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# Galectin-9 Induces Apoptosis Through the Calcium-Calpain-Caspase-1 Pathway<sup>1</sup>

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Galectin-9 (Gal-9) induced the apoptosis of not only T cell lines but also of other types of cell lines in a dose- and time-dependent manner. The apoptosis was suppressed by lactose, but not by sucrose, indicating that  $\beta$ -galactoside binding is essential for Gal-9-induced apoptosis. Moreover, Gal-9 required at least 60 min of Gal-9 binding and possibly de novo protein synthesis to mediate the apoptosis. We also assessed the apoptosis of peripheral blood T cells by Gal-9. Apoptosis was induced in both activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, but the former were more susceptible than the latter. A pan-caspase inhibitor (Z-VAD-FMK) inhibited Gal-9-induced apoptosis. Furthermore, a caspase-1 inhibitor (Z-YVAD-FMK), but not others such as Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), and Z-AEVD-FMK (caspase-10 inhibitor), inhibited Gal-9-induced apoptosis. We also found that a calpain inhibitor (Z-LLY-FMK) suppresses Gal-9-induced apoptosis, that Gal-9 induces calcium (Ca<sup>2+</sup>) influx, and that either the intracellular Ca<sup>2+</sup> chelator BAPTA-AM or an inositol trisphosphate inhibitor 2-aminoethoxydiphenyl borate inhibits Gal-9-induced apoptosis. These results suggest that Gal-9 induces apoptosis via the Ca<sup>2+</sup>-calpain-caspase-1 pathway, and that Gal-9 plays a role in immunomodulation of T cell-mediated immune responses. *The Journal of Immunology*, 2003, 170: 3631–3636.

**G**alectin-9 (Gal-9)<sup>3</sup> is a  $\beta$ -galactoside binding lectin that belongs to a growing animal lectin family of galectins (1, 2). We cloned ecalectin as a T cell-derived eosinophil chemoattractant (3) and showed that Gal-9 is identical with ecalectin (1). Gal-9 has N- and C-terminal carbohydrate recognition domains (4, 5) that are connected by a linker peptide (2, 6–8).

Galectins modulate a variety of biological functions, such as cell activation, proliferation, adhesion, and apoptosis (9–11). Like other galectins, Gal-9 exhibits various biological functions, such as cell aggregation and chemoattraction of eosinophils, as well as apoptosis of murine thymocytes and T cells, and human melanoma cells (3, 12–15).

Apoptosis is induced by several stimuli, including galectins, and apoptosis pathways have been intensively studied. Accordingly, several pathways have been proposed. Although the activation of caspase-3 and -7 is essentially required for all pathways, activation of a different caspase is involved upstream. For example, caspase-1 (16–18), caspase-8 (19, 20), caspase-9 (21), and caspase-10 (22, 23) are required for apoptosis mediated through association with glucocorticoid (GC), Fas/TNF, mitochondria or perforin, respectively. Gal-1 induces the apoptosis of T cells (24), thymocytes

(25), and prostate cancer (26) through the down-regulation of Bcl-2 (27) and caspase activation. Gal-1 does not require an increase in intracellular Ca<sup>2+</sup> to induce apoptosis (28). Gal-7 is a proapoptotic protein that functions intracellularly upstream of c-Jun N-terminal kinase activation and cytochrome *c* release (29, 30). Although Gal-9 can trigger the apoptosis of murine thymocytes and melanoma (12, 15), the pathway has not been clarified. The purpose of the present study was to show that human Gal-9 induces the apoptosis of T cells via the Ca<sup>2+</sup>-calpain-caspase-1 pathway.

## Materials and Methods

### Cell culture

MOLT-4 (T cells), Jurkat (T cells), BALL-1 (B cells), THP-1 (monocytes), and HL-60 (myelocytes) obtained from American Type Culture Collection (Manassas, VA) were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS at 37°C in 5% CO<sub>2</sub>. The activity of Gal-9 was inhibited by adding 30 mM lactose to the culture medium. Sucrose at the same concentration served as the control.

