST-8 reagent in the Cell Counting Kit-8 was added to the cells (10 μl/well), followed by incubation for 2 h. The viable cell number was determined by measuring the difference in absorbance (450, 630 nm) with an enzyme-linked immunoadsorbent assay autoreader.

Peptidase activity of caspase-3—Cell lysates were prepared by vigorously vortexing in 20 mM tris-HCl (pH 7.2)/0.15 M NaCl/5 mM EDTA/5 mM benzamidine/0.5% Triton X-100/5 mM 2-mercaptoethanol. The peptidase activity of activated caspase-3 in the lysates was assayed using a caspase-3 substrate, Ac-DEVD-AFC (excitation at 400 nm, emission at 500 nm), at 37°C.

Immunoblotting—For AIF, cytochrome *c* and Smac/DIABLO analysis, Jurkat T cells treated with various stimulants were washed with 20 mM tris-HCl (pH 7.2)/0.25 M sucrose. The cells were resuspended in the above buffer, and then treated with digitonin (20 μg/ml) to puncture them on ice for 15 min. The cell suspensions were centrifuged at 15 000 rpm for 15 min at 4°C and the resulting supernatants were used as cytosolic fractions. Proteins in the cytosolic fractions were separated by 12.5% SDS-PAGE and then blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked with 5% skim milk/TBS, followed by incubation with the indicated primary antibody at room temperature overnight. After being washed with 1% skim milk/TBS, the membranes were incubated for 1 h with an appropriate HRP-conjugated secondary antibody (Amersham Bioscience, Buckinghamshire, England). Both the first and second antibodies were diluted with the immune reaction buffer (Can-Get-Signal™, TOYOBO, Kyoto, Japan). Blots were visualized with an Enhanced Chemiluminescence (ECL) Kit (Amersham Bioscience) by exposing the membranes to X-ray film (Kodak).

Measurement of mitochondrial membrane potential—After treatment with the stimulants, Jurkat T cells (2×10^5 cells/well) were washed with warm PBS and then incubated with 50 nM $DiOC_2(3)$ at 37°C for 30 min under a 5% CO_2 and 95% atmosphere. Then the cells were washed with warm PBS, and the shift in the fluorscence intensity was analysed by flow cytometry (FACSCalibur, BD Bioscience). Loss of the mitochondrial membrane potential was indicated by decreased $DiOC_2(3)$ staining.

Ca^{2+} mobilization—Jurkat T cells in culture medium were loaded with Fluo 3-AM (an intracellular Ca^{2+} indicator; final concentration, 10 μM) at 37°C for 30 min. After washing and resuspension in fresh medium, intracellular Ca^{2+} uptake was measured by flow cytometry (FACSCalibur, BD Bioscience). A stimulant was applied to the cells, and then continuous recording of fluorescence was started again until another stimulus was applied. A23187 (Ca^{2+} ionophore; final concentration, 5 ng/ml) was used as a positive control.

Statistical analysis—Data are presented as means ± S.D. with the number of experiments indicated. Most data were analysed with the Mann–Whitney U test or one-way analysis of variance (ANOVA) in combination with Dunnett's multiple comparison test.

RESULTS

Induction of Jurkat T-cell Apoptosis by Galectin-9—Jurkat T cells were incubated with galectins (0.1–2 μM) for 6 h and then stained with annexin V/7-AAD to examine the cell surface exposure of phosphatidylserine (PS), which is an apoptotic cellular response. In the present study, a tandem-repeat galectin-9 with the shortest linker was used (*40*) as it induced PS exposure at 0.1–1 μM (Fig. 1A and C-1). In the galectin family, another tandem-repeat galectin, galectin-8, also exhibited PS exposure activity at 0.5–2 μM (Fig. 1A and C-1). However, galectins-1 and -3 exhibited no or only weak activity at the concentrations examined (Fig. 1A). After prolonged treatment with galectins for 12 h, DNA fragmentation, an executive apoptotic cellular event, was measured by the TUNEL method and agarose gel electrophoresis (Fig. 1B and C-2). As a result, galectin-9 was found to induce DNA-ladder formation in Jurkat T cells. Although galectin-8 induced evident PS exposure at high concentrations, apparent DNA-ladders were not observed (Fig. 1C-2). Other galectins, i.e. galectins-1 and -3, showed no effect on Jurkat T-cell apoptosis at these concentrations (data not shown). Thus, in the galectin family, galectin-9 is the most potent apoptosis inducer in Jurkat T cells. These findings also indicate that evaluation of galectin-induced apoptosis by means of annnexin V-staining was valid, but not sufficient.

The time courses of the cellular responses (2–12 h) with galectin-9 and actinomycin D were also examined. The early apoptotic reaction, i.e. PS exposure, in the cells occurred at 2 h with both stimulants and increased to 6 h time-dependently (Fig. 1D-1). Apparent DNA-fragmentation occurred at 4 h with

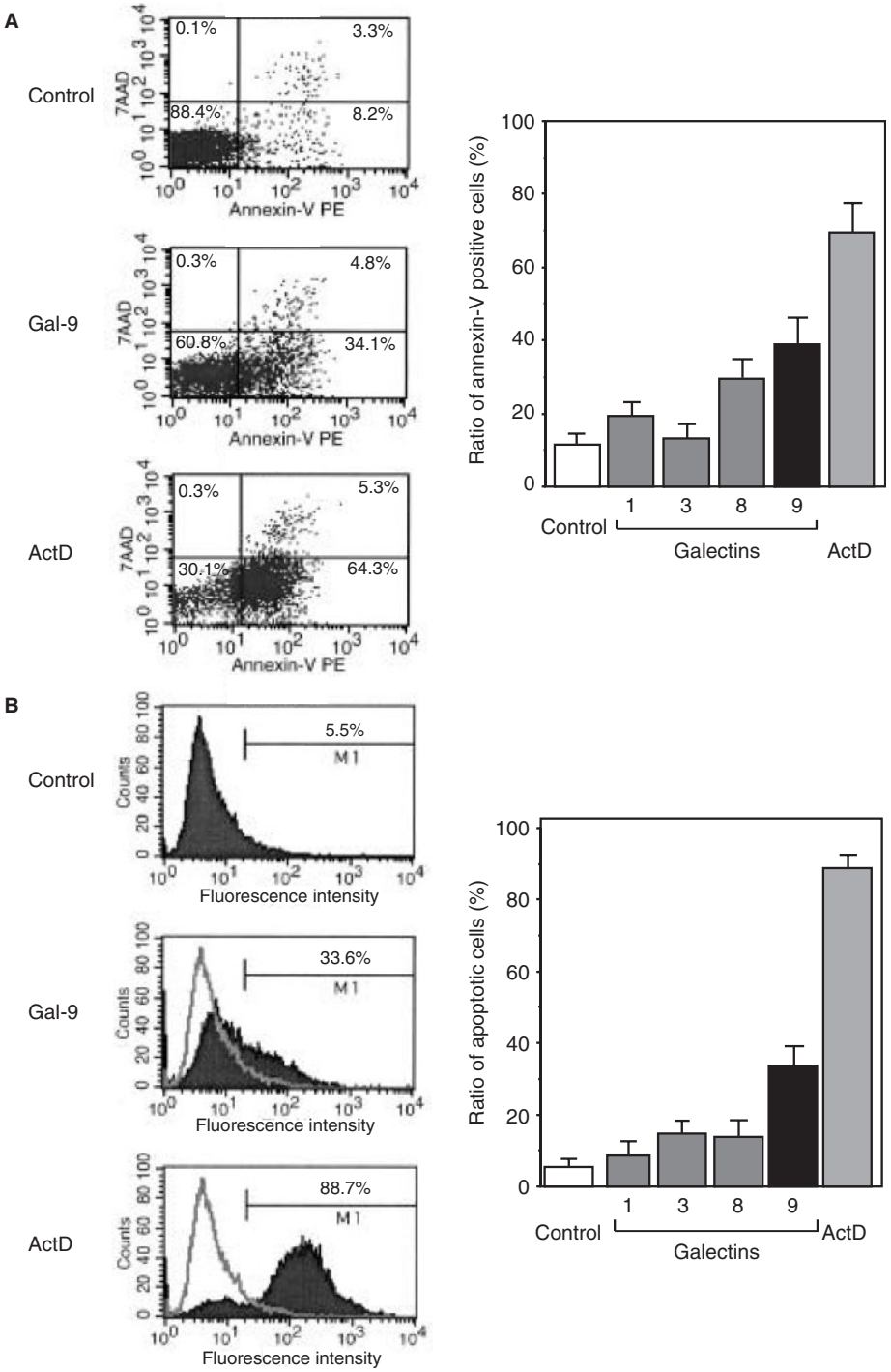

Fig. 1. **Apoptosis-inducing activity of galectins in Jurkat T cells.** (**A**) Jurkat T cells were incubated with galectin-1 (1 μM), galectin-3 (1 μM), galectin-8 (1 μM), galectin-9 (0.25 μM), or actinomycin D (2 μg/ml) on a 48-well culture plate for 6 h. After washing with 100 mM lactose to dissociate cell aggregates, the cells were stained with annexin V/7-AAD and then analyzed by flow cytometry. The *upper* and *lower* right quadrants of each plot show annexin V/7-AAD double-positive cells and annexin V single-positive cells, respectively. Data are expressed as means ± S.D. of the percentages of annexin V-positive cells determined in triplicate. (**B**) After treatment of Jurkat T cells with galectins as described previously (**A**) for 12 h, DNA fragmentation was evaluated by the TUNEL assay method as described under Materials and Methods. Data represent the means ± S.D. of triplicate measurements.

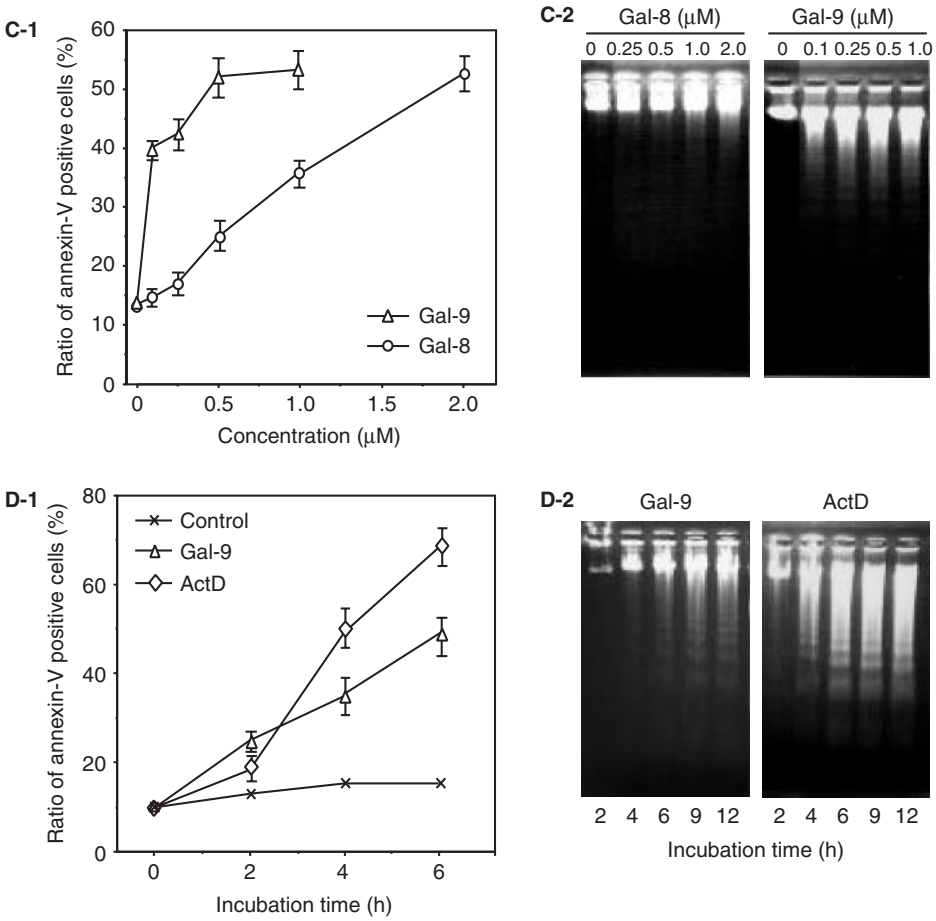

Fig. 1. **Continued** (**C-1**) Jurkat T cells were incubated with galectin-8 (0.1–2 μM) or galectin-9 (0.1–1 μM) for 6 h. The resulting cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Data are means ± S.D. of triplicate measurements. (**C-2**) After treatment of Jurkat T cells with galectin-8 (0.25–2 μM) or galectin-9 (0.1–1 μM) for 12 h, DNA fragments were separated by 2% agarose gel electrophoresis and then visualized by ethidium bromide-staining. The results are representative of three independent experiments. (**D-1**) Jurkat T cells were incubated with galectin-9 (0.25 μM) or actinomycin D (2 μg/ml) for 2–6 h. After washing, the cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Data are means ± S.D. of triplicate measurements. (**D-2**) After incubation as described previously (**D-1**) for 2–12 h, DNA fragments in treated cells were separated by 2% agarose gel electrophoresis and then stained. The results are representative of three independent experiments.

actinomycin D, but occurred later at 6 h with galectin-9 (Fig. 1D-2).

Because the galectin family binds to sugar chains containing β-galactosides such as lactose or lactosamine units, the effects of plant lectins exhibiting similar or different sugar specificities were compared to that of galectin-9 in Jurkat T cells. Among typical plant lectins, WGA, RCA-120 and Con A, which can recognize hapten-sugars, i.e. *N*-acetylglucosamine, galactose/lactose and mannose, respectively, RCA-120 induced marked PS exposure and DNA-ladder formation at low concentration (8 nM) (Fig. 1E-1 and 1E-2).

In addition, the proapoptotic activities of both galectin-9 and RCA-120 were efficiently inhibited on the addition of a common hapten sugar, lactose, but not sucrose (Fig. 1E-1 and 1E-2). These results indicate that sugar-binding activity is indispensable for galectin-9-and RCA-120-induced Jurkat T-cell apoptosis. Furthermore, we examined the effects of galectin family members on cell proliferation during prolonged treatment with galectins for 24 h. After 24 h culture, the resulting viable cell numbers were determined. As a result, galectin-9 was found to cause significant cell death and/or cell growth arrest in a dose-dependent manner in Jurkat T cells (Fig. 2). This growth inhibition was completely abrogated on the addition of lactose. Galectin-8 also caused distinct growth inhibition at high concentrations (1–2 μM). Thus, galectin-8 may play a sufficient role in the regulation of total cell growth.

Proapoptotic Activity of Galectin-9 Mutants—Galectin-9 is a tandem-repeat type galectin consisting of two CRDs connected by a linker peptide. The divalent structure of CRDs seems to be important for PS exposure and/or DNA-fragmentation in Jurkat T cells. We investigated the roles of the two CRDs of galectin-9 in apoptotic cellular responses. We produced single N-terminal and C-terminal CRDs (galectin-9NT and galectin-9CT), and synthetic tandem-repeat galectin-9 derivatives consisting of two N-terminal CRDs or two C-terminal CRDs (galectin-9NN and galectin-9CC).

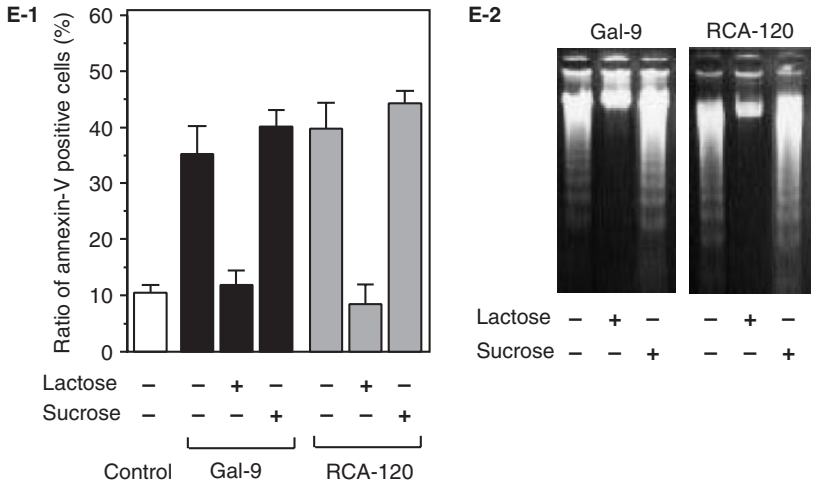

Fig. 1. **Continued** (**E-1**) Jurkat T cells were incubated with galectin-9 (0.25 μM) or RCA-120 (8 nM) in the presence of lactose (25 mM) or sucrose (25 mM) for 6 h. After incubation, the cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Data are means ± S.D. of duplicate measurements. **(E-2)** After incubation as described previously **(E-1)** for 12 h, DNA fragments in treated cells were separated by 2% agarose gel electrophoresis and then stained. The results are representative of two independent experiments.

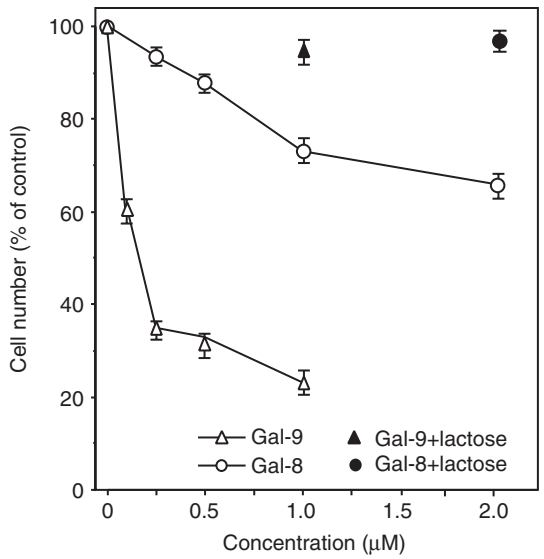

Fig. 2. **Growth inhibition of Jurkat T cells by galectins.** Jurkat T cells were cultured with galectin-8 (0.25–2 μM) or galectin-9 (0.1–1 μM) in the presence or absence of 25 mM lactose for 24 h. Viable cell numbers were determined with a WST-8 Reagent Kit. Relative viable cell numbers were calculated taking the number of viable cells in the absence of a stimulant as 100%. Data represent the means ± S.D. of triplicate measurements.

The proapoptotic activities of these mutant proteins were compared with that of wild-type galectin-9 (galectin-9NC) with a short linker. Both PS exposure and DNA fragmentation occurred on treatment with galectins-9NN and -9CC. Galectin-9CC exhibited somewhat lower apoptotic activity than that of galectins-9NN and -9NC at 0.1–0.25 μM (Fig. 3A-1 & 3B-1). On the other hand, neither galectin-9NT nor galectin-9CT showed proapoptotic activity toward DNA fragmentation at high concentration (2 μM) (Fig. 3A-2 & 3B-2). These results indicate that the tandem-repeat structure consisting of two CRDs is indispensable for both cellular responses induced by galectin-9.