### Expression and purification of recombinant Gal-9 (rGal-9)

Recombinant Gal-9 was expressed and purified as previously described (31, 32). In brief, *Escherichia coli* BL-21 cells containing a Gal-9 expression plasmid were cultured in Luria-Bertoni medium (Life Technologies, Gaithersburg, MD) containing 100  $\mu$ g/ml ampicillin. Isopropyl- $\beta$ -D-thiogalactopyranoside (Wako, Osaka, Japan) was added to induce the expression of fusion proteins. Adsorbed protein was eluted from *E. coli* extract by lactose agarose affinity chromatography (Seikagaku, Tokyo, Japan) using 200 mM lactose. Fractions were collected and analyzed by SDS-PAGE after staining with Coomassie brilliant blue R 250. Fractions containing rGal-9 were pooled and dialyzed against PBS containing 0.1 mM DTT.

### Apoptosis assay

Cultured cells were incubated with rGal-9 for 24 h, then pelleted cells sedimented by centrifugation were resuspended in 300  $\mu$ l of PBS and 700  $\mu$ l of 100% ethanol. The cells were washed with PBS and incubated with 50  $\mu$ g/ml ribonuclease A (Sigma-Aldrich) for 30 min at 37°C, then with 50  $\mu$ g/ml of propidium iodide (PI; Sigma-Aldrich) for 10 min. Stained cells were analyzed by flow cytometry (33, 34).

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<sup>3</sup> Abbreviations used in this paper: Gal-9, galectin-9; 2-APB, 2-aminoethoxydiphenyl borate; DEX, dexamethasone; GC, glucocorticoid; IP<sub>3</sub>, inositol trisphosphate; PI, propidium iodide.

The apoptosis assay proceeded using the MEBCYTO apoptosis kit (MBL, Nagoya, Japan) according to the manufacturer's instructions. In brief, cells were washed and resuspended in binding buffer. Annexin V-FITC and PI were added to the cell suspension, and then the mixture was incubated for 15 min in the dark at room temperature. Thereafter, the suspension was analyzed using a flow cytometer (EPICS XL-MCL; Coulter, Miami, FL).

To assess the Gal-9-mediated apoptotic pathway, cells were cultured with cycloheximide (a protein synthesis inhibitor; Sigma-Aldrich) in the presence or the absence of 1  $\mu$ M Gal-9 for 24 h to determine whether de novo protein synthesis is required for Gal-9-mediated apoptosis (35).

Cells were incubated with Gal-9 in the presence of 10  $\mu$ M Z-VAD-FMK (pan-caspase inhibitor), Z-YVAD-FMK (caspase-1 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), Z-AEVD-FMK (caspase-10 inhibitor), or Z-LLY-FMK (calpain inhibitor; BioVision, Mountain View, CA) to examine the involvement of caspase or calpain in Gal-9-induced apoptosis. Z-FA-FMK (BioVision) served as the control (36, 37).

To clarify the requirement for  $Ca^{2+}$  flux in Gal-9-mediated apoptosis, apoptosis was induced by Gal-9 in the presence or the absence of an extracellular  $Ca^{2+}$  chelator 4 mM EGTA (Dojindo, Kumamoto, Japan) (38), or cells were also preincubated with either intracellular  $Ca^{2+}$  chelator 30  $\mu$ M BAPTA-AM (Dojindo) for 10 min (39) or an inositol triphosphate ( $IP_3$ ) inhibitor 30  $\mu$ M 2-aminoethoxydiphenyl borate (2-APB; Calbiochem, San Diego, CA) for 10 min (40, 41), followed by treatment with 1  $\mu$ M Gal-9.

The following proapoptotic reagents were purchased from the indicated suppliers: dexamethasone (DEX; BioVision), anti-Fas Ab (clone CH-11; MBL), TNF- $\alpha$  (Genzyme, Cambridge, MA), C2 ceramide (Sigma-Aldrich), and etoposide (BioVision).

#### T cell analysis

Twenty-four-well plates were coated with 3  $\mu$ g/well anti-CD3 Ab (Immunotech, Marseilles, France). The mononuclear leukocyte fraction was isolated with Histopaque (Sigma-Aldrich). CD4- or CD8-positive T cells were isolated using a CD4-positive selection kit and Dynabeads M-450 CD8 (DynaL Biotech, Oslo, Norway) as previously described by the manufacturer. T cells were activated by incubation on anti-CD3-coated plates for 24 h at 37°C, followed by rGal-9 treatment.

#### $Ca^{2+}$ mobilization

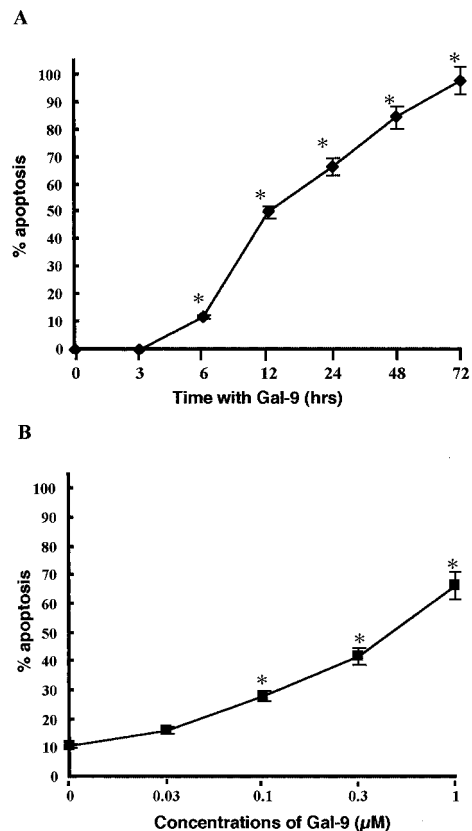
Cells ( $1 \times 10^7$ /ml) in culture medium (10% FCS and 10 mM HEPES, pH 7.2) were loaded with fluo-3/AM (final concentration, 10  $\mu$ M), an intracellular  $Ca^{2+}$  indicator (Dojindo), at 37°C for 30 min (42). The cells were washed and resuspended in culture medium. Intracellular  $Ca^{2+}$  was measured using a flow cytometer. A stimulus was applied to the cells 1 min after measuring the fluorescence intensity of resting cells, then continuous recording was started again until another stimulus was applied. To assay lactose inhibition, cells were stimulated in the presence of 30 mM lactose, and 30 mM sucrose served as the control (43). A23187 ( $Ca^{2+}$  ionophore; final concentration, 5 ng/ml; Wako) served as the positive control.

## Results

#### Induction of apoptosis by Gal-9

We examined whether Gal-9 could induce apoptosis of the cell lines. MOLT-4 was incubated with rGal-9 (1  $\mu$ M) for various incubation periods (3, 6, 12, 24, 48, and 72 h) and was stained with PI to differentiate apoptotic cells. Fig. 1A showed that rGal-9 induces the apoptosis of MOLT-4 cells in a time-dependent manner. Apoptosis was significantly, but weakly, induced by 1  $\mu$ M Gal-9 at 6 h and strongly induced after 12 h of incubation. Moreover, MOLT-4 cells were incubated with various concentrations of rGal-9 (0.03, 0.1, 0.3, and 1.0  $\mu$ M) for 24 h to clarify the dose dependency. Fig. 1B shows that apoptosis was induced in a dose-dependent fashion. At least 0.1  $\mu$ M Gal-9 was required for significant induction of apoptosis.

We also examined the proapoptotic activity of Gal-9 by annexin V staining, which detects phosphatidylserine that is externalized during apoptosis. We found that the numbers of apoptotic cells detected with annexin V were increased from 13.2 to 69.1% by 1  $\mu$ M Gal-9, confirming that Gal-9 exhibits proapoptotic activity against MOLT-4.