Inhibitory Effects of Caspase Inhibitors on Jurkat T-cell Apoptosis—In the previous work, we demonstrated that caspase-1 is a mediator of the apoptosis of another T-cell line, MOLT-4, by galectin-9 (*25*). Thus, we examined the effects of caspase inhibitors on galectin-9-induced Jurkat T-cell apoptosis. RCA-120 and anti-Fas antibodies were used as positive apoptosis inducers. Cells were precultured with various caspase inhibitors (10 or 20 μM), i.e. a caspase-1 inhibitor (zYVAD-fmk), a caspase-3 inhibitor (zDEVD-fmk), a caspase-8 inhibitor (zIETD-fmk), a caspase-9 inhibitor (zLEHD-fmk), and a broad-specific pan-caspase inhibitor (zVAD-fmk), for 30 min prior to the addition of galectin-9 or another stimulant. After 6 h culture, PS exposure in the cells was examined and the inhibitory effect of each caspase inhibitor was evaluated (Fig. 4A). Caspase inhibitors including pan-caspase inhibitor (zVAD-fmk) exhibited no or only a weak inhibitory effect on galectin-9-induced PS exposure. The PS exposure induced by anti-Fas antibodies was significantly inhibited by all these caspase inhibitors. The effect of RCA-120 was moderately or significantly inhibited by these caspase inhibitors, especially the pan-caspase inhibitor. When the inhibitory effects of caspase inhibitors on cell death were assessed, the disappearance of DNA ladders was visually observed on agarose gel electrophoresis for anti-Fas antibody- or RCA-120-treated cells (data not shown). However, the effect of a specific caspase inhibitor on galectin-9-induced DNA ladder formation was restricted. Then, TUNEL assaying and agarose gel electrophoresis were performed to evaluate the effect of the pan-caspase inhibitor (zVAD-fmk) on Jurkat-T-cell apoptosis. As a result, RCA-120- and anti-Fas antibody-induced DNA ladder formation was found to be significantly inhibited by the

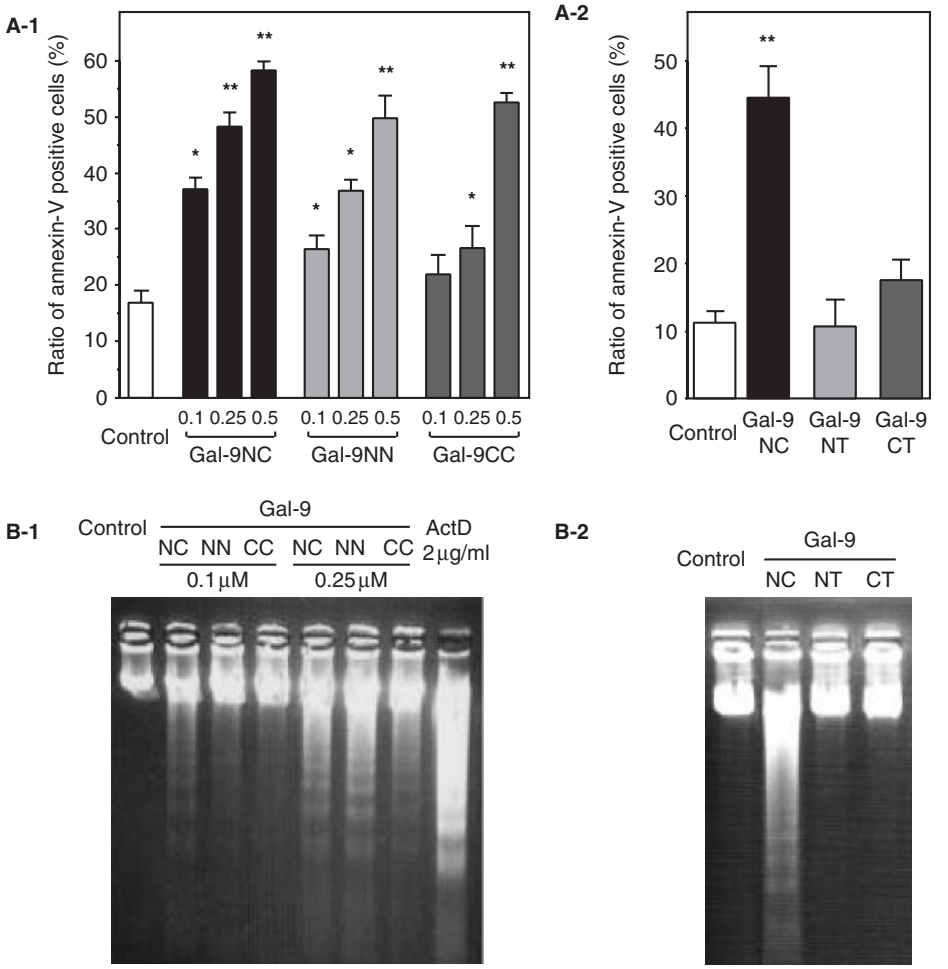

Fig. 3. **Apoptosis-inducing activity of galectin derivatives in Jurkat T cells.** (**A-1**) Jurkat T cells were incubated with synthetic tandem-repeat galectins, i.e. galectin-9NC (galectin-9), galectin-9NN or galectin-9CC, at 0.1–0.5 μM on a 48-well culture plate for 6 h. After washing, the cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Data are means ± S.D. of triplicate measurements. $^{*}P<0.05$, $^{**}P<0.01$. (**A-2**) Jurkat T cells were incubated with tandem-repeat galectin-9NC (0.25 μM), a synthetic single CRD of galectin-9, i.e. galectin-9NT (2 μM) or galectin-9CT (2 μM), for 6 h. After washing, the cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Data are means ± S.D. of triplicate measurements. $^{**}P<0.01$. (**B-1**) After treatment of Jurkat T-cells with tandem-repeat galectin-9 derivatives (0.1 or 0.25 μM) for 12 h, DNA fragments were separated by 2% agarose gel electrophoresis and then stained. The results are representative of three independent experiments. (**B-2**) After treatment of Jurkat T cells with tandem-repeat galectin-9NC (0.25 μM), a synthetic single CRD of galectin-9, i.e. galectin-9NT (2 μM) or galectin-9CT (2 μM), for 12 h, DNA fragments were separated by 2% agarose gel electrophoresis and then stained. The results are representative of three independent experiments.

pan-caspase inhibitor, while galectin-9-induced DNA ladders were only partially inhibited (Fig. 4B and 4C). In addition, both PS exposure and DNA-fragmentation induced by actinomycin D were significantly inhibited by the caspase inhibitors (data not shown). These results indicate that actinomycin D and RCA-120 mainly utilize a caspase-dependent pathway like the Fas-associated death pathway, while galectin-9 partly uses a caspase-dependent pathway.

Activation of Caspase-3 and Release of Mitochondrial Factors—The activation of a downstream caspase-3 was examined in Jurkat T cells treated with various stimulants. The peptidase activity of activated caspase-3 was measured using Ac-DEVD-AFC as a synthetic fluorogenic substrate. In the cases of RCA-120 and actinomycin D, the peptidase activity of caspase-3 was induced time-dependently (Fig. 5A). On the other hand, galectin-9 induced the caspase-3 activation slowly. Thus, the activation of caspase-3 by galectin-9 was delayed and only partial in Jurkat T cells. Some mitochondrial factors, including Smac/DIABLO, cytochrome *c* and AIF, are associated with caspase activation and/or the cell death pathway. The release of these mitochondrial factors into the cytoplasm was also examined by Western blotting (Fig. 5B). Smac/DIABLO and cytochrome *c* are thought to be mitochondrial proteins involved in caspase-dependent cell death. Smac/DIABLO release significantly increased in a time-dependent manner in galectin-9-, RCA-120- or actinomycin D-treated cells. Cytochrome *c* was released

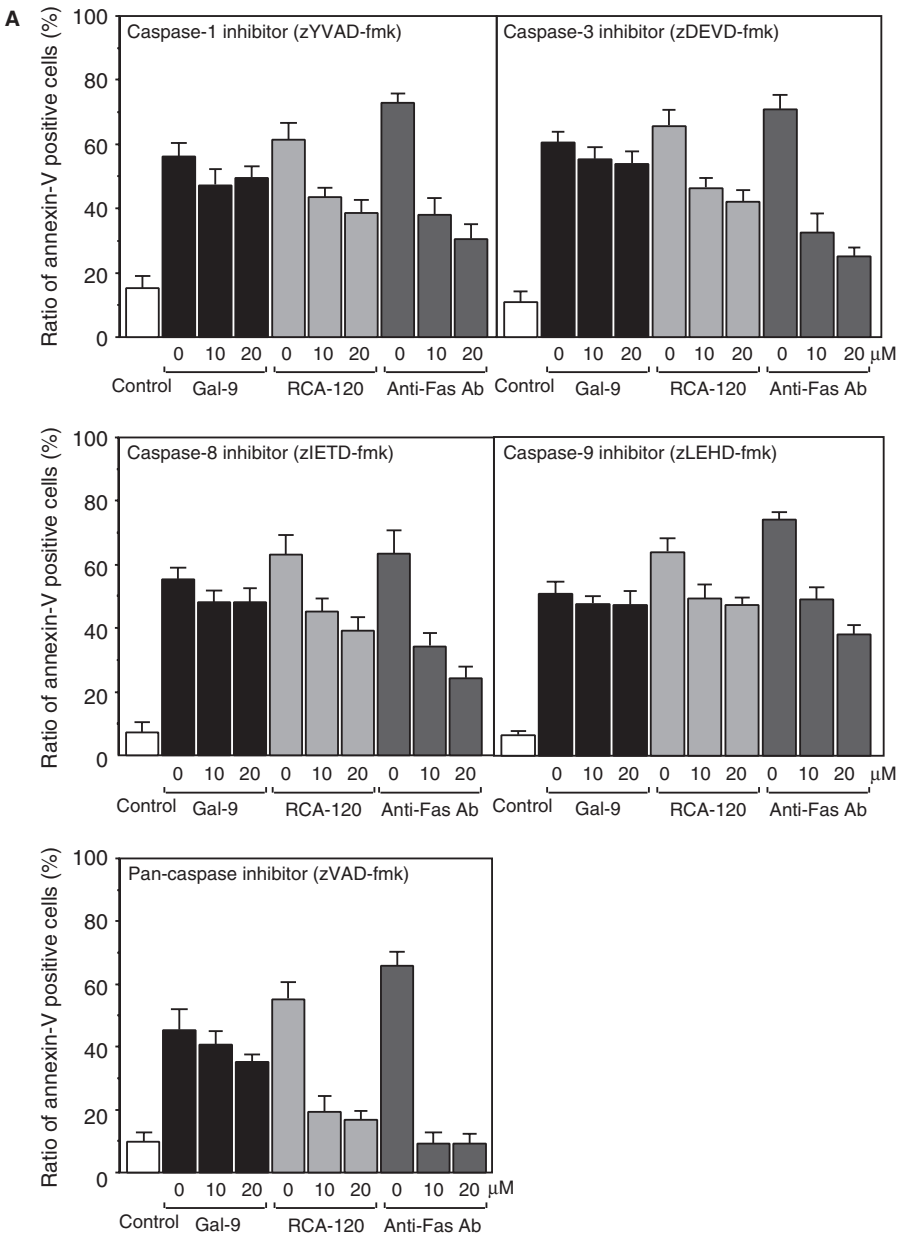

Fig. 4. **Effects of caspase inhibitors on Jurkat-T cell apoptosis.** (**A**) Jurkat T cells were preincubated with or without various caspase inhibitors (10 or 20 μM) for 30 min, and then treated with galectin-9 (0.25 μM), RCA-120 (8 nM), or anti-Fas Ab (100 ng/ml) for 6 h. After washing, the cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Data are means ± S.D. of triplicate measurements.

significantly in RCA-120-, actinomycin D-treated cells, and a little in galectin-9-treated cells. In addition, mitochondria contain a caspase-independent death effector, AIF, which induces chromatin condensation and large-scale DNA fragmentation (50 kbp) when it is released into the cytoplasm. The release of AIF occurred very rapidly and significantly within 2 h after the addition of galectin-9. In RCA-120- or actinomycin D-treated cells, AIF release was observed time-dependently. Therefore, galectin-9 mediates Jurkat T-cell responses by utilizing both caspase-dependent and-independent mechanisms.