**FIGURE 1.** Gal-9 induces apoptosis in a time- (A) and dose- (B)-dependent manner. MOLT-4 cells were incubated with 1  $\mu$ M rGal-9 for various incubation periods (A). The cells were also incubated with various concentrations of rGal-9 for 24 h (B). The proapoptotic activity of Gal-9 against MOLT-4 was assessed by PI labeling as described. Data are the mean  $\pm$  SEM of four experiments. ANOVA was used for statistical analysis. \*,  $p < 0.05$ .

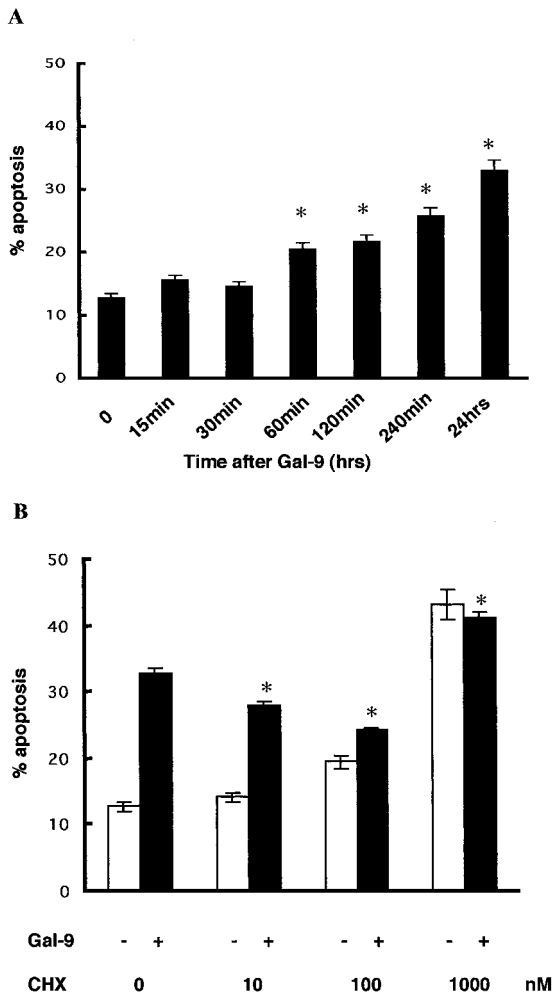
We further examined the minimal time of Gal-9 binding required to induce the apoptosis. Cells were treated with Gal-9 for various periods (0, 15, 30, 60, 120, and 240 min) and were further cultured in the absence of Gal-9 for a total of 24 h, followed by assessment of apoptosis. Fig. 2A showed that Gal-9-mediated apoptosis in MOLT-4 requires at least 60 min of Gal-9 binding to induce detectable Gal-9-mediated apoptosis, although a longer period of incubation is more effective for the induction.

Furthermore, to examine the requirement for synthesis of a Gal-9-inducible factor, cells were exposed for 24 h to various concentrations of cycloheximide (0, 10, 100, and 1000 nM) in the presence or the absence of 1  $\mu$ M Gal-9. The results were shown in Fig. 2B. Cycloheximide suppressed Gal-9-induced apoptosis in a dose-dependent manner, suggesting that Gal-9 required de novo protein synthesis to exhibit its proapoptotic activity.

#### Gal-9 causes apoptosis of various cells

We examined whether such Gal-9 proapoptotic activity is applicable to other cell lines that were cultured with or without 1  $\mu$ M rGal-9 for 24 h and assessed for apoptosis. Fig. 3A shows that the apoptosis not only of T cells but also of B cells (BALL-1), monocytes (THP-1), and myelocytes (HL-60) is induced by Gal-9.

Next, we examined the effects of lactose on the ability of Gal-9 to induce apoptosis. Fig. 3A shows that the proapoptotic activity of Gal-9 is almost completely inhibited by 30 mM lactose, but not by sucrose, indicating that  $\beta$ -galactoside binding activity is essentially required for Gal-9-induced apoptosis.