Since we detected some mitochondrial protein release into the cytoplasm during the galectin-9-treatment, we next examined alteration of the mitochondrial membrane potential of Jurkat T cells undergoing galectin-9 stimulation. As a result, we found that loss of the mitochondrial membrane potential occurred in a time-dependent manner in galectin-9- and RCA-120-stimulated cells like in actinomycin D-treated cells (Fig. 6A and 6B). However, other galectins including galectin-8 had no effect on the mitochondrial membrane potential at high concentration (2 μM) (data not shown). Thus, the alteration of the mitochondrial membrane

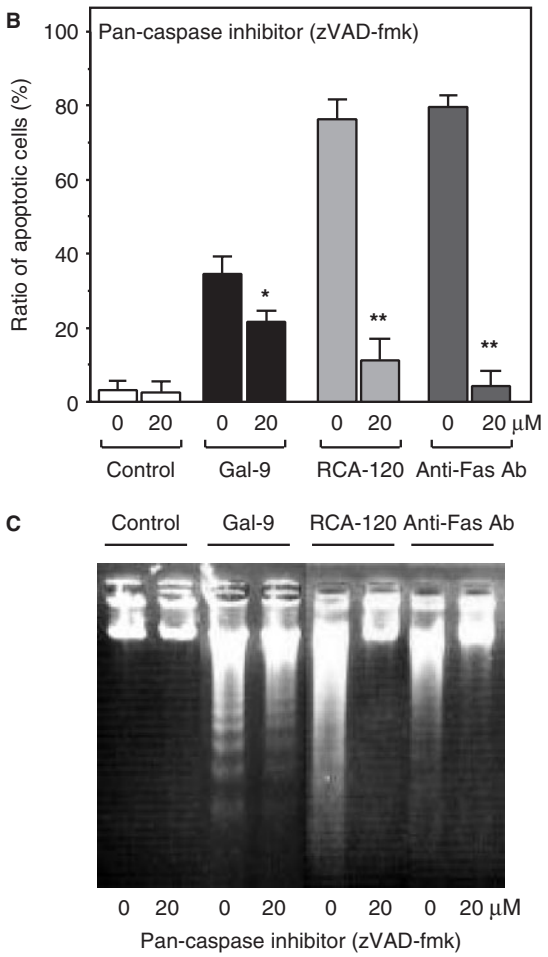

Fig. 4. **Continued.** (**B**) Jurkat T cells were preincubated with or without a pan-caspase inhibitor (zVAD-fmk: 20 μM), and then treated with galectin-9 (0.25 μM), RCA-120 (8 nM), or anti-Fas Ab (100 ng/ml) for 12 h. DNA fragmentation was evaluated by the TUNEL assay method as described under Materials and Methods. Data represent the means ± S.D. of triplicate measurements. $*P < 0.05$, $**P < 0.01$. (**C**) Jurkat T cells were preincubated with or without a pan-caspase inhibitor (zVAD-fmk: 20 μM), and then treated with galectin-9 (0.25 μM), RCA-120 (8 nM), or anti-Fas Ab (100 ng/ml) for 12 h. DNA fragments were separated by 2% agarose gel electrophoresis and then stained. The results are representative of three independent experiments.

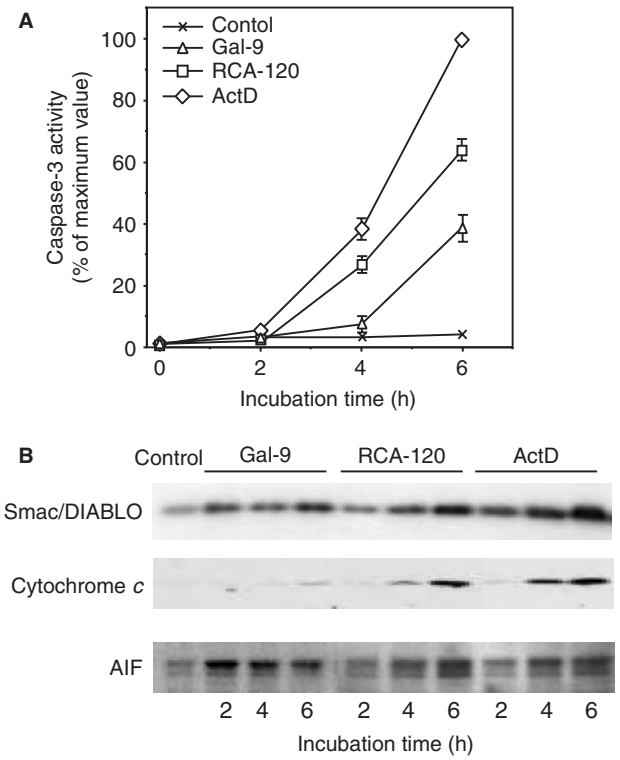

Fig. 5. **Activation of caspase-3 and release of mitochondrial proapoptotic factors in Jurkat T cells.** **A**: Jurkat T cells were incubated with galectin-9 (0.25 μM), RCA-120 (8 nM), or actinomycin D (2 μg/ml) for 2–6 h. At the indicated times, cells were harvested, and then cytosolic fractions were prepared. The peptidase activity in the cytosolic fractions was measured using a fluorogenic peptide substrate, Ac-DEVD-AFC, for active caspase-3. Relative peptidase activity was calculated taking the caspase activity in the cell lysate treated with actinomycin D for 6 h as 100%. The results are representative of two independent experiments. (**B**) The cytosolic fractions (20 μg protein equivalent/lane) were subjected to 12.5% SDS-PAGE, and then the protein bands of Smac/DIABLO, cytochrome *c* and AIF released from mitochondria were visualized by Western blot analysis using specific antibodies to each protein factor. The results are representative of three independent experiments.

potential may be involved in galectin-9-induced Jurkat T-cell apoptosis.

Ca^{2+} Uptake Induced by Galectin-9—We examined Ca^{2+} mobilization in Jurkat T cells stimulated with various stimulants including galectin family members, because we showed that Ca^{2+} influx is a trigger for the apoptosis of MOLT-4 by galectin-9 in the previous work (*25*). The results revealed that galectin-9 and RCA-120 induced Ca^{2+} uptake in the cytoplasm, and this Ca^{2+} mobilization was clearly inhibited by the addition of lactose (Fig. 7A). In the galectin family, galectin-8 moderately induced Ca^{2+} uptake at high concentration (5 μM). To determine the effect of extracelluar Ca^{2+}, the Fluo 3-AM assay was also performed in Ca^{2+}-free medium containing EGTA, a Ca^{2+} chelator. We found that galectin-9-induced Ca^{2+} uptake decreased to 50% in the Ca^{2+}-free medium, i.e. it was not completely suppressed (Fig. 7B-1 and 7B-2). Thus, Ca^{2+} uptake may be induced by galectin-9 extracellularly and intracellularly. To clarify the relation between Ca^{2+} uptake and apoptosis, PS exposure and DNA fragmentation were assayed in the presence of an intracellular Ca^{2+}-chelator, BAPTA-AM, in both Jurkat and MOLT-4 cells. Although PS exposure was not or only weakly inhibited by BAPTA-AM in both types of cells, interestingly, DNA fragmentation exhibited a distinct decrease in MOLT-4 cells, but not in Jurkat cells (Fig. 8A and 8B). In addition, calpain inhibitor III was also effective for MOLT–4 cells, but not for Jurkat T cells. This indicates that Jurkat T cells utilize a different apoptosis pathway from the Ca^{2+}-calpain-caspase-1-dependent pathway in MOLT-4 cells, as described previously (*25*).

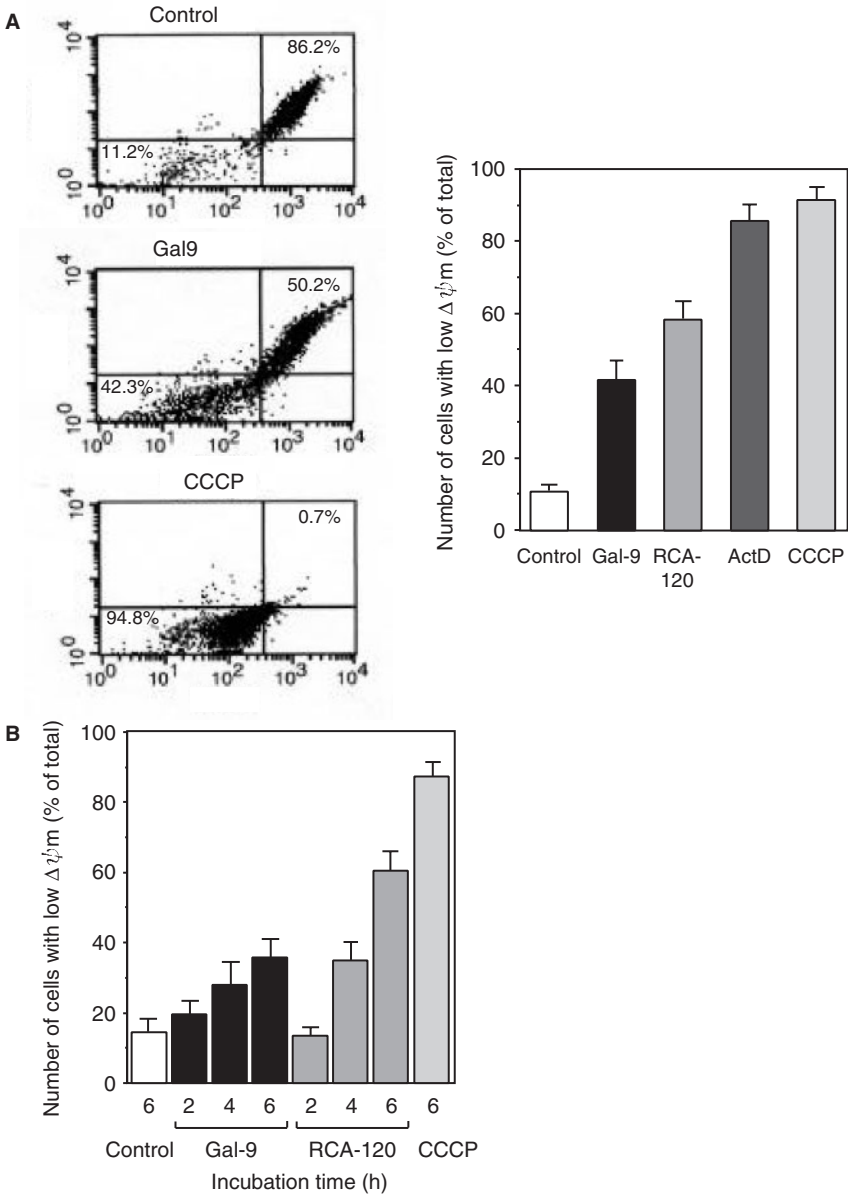

Fig. 6. **Effects of various stimulants on the mitochondrial membrane potential.** (**A**) Jurkat T cells were incubated with galectin-9 (0.25 μM), RCA-120 (8 nM), or actinomycin D (2 μg/ml) for 6 h. After washing, alteration of the mitochondrial membrane potential of the cells was determined by the $DiOC_2(3)$-staining method as described under Materials and Methods. CCCP (final concentration, 50 μM) was used as a positive control reagent for disruption of the mitochondrial membrane potential. The lower left quadrant of each plot show the cells disrupted on mitochondrial membrane potential. Data represent the means ± S.D. of triplicate measurements. (**B**) Jurkat T cells were incubated with galectin-9 (0.25 μM) or RCA-120 (8 nM) for 2–6 h. After washing, alteration of the mitochondrial membrane potential of the cells was assayed. Data represent the means ± S.D. of triplicate measurements.

N-Glycans are targets for Jurkat T-cell apoptosis by lectins—There have been some reports that *O*-glycans and/or *N*-glycans are necessary for galectin-1-induced T-cell death. In order to identify target glycans for galectin-9 in Jurkat T cells, the effects of glycan synthesis inhibitors, i.e. deoxymannojirimycin (DMNJ) and benzyl-2-acetamide-2-deoxy-D-galactopyranoside (BG), were examined. Mannosidase I inhibitor DMNJ inhibits *N*-linked oligosaccharide elongation, and BG inhibits *O*-linked oligosaccharide chain processing. Although we did not detect a distinct inhibitory effect of BG (Fig. 9B-1 and 9B-2), DMNJ-treatment resulted in a dramatic reduction in both PS exposure and DNA fragmentation in galectin-9- or RCA-120-treated Jurkat T cells (Fig. 9A-1 and 9A-2). This indicates that *N*-glycans are primary targets for galectin-9 and RCA-120, which are responsible for the cellular responses.

CD7, CD29 and CD45 have been identified as specific T-cell surface receptors for galectin-1 and/or galectin-3, which are responsible for the cell death. Because Jurkat

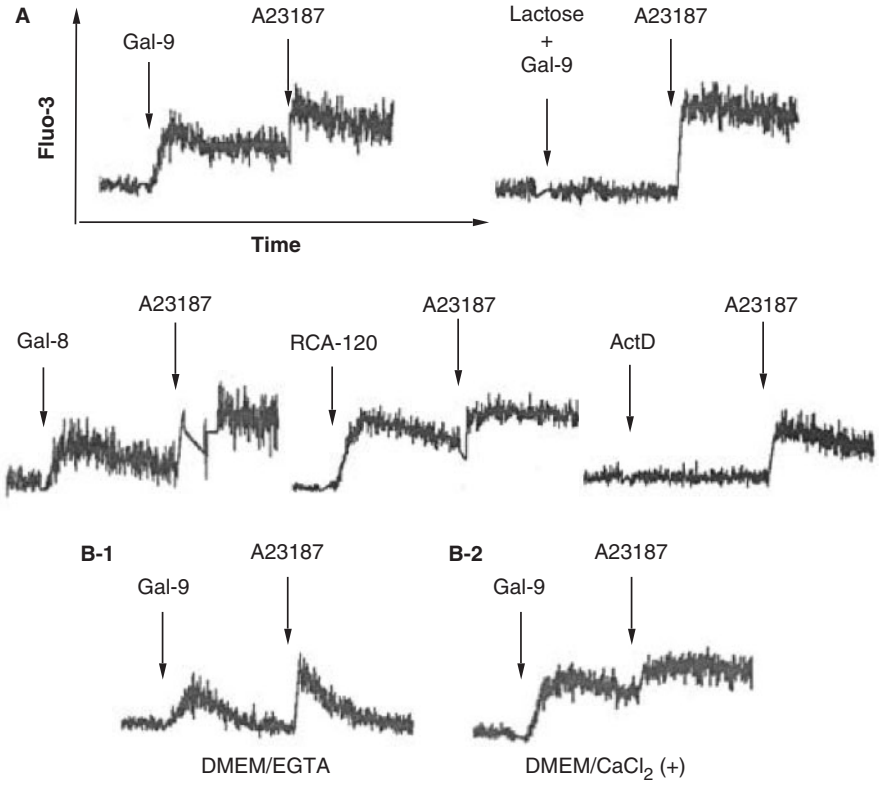

Fig. 7. **Intracellular Ca^{2+} uptake by galectins in Jurkat T cells.** (**A**) Jurkat T cells were preincubated with Fluo 3-AM (10 μM) for 30 min. After incubation, the cells were washed with fresh medium and then galectin-8 (5 μM), galectin-9 (0.25 μM), RCA-120 (8 nM), or actinomycin D (2 μg/ml) was added to the cell suspensions. Intracellular Ca^{2+} uptake was measured by flow cytometry as described under Materials and Methods. Ca^{2+} ionophore A23187 (final concentration, 5 ng/ml), was used as a positive control reagent. In the case of galectin-9, the effect of 25 mM lactose was also examined. Data are representative of three independent experiments. (**B**) Jurkat T cells were preincubated with Fluo 3-AM (10 μM) in Ca^{2+}-free DMEM containing a Ca^{2+} chelator, EGTA (1 mM) **(B-1)** or $CaCl_2$ (1 mM) **(B-2)**, for 30 min. After incubation, the cells were washed with the same fresh medium and then galectin-9 (0.25 μM) was added to the cell suspensions. Intracellular Ca^{2+} uptake was measured by flow cytometry. Data are representative of three independent experiments.

and MOLT-4 are CD7, CD29 and CD45-expressing T cells, and susceptible to galectin-9 as to cell apoptosis (Fig. 10A and 10B), we examined whether these CD antigens are important for galectin-9-induced Jurkat cell death or not. However, specific antibodies against CD7, CD29 and CD45 did not exhibit any inhibitory or stimulatory effect on DNA fragmentation in galectin-9-treated Jurkat T cells (Fig. 10C). Thus, CD7 may be not a specific receptor for galectin-9, like CD29 and CD45, which is associated with the cell death pathway in Jurkat T cells.

DISCUSSION

In this study, we analysed the properties of a tandem-repeat galectin-9 as to the apoptotic death of Jurkat T cells, and compared them with those of other galectins and plant lectins. Of course, galectin-1 is a well-known apoptosis inducer for T-cell lines at high concentration (5–20 μM) (*27, 43*). In the galectin family, galectin-9 induces apoptotic cell death at low concentration (0.1–0.5 μM), but other galectins have no or only a weak effect even at high concentration (1–2 μM) in Jurkat T cells. Another tandem-repeat galectin, galectin-8, induced exposure of phosphatidylserine (PS) and Ca^{2+} uptake in Jurkat T cells at high concentration (2–5 μM), but did not induce DNA fragmentation. Although PS exposure is an early event in apoptotic cell death and a conventional indicator for screening of proapototic factors, PS mobilization may not be a direct signal leading to apoptotic cell death in galectin-mediated cell reactions. It has been reported that galectins-1, -2 and -4 induced PS exposure in leucocytes without accompanying apoptosis (*44, 45*). A secreted glycoprotein, milk fat globule-EGF-factor 8 (MFG-E8), was found to specially bind to apoptotic cells by recognizing aminophospholipids such as PS and to carry them to phagocytes for engulfment (*46, 47*). Since galectin-8 and -9 can induce PS exposure in various leukaemia cells, without accompanying DNA fragmentation (data not shown), galectin-induced PS exposure may be the most important cell response and signal for maintenance of the cell population among leucocytes.

Tandem-linked CRD mutants of galectin-9, i.e. galectin-9NN and galectin-9CC, exhibited almost the same proapoptotic activity as to that of galectn-9NC.