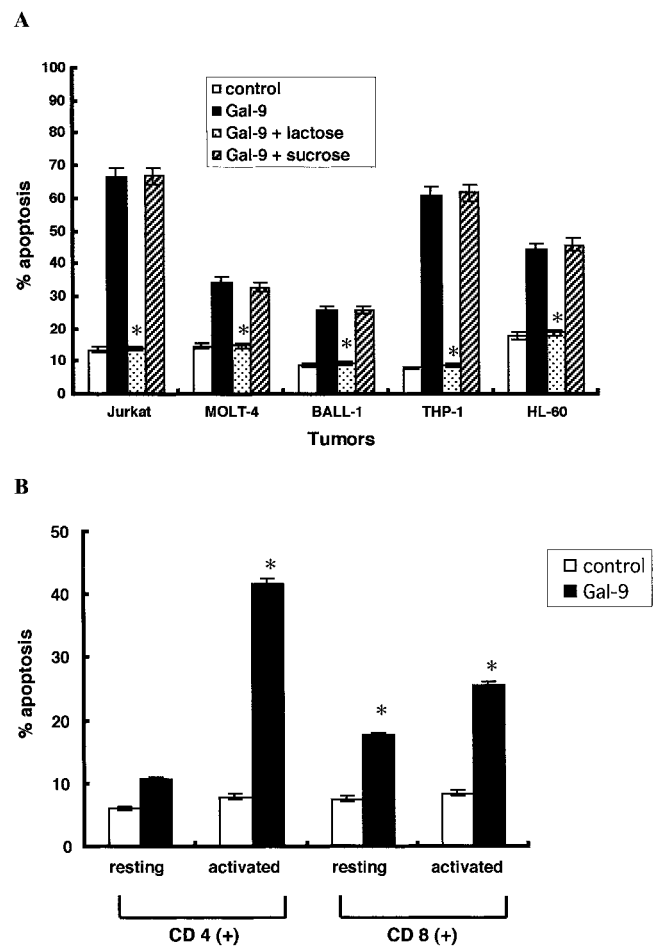
**FIGURE 2.** Gal-9-mediated apoptosis requires Gal-9 binding (A) and de novo protein synthesis (B). The proapoptotic activity of Gal-9 was assessed as described. Data are represented as the mean  $\pm$  SEM of three experiments. \*,  $p < 0.05$ .

We further assessed the Gal-9-induced apoptosis of human peripheral blood T cells separated into CD4<sup>+</sup> and CD8<sup>+</sup> T cells and activated with or without anti-CD3 Ab. The cells were then incubated in the presence of 1  $\mu$ M rGal-9. Fig. 3B shows that Gal-9 induced more obvious apoptosis in both CD3-activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells than in nonactivated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Moreover, we found that CD4<sup>+</sup> T cells were more susceptible to Gal-9 than CD8<sup>+</sup> T cells (Fig. 3B).

*Caspase inhibition*

We examined whether caspase activation is involved in Gal-9-induced apoptosis. MOLT-4 cells were incubated with the pan-caspase inhibitor, Z-VAD-FMK, then with rGal-9. Fig. 4A shows that Gal-9-induced apoptosis was almost completely suppressed by the caspase inhibitor. We also found that apoptosis induced by other stimuli, such as DEX, anti-Fas Ab, TNF- $\alpha$ , and C2 ceramide, was suppressed by the pan-caspase inhibitor (data not shown).