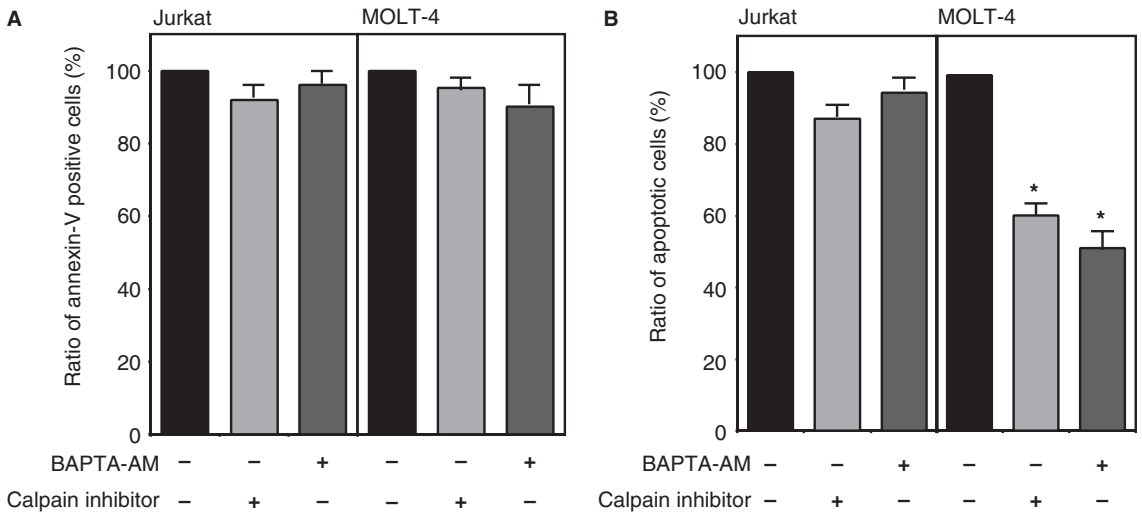

Fig. 8. **Intracellular Ca^{2+} and calpain are not required for Jurkat T-cell death caused by galectin-9.** (**A**) Jurkat or MOLT–4 cells were preincubated with an intracellular Ca^{2+} chelator, BAPTA-AM (10 μM), or a calpain inhibitor, calpain inhibitor III (20 μM) for 30 min, and then treated with galectin-9 (0.25 μM) for 6 h. After washing, the cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Relative annexin V-positibe cell numbers were calculated taking the number of annexin V-positive cells treated with galectin-9 in the absence of the Ca^{2+} chelator and calpain inhibitor as 100%. Data are means ± S.D. of triplicate measurements. (**B**) After pretreatment of Jurkat or MOLT-4 cell with a Ca^{2+} chelator or calpain inhibitor as described previously (**A**), the cells were treated with galectin-9 (0.25 μM) for 12 h. DNA fragmentation was evaluated by the TUNEL assay method. Relative apoptotic cell numbers were calculated taking the number of apoptotic cells treated with galectin-9 in the absence of the Ca^{2+} chelator and calpain inhibitor as 100%. Data represent the means ± S.D. of triplicate measurements. $^*P < 0.05$.

While single CRDs of galectin-9, i.e. galectin-9NT and galectin-9CT, had lost the activity. Our previous work showed that synthetic galectins-9NC, -9NN and -CC exhibited similar eposhinophil chemoattractant activity (ECA), and that the ECA activity of single CRDs of galectin-9 was decreased (*40*). These results indicate that the stable dimeric structure of CRDs is indispensable for galectin-9 for both the ECA and proapoptotic activities, and the abilities of galectins-9NT and -9CT are almost equal. On the other hand, galectin-8 exhibited no proapoptotic activity as to Jurkat T cells and low ECA activity (*41*). Thus, its binding specificity and/or affinity towards cell surface receptors in the tandem-repeat galectins, seem to be important to induce the unique cellular responses. Some researchers thought the structural feature of the pentameric form of galectin-3 was one of the reasons why it killed cells more efficiently than a dimeric form of galectin-1 during T-cell death (*48*). In fact, the minimal concentrations of galectins required for the aggregation of Jurkat T cells fixed with glutaraldehyde were observed to be low for galectin-9 (12.5–25 nM), but high for galectin-1 (800–1600 nM) and galectin-3 (3200 nM) in culture medium (data not shown). So we propose that the stable dimeric CRD structural feature may allow galectin-9 to bind or cross-link a great number of T-cell surface receptors and a longer occupancy time on saccharide ligands, compared with galectins-1 and -3 at equivalent concentration.

Anti-Fas antibodies and actinomycin D are typical apoptosis inducers in T cell lines. Anti-Fas antibodies induce apoptotic cell death through sequential activation of caspases-8, -9 and -3 (*49–51*). Actinomycin D inhibits the proliferation of cells by forming a stable complex with double-stranded DNA, and then this stress may induce cell death via a caspase-dependent pathway. In our experiments, broad-specific inhibitor zVAD-fmk exhibited only partial inhibitory activity toward both PS exposure and DNA fragmentation in galectin-9-treated Jurkat T cells. Furthermore, activation of caspase-3 and release of mitochondrial components, i.e. Smac/DIABLO and cytochrome *c*, which are thought to be factors resulting in caspase activation (*52–54*), were also observed in the cells treated with galectin-9. On the other hand, we detected rapid AIF release within 2 h treatment with galectin-9. AIF, which is located in the mitochondrial intermembrane space, induces caspase-independent cell death on moving into the nucleus and interacting with DNA (*54, 55*). Overall, galectin-9 may induce Jurkat T-cell apoptosis through both caspase-dependent and caspase-independent pathways. In addition, we found that loss of the mitochondrial membrane potential occurred in galectin-9-treated T cells. Several studies have revealed that galectin-1 induced T-cell death via nuclear translocation of EndoG through a cytochrome *c*-independent pathway, that cytochome *c* was important in galectin-2-mediated T-cell death, and that the Bcl-2 family also regulated galectin-2- or galectin-3-induced T-cell apoptosis (*32, 33, 56*). Although we could not detect the change of EndoG release in galectin-9-treated Jurkat T cells (data not shown), multiple mitochondrial factors may play different roles in galectin family-induced cell death. It remains to be elucidated that whether more apoptotic factors such as Bcl family members be involved in galectin-9-mediated cell death.

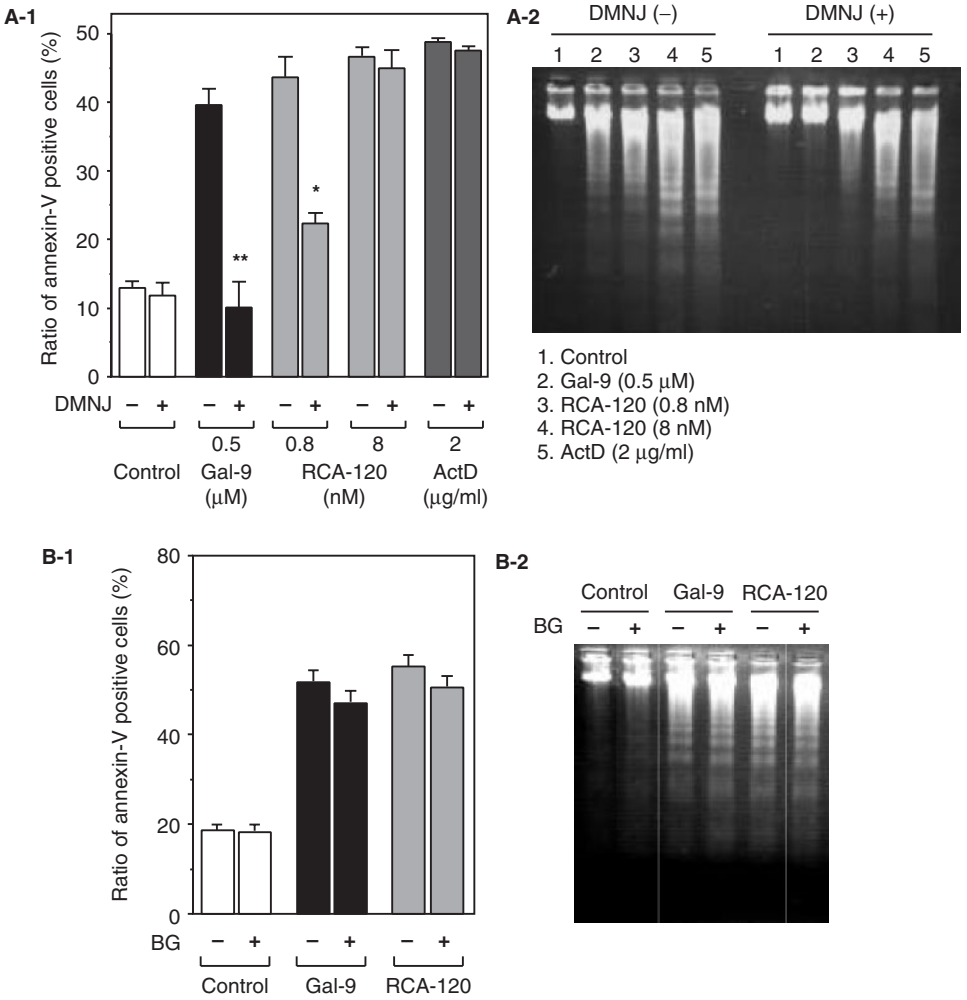

Fig. 9. **Effect of a *N*-glycan synthesis inhibitor on Jurkat T-cell apoptosis. (A-1)** Jurkat T cells (6×10^5 cells/well) were precultured in the presence of *N*-glycan synthesis inhibitor DMNJ (2 mM) for 64 h. The cells were harvested, washed with fresh medium, and then treated with galectin-9 (0.5 μM), RCA-120 (0.8 or 8 nM), or actinomycin D (2 μg/ml) for 6 h. After washing, the cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Data are means ± S.D. of triplicate measurements. $^*P < 0.05$, $^{**}P < 0.01$. **(A-2)** After preculture of Jurkat T cells as described previously (A-1) for 64 h, the cells were harvested, washed with fresh medium, and then treated with galectin-9 (0.5 μM), RCA-120 (0.8 or 8 nM), or actinomycin D (2 μg/ml) for 12 h. DNA fragments were separated by 2% agarose gel electrophoresis and then stained. Data are representative of three independent experiments. **(B-1)** Jurkat T cells (6×10^5 cells/well) were precultured in the presence of *O*-glycan synthesis inhibitor BG (1 mM) for 64 h. The cells were harvested, washed with fresh medium, and then treated with galectin-9 (0.5 μM) or RCA-120 (0.8 nM) for 6 h. After washing, the cells were stained with annexin V/7-AAD and then analysed by flow cytometry. Data are means ± S.D. of triplicate measurements. **(B-2)** After preculture of Jurkat T cells as described previously (B-1) for 64 h, the cells were harvested, washed with fresh medium, and then treated with galectin-9 (0.5 μM) or RCA-120 (0.8 nM) for 12 h. DNA fragments were separated by 2% agarose gel electrophoresis and then stained. Data are representative of three independent experiments.

We previously reported that intracellular Ca^{2+} uptake is essential for the apoptotic cell death of MOLT-4 cells (*25*). In Jurkat T cells, intracellular Ca^{2+} uptake was significantly induced by galectin-9. However, an intracellular Ca^{2+} chelator, BAPTA-AM, and a calpain inhibitor, calpain inhibitor III, in the culture medium had no significant effect on PS exposure or DNA fragmentation in galectin-9-treated Jurkat T cells. These findings indicate that there is no direct relation between Ca^{2+} mobilization and calpain activation, and the apoptotic death of Jurkat T cells, and that the galectin-9-mediated apoptotic pathway in Jurkat T cells may differ from the Ca^{2+}-calpain-caspase-1-dependent pathway in MOLT-4 T cells.

Among plant lectins, RCA-120, a tetramer plant lectin consisting of two CRDs and two ribosome-inactivating enzyme domains, belongs to the type-2 ribosome-inactivating protein (RIP) family. It is a RIP exhibiting no or low cytotoxicity that is structurally similar to the most toxic plant lectin ricin (*57*). In our experiment, we found that both galectin-9 and RCA-120, which recognize lactose as a common hapten sugar, induced significant apoptosis of Jurkat T cells.

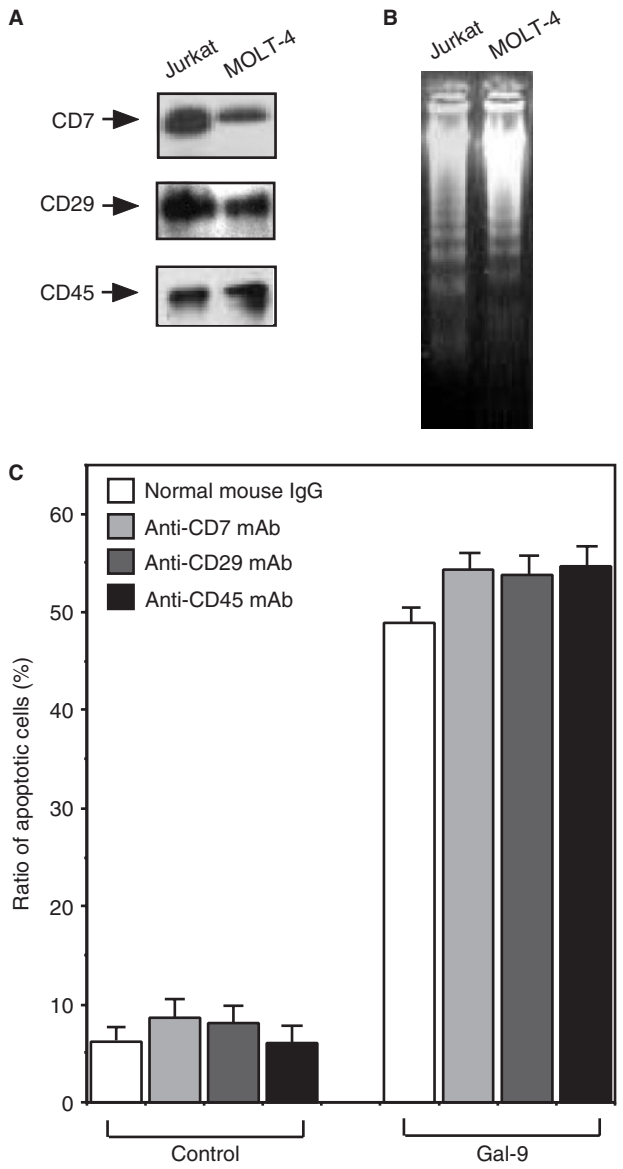

Fig. 10. **CD7 CD29 and CD45 are not the main targets for galectin-9-induced Jurkat T-cell death**. (**A**) Whole cell lysates (50 μg protein equivqlent/lane) of Jurkat or MOLT-4 cells were subjected to 12.5% SDS-PAGGE. Then, Western blot analysis was performed using anti-CD7 (H-126) Ab, anti-CD29 (M-107) Ab and anti-CD45 (135-4C5) Ab. The results are representative of two independent experiments. (**B**) Jurkat or MOLT-4 cells were incubated with galectin-9 (0.25 μM) for 12 h. DNA fragments were separated by 2% agarose gel electrophoresis. The results are representative of two independent experiments. (**C**) Jurkat T cells were preincubated with CD7 (M-T701) mAb, CD29 (TDM29) mAb or CD45 (T29/33) mAb (20 μg/ml) in the culture medium for 30 min at 37°C, and then the cells were incubated with galectin-9 (0.25 μM) for 12 h at 37°C. After washing, DNA fragmentation was evaluated by the TUNEL assay method. Data represent the means ± S.D. of triplicate measurements.

Although both *N*-glycans and *O*-glycans have been reported to take part in galectin-1-induced cell death (*27, 58*), mannosidase I inhibitor DMNJ, which blocks the synthesis of all complex *N*-glycans, significantly inhibited galectin-9 and RCA-120 mediated-PS exposure and DNA fragmentation. However, *O*-glycan synthesis inhibitor BG had no effect on either PS exposure or DNA fragmentation. Actually, galectin-9 binds preferentially to tri- and/or tetra-antennary *N*-linked glycans with *N*-acetyllactosamine units (*40*). These findings suggest that the *N*-glycans of target proteins might be essential for galectin-9 and RCA-120-induced cell death in Jurkat T cells.

Among CD antigens, i.e. CD2, CD3, CD4, CD7, CD43 and CD45, CD7 may be an essential component of a proapoptotic complex of receptors that sends the galectin-1 death signal (*43, 59, 60*). In addition, it was reported that oligosaccharide-mediated clustering of CD45 facilitates galectin-1-induced cell death (*58*). Galectin-3 also induces Type II T-cell apoptosis after binding CD7, with or without CD29 (β1 integrin) (*34*), while galectin-2 lacks this reactivity to CD3 and CD7, but exhibits affinity to β1 integrin (*32*). In this study, although the target(s) of galectin-9 responsible for Jurkat -T-cell death was not identified, the data revealed that CD7, CD29 and CD45 are not the main targets of galectin-9. Recently, it was reported that neither CD7 nor CD29 are required for galectin-3-induced death of T cells (*48*). Also, galectin-9 was identified as one of the ligands of Tim-3 protein, which is specifically expressed on Th1 cells, and triggers negative regulation of Th1 responses (*61*). Therefore, the target receptors may be different for each galectin and in each cell type examined. We previously reported that galectin-9 induces apoptosis in $CD4^+$ and $CD8^+$ T cells activated with anti-CD3 Ab, but not in the resting T cells from human peripheral blood (*25*). However, PS exposure was induced markedly by galectin-9 in both activated and resting $CD4^+$ and $CD8^+$ T cells, and granulocytes (data not shown). Thus, T cell apoptosis by galectin-9 may occur preferentially in activated T cells and Th1 cells *in vivo*. While resting T cells and another lymphocyte subsets can also respond to galectin-9, which may result in modulation of the lymphocyte functions except for apoptosis *in vivo*. Further study is needed to elucidate the roles of galectin family members in immunoregulation and in the leukocytes survival by means of identification of functional receptor(s) for each galectin.

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REFERENCES

1. Barondes, S.H., Castronovo, V., Cooper, D.N. *et al.* (1994a) Galectins: a family of animal β-galactoside-binding lectins. *Cell* **76**, 597–598
2. Barondes, S.H., Cooper, D.N., Gitt, M.A. *et al.* (1994b) Galectins: structure and function of a large family of animal lectins. *J. Biol. Chem.* **269**, 20807–20810
3. Cooper, D.N. and Barondes, S.H. (1999) God must love galectins; he made so many of them. *Glycobiology* **9**, 979–984