We then investigated other caspase inhibitors for specific upstream caspases, namely, Z-YVAD-FMK for caspase-1, Z-IETD-FMK for caspase-8, Z-LEHD-FMK for caspase-9, and Z-YVAD-FMK for caspase-10. Fig. 4A also shows that only Z-YVAD-FMK (caspase-1 inhibitor) suppressed Gal-9-induced apoptosis, whereas other caspase inhibitors for caspase-8, -9, and -10 did not. We confirmed that caspase-1 and caspase-9 inhibitors suppressed



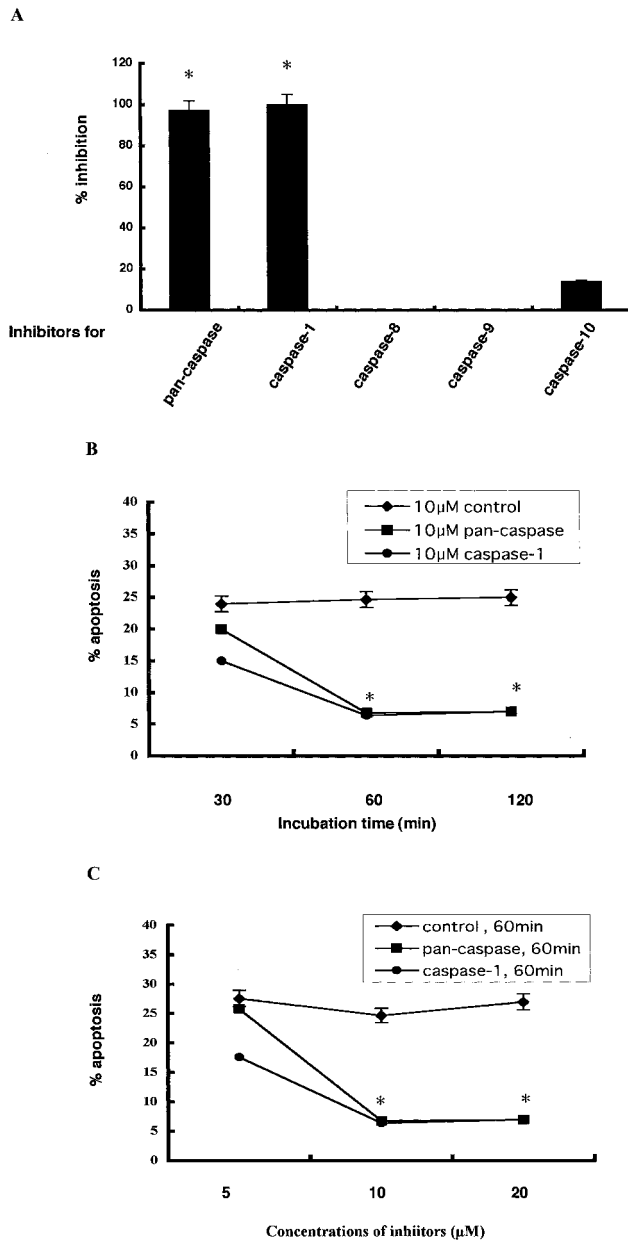
**FIGURE 3.** Gal-9 induces apoptosis of various cells. Proapoptotic activity of Gal-9 was assessed as described. A, Jurkat, MOLT-4, BALL-1, THP-1, and HL-60 were incubated with or without 30 mM lactose or sucrose, followed by 1  $\mu$ M Gal-9, then apoptotic cells were assessed by PI. □, Percentage of apoptosis without Gal-9. B, Separated CD4<sup>+</sup> or CD8<sup>+</sup> T cells were activated with anti-CD3 Ab, and proapoptotic activities of Gal-9 were assessed by PI. The percent apoptosis of the cells treated with or without Gal-9 is shown. Data are the mean  $\pm$  SEM of three experiments. \*,  $p < 0.05$ .

DEX-mediated apoptosis, that caspase-8 and caspase-10 inhibitors suppressed apoptosis induced by anti-Fas Ab and TNF- $\alpha$ , and that the caspase-9 inhibitor suppressed apoptosis induced by C2 ceramide (data not shown). Fig. 4, B and C, show that both the pan-caspase and caspase-1 inhibitors suppressed Gal-9-induced apoptosis time- and dose-dependently. Such suppression was reproducible in other cell lines (data not shown). The present results suggested that Gal-9 probably induces apoptosis through caspase-1 activation, and that caspase-8, caspase-9, and caspase-10 are not involved in Gal-9-mediated apoptosis.