4. Kasai, K. and Hirabayashi, J. (1996) Galectins: a family of animal lectins that decipher glycocodes. *J. Biochem. (Tokyo)* **119**, 1–8
5. Dunphy, J.L., Balic, A., Barcham, G.J. *et al.* (2000) Isolation and characterization of a novel inducible mammalian galectin. *J. Biol. Chem.* **275**, 32106–32113
6. Yang, R.Y., Hsu, D.K., Yu, L. *et al.* (2001) Cell cycle regulation by galectin-12, a new member of the galectin superfamily. *J. Biol. Chem.* **276**, 20252–20260
7. Visegrady, B., Than, N.G., Kilar, F. *et al.* (2001) Homology modelling and molecular dynamics studies of human placental tissue protein 13 (galectin-13). *Protein Eng.* **14**, 875–880
8. Gray, C.A., Adelson, D.L., Bazer, F.W. *et al.* (2004) Discovery and characterization of an epithelial-specific galectin in the endometrium that forms crystals in the trophectoderm. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 7982–7987
9. Cooper, D.N. (2002) Galectinomics: finding themes in complexity. *Biochim. Biophys. Acta* **1572**, 209–231
10. Hirabayashi, J., Hashidate, T., Nishi, N. *et al.* (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim. Biophys. Acta* **1572**, 232–254
11. Leonidas, D.D., Vatzaki, E.H., Vorum, H. *et al.* (1998) Structural basis for the recognition of carbohydrates by human galectin-7. *Biochemistry* **37**, 12930–12940
12. Ahmad, N., Gabius, H.J., Andre, S. *et al.* (2004) Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. *J. Biol. Chem.* **279**, 10841–10847
13. Rabinovich, G.A., Baum, L.G., Tinari, N. *et al.* (2002) Galectins and their ligands: Amplifiers, silencers or tuners of the inflammatory response?. *Trends Immunol.* **23**, 313–320
14. Liu, F.T. and Rabinovich, G.A. (2005) Galectins as modulators of tumour progression. *Nat. Rev. Cancer* **5**, 29–41
15. Perillo, N.L., Marcus, M.E., and Baum, L.G. (1998) Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *J. Mol. Med.* **76**, 402–412
16. Rabinovich, G.A. (1999) Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy. *Cell Death Differ.* **6**, 711–721
17. Liu, F.T. (2000) Galectins: a new family of regulators of inflammation. *Clin. Immunol.* **97**, 79–88
18. Wada, J. and Kanwar, Y. S. (1997) Identification and characterization of galectin-9, a novel β-galactoside-binding mammalian lectin. *J. Biol. Chem.* **272**, 6078–6086
19. Matsumoto, R., Matsumoto, H., Seki, M. *et al.* (1998) Human ecalectin, a variant of human galectin-9, is a novel eosinophil chemoattractant produced by T lymphocytes. *J. Biol. Chem.* **273**, 16976–16984
20. Saita, N., Goto, E., Yamamoto, T. *et al.* (2002) Association of galectin-9 with eosinophil apoptosis. *Int. Arch. Allergy Immunol.* **128**, 42–50
21. Irie, A., Yamauchi, A., Kontani, K. *et al.* (2005) Galectin-9 as a prognostic factor with antimetastatic potential in breast cancer. *Clin. Cancer Res.* **11**, 2962–2968
22. Dai, S.Y., Nakagawa, R., Itoh, A. *et al.* (2005) Galectin-9 induces maturation of human monocyte-derived dendritic cells. *J. Immunol.* **175**, 2974–2981
23. Wada, J., Ota, K., Kumar, A. *et al.* (1997) Developmental regulation, expression, and apoptotic potential of galectin-9, a β-galactosides binding lectin. *J. Clin. Invest.* **99**, 2452–2461
24. Kageshita, T., Kashio, Y., Yamauchi, A. *et al.* (2002) Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. *Int. J. Cancer* **99**, 809–816
25. Kashio, Y., Nakamura, K., Abedin, M.J. *et al.* (2003) Galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway. *J. Immunol.* **170**, 3631–3636
26. Liu, F.T. (2005) Regulatory roles of galectins in the immune response. *Int. Arch. Allergy Immunol.* **136**, 385–400
27. Perillo, N.L., Pace, K.E., Seilhamer, J.J. *et al.* (1995) Apoptosis of T cells mediated by galectin-1. *Nature* **378**, 736–739
28. Perillo, N.L., Uittenbogaart, C.H., Nguyen, J.T. *et al.* (1997) Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes. *J. Exp. Med.* **185**, 1851–1858
29. Fouillit, M., Joubert-Caron, R., Poirier, F., Bourin, P., Monostori, E., Levi-Strauss, M., Raphael, M., Bladier, D., and Caron, M. (2000) Regulation of CD45-induced signaling by galectin-1 in Burkitt lymphoma B cells. *Glycobiology* **10**, 413–419
30. Wells, V., Davies, D., and Mallucci, L. (1999) Cell cycle arrest and induction of apoptosis by beta galactoside binding protein (beta GBP) in human mammary cancer cells. A potential new approach to cancer control. *Eur. J. Cancer* **35**, 978–983
31. Ellerhorst, J., Nguyen, T., Cooper, D.N., Estrov, Y., Lotan, D., and Lotan, R. (1999) Induction of differentiation and apoptosis in the prostate cancer cell line LNCaP by sodium butyrate and galectin-1. *Int. J. Oncol.* **14**, 225–232
32. Sturm, A., Lensch, M., Andre, S., Kaltner, H., Wiedenmann, B., Rosewicz, S., Dignass, A.U., and Gabius, H.J. (2004) Human galectin-2: novel inducer of T cell apoptosis with distinct profile of caspase activation. *J. Immunol.* **173**, 3825–3837
33. Yang, R.Y., Hsu, D.K., and Liu, F.T. (1996) Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6737–6742
34. Fukumori, T., Takenaka, Y., Yoshii, T., Kim, H.R., Hogan, V., Inohara, H., Kagawa, S., and Raz, A. (2003) CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis. *Cancer Res.* **63**, 8302–8311
35. Fukumori, T., Takenaka, Y., Oka, N., Yoshii, T., Hogan, V., Inohara, H., Kanayama, H.O., Kim, H.R., and Raz, A. (2004) Endogenous galectin-3 determines the routing of CD95 apoptotic signaling pathways. *Cancer Res.* **64**, 3376–3379
36. Oka, N., Nakahara, S., Takenaka, Y., Fukumori, T., Hogan, V., Kanayama, H.O., Yanagawa, T., and Raz, A. (2005) Galectin-3 inhibits tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by activating Akt in human bladder carcinoma cells. *Cancer Res.* **65**, 7546–7553
37. Kuwabara, I., Kuwabara, Y., Yang, R.Y., Schuler, M., Green, D.R., Zuraw, B.L., Hsu, D.K., and Liu, F.T. (2002) Galectin-7 (PIG1) exhibits pro-apoptotic function through JNK activation and mitochondrial cytochrome c release. *J. Biol. Chem.* **277**, 3487–3497
38. Arbel-Goren, R., Levy, Y., Ronen, D., and Zick, Y. (2005) Cyclin-dependent kinase inhibitors and JNK act as molecular switches, regulating the choice between growth arrest and apoptosis induced by galectin-8. *J. Biol. Chem.* **280**, 19105–19114
39. Hotta, K., Funahashi, T., Matsukawa, Y., Takahashi, M., Nishizawa, H., Kishida, K., Matsuda, M., Kuriyama, H., Kihara, S., Nakamura, T., Tochino, Y., Bodkin, N.L., Hansen, B.C., and Matsuzawa, Y. (2001) Galectin-12, an adipose-expressed galectin-like molecule possessing apoptosis-inducing activity. *J. Biol. Chem.* **276**, 34089–34097
40. Sato, M., Nishi, N., Shoji, H., Seki, M., Hashidate, T., Hirabayashi, J., Kasai, K., Hata, Y., Suzuki, S., Hirashima, M., and Nakamura, T. (2002) Functional analysis of the carbohydrate recognition domains and a linker peptide of galectin-9 as to eosinophil chemoattractant activity. *Glycobiology* **12**, 191–197

41. Matsushita, N., Nishi, N., Seki, M., Matsumoto, R., Kuwabara, I., Liu, F.T., Hata, Y., Nakamura, T., and Hirashima, M. (2000) Requirement of divalent galactoside-binding activity of ecalectin/galectin-9 for eosinophil chemoattraction. *J. Biol. Chem.* **275**, 8355–8360
42. Nishi, N., Itoh, A., Fujiyama, A., Yoshida, N., Araya, S., Hirashima, M., Shoji, H., and Nakamura, T. (2005) Development of highly stable galectins: truncation of the linker peptide confers protease-resistance on tandem-repeat type galectins. *FEBS Lett.* **579**, 2058–2064
43. Pace, K.E., Lee, C., Stewart, P.L., and Baum, L.G. (1999) Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. *J. Immunol.* **163**, 3801–3811
44. Dias-Baruffi, M., Zhu, H., Cho, M., Karmakar, S., McEver, R.P., and Cummings, R.D. (2003) Dimeric galectin-1 induces surface exposure of phosphatidylserine and phagocytic recognition of leukocytes without inducing apoptosis. *J. Biol. Chem.* **278**, 41282–41293
45. Stowell, S., Karmakar, S., Stowell, C., Dias-Baruffi, M., McEver, R.P., and Cummings, R.D. (2006) Human Galectins-1, -2, and -4 induce surface exposure of phosphatidylserine in activated human neutrophils but not activated T cells. *Blood* (in press)
46. Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., and Nagata, S. (2002) Identification of a factor that links apoptotic cells to phagocytes. *Nature* **417**, 182–187
47. Hanayama, R., Tanaka, M., Miyasaka, K., Aozasa, K., Koike, M., Uchiyama, Y., and Nagata, S. (2004) Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* **304**, 1147–1150
48. Stillman, B.N., Hsu, D.K., Pang, M., Brewer, C.F., Johnson, P., Liu, F.T., and Baum, L.G. (2006) Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J. Immunol.* **176**, 778–789
49. Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E., and Dixit, V.M. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death–inducing signaling complex. *Cell* **85**, 817–827
50. Enari, M., Talanian, R.V., Wong, W.W., and Nagata, S. (1996) Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* **380**, 723–726
51. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., and Peter, M.E. (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* **17**, 1675–1687
52. Krippner, A., Matsuno-Yagi, A., Gottlieb, R.A., and Babior, B.M. (1996) Loss of function of cytochrome c in Jurkat cells undergoing fas-mediated apoptosis. *J. Biol. Chem.* **271**, 21629–21636
53. Wu, G., Chai, J., Suber, T.L., Wu, J.W., Du, C., Wang, X., and Shi, Y. (2000) Structural basis of IAP recognition by Smac/DIABLO. *Nature* **408**, 1008–1012
54. Saelens, X., Festjens, N., Vande Walle, L., van Gurp, M., van Loo, G., and Vandenabeele, P. (2004) Toxic proteins released from mitochondria in cell death. *Oncogene* **23**, 2861–2874
55. Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M., and Kroemer, G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441–446
56. Hahn, H.P., Pang, M., He, J., Hernandez, J.D., Yang, R.Y., Li, L.Y., Wang, X., Liu, F.T., and Baum, L.G. (2004) Galectin-1 induces nuclear translocation of endonuclease G in caspase- and cytochrome c-independent T cell death. *Cell Death Differ.* **11**, 1277–1286
57. Baenziger, J.U. and Fiete, D. (1997) Structural determinants of Ricinus communis agglutinin and toxin specificity for oligosaccharides. *J. Biol. Chem.* **254**, 9795–9799
58. Nguyen, J.T., Evans, D.P., Galvan, M., Pace, K.E., Leitenberg, D., Bui, T.N., and Baum, L.G. (2001) CD45 modulates galectin-1-induced T cell death: regulation by expression of core 2 O-glycans. *J. Immunol.* **167**, 5697–5707
59. Pace, K.E., Hahn, H.P., Pang, M., Nguyen, J.T., and Baum, L.G. (2000) CD7 delivers a pro-apoptotic signal during galectin-1-induced T cell death. *J. Immunol.* **165**, 2331–2334
60. Elola, M.T., Chiesa, M.E., Alberti, A.F., Mordoh, J., and Fink, N.E. (2005) Galectin-1 receptors in different cell types. *J. Biomed. Sci.* **12**, 13–29
61. Zhu, C., Anderson, A.C., Schubart, A., Xiong, H., Imitola, J., Khoury, S.J., Zheng, X.X., Strom, T.B., and Kuchroo, V.K. (2005) The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat. Immunol.* **6**, 1245–1252