*Ca<sup>2+</sup>-calpain pathway*

Since calpain, a Ca<sup>2+</sup>-dependent protease, is required for caspase-1 activation (44, 45), we examined whether calpain-caspase-1 activation is involved in Gal-9-induced apoptosis in MOLT-4. Fig. 5, A and B, show that the calpain inhibitor, Z-LLY-FMK, inhibits Gal-9-induced apoptosis time- and dose-dependently.

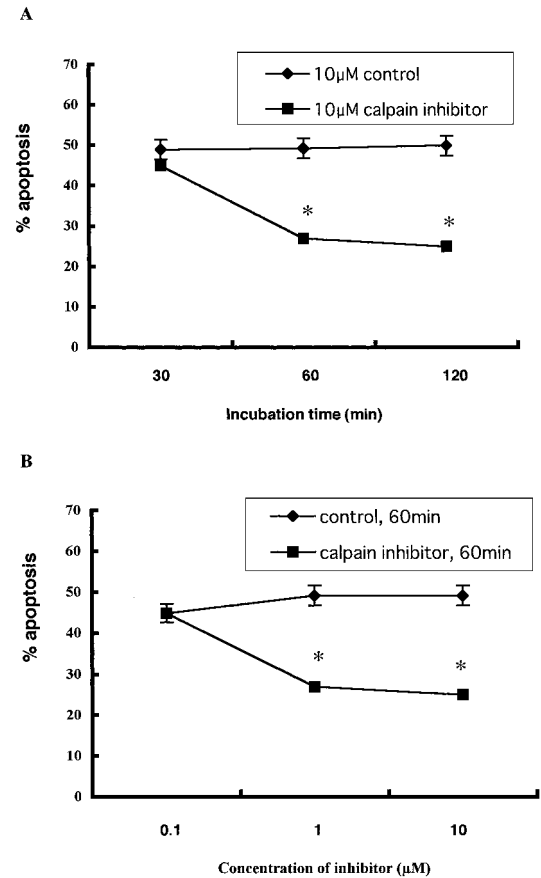
We performed Fluo-3 assays to determine whether Gal-9 causes a Ca<sup>2+</sup> influx in MOLT-4. Fig. 6 shows that a Ca<sup>2+</sup> influx was generated in MOLT-4 within 10–20 s after adding Gal-9, and that



**FIGURE 4.** Suppression of Gal-9-mediated apoptosis by caspase inhibitors (A) in a time- (B) and dose (C)-dependent manner. Cells were incubated with 10 μM Z-FA-FMK (control), Z-VAD-FMK (pan-caspase inhibitor), Z-YVAD-FMK (caspase-1 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), or Z-AEVD-FMK (caspase-10 inhibitor) for 1 h, followed by 1 μM rGal-9. Results were shown as the percent inhibition of Gal-9-induced apoptosis. Data are the mean ± SEM of three experiments. \*,  $p < 0.05$ .

lactose, but not sucrose, suppressed the Gal-9-induced  $Ca^{2+}$  influx. A23187 (final concentration, 5 ng/ml), as a control, induced an evident  $Ca^{2+}$  influx in MOLT-4 (Fig. 6). We also confirmed the involvement of calpain and  $Ca^{2+}$  influx in the Gal-9-induced apoptosis of other cell lines (data not shown).

$Ca^{2+}$  signaling is originated with extracellular and intracellular  $Ca^{2+}$  flux. To examine whether  $Ca^{2+}$  flux is actually required for Gal-9-mediated apoptosis, we assessed the apoptosis in MOLT-4 preincubated with either the  $IP_3$  inhibitor, 2-APB, or the intracellular  $Ca^{2+}$  chelator, BAPTA-AM. To block extracellular  $Ca^{2+}$  influx, apoptosis was induced by Gal-9 in the presence or the absence of the extracellular  $Ca^{2+}$  chelator, EGTA. Fig. 7 shows that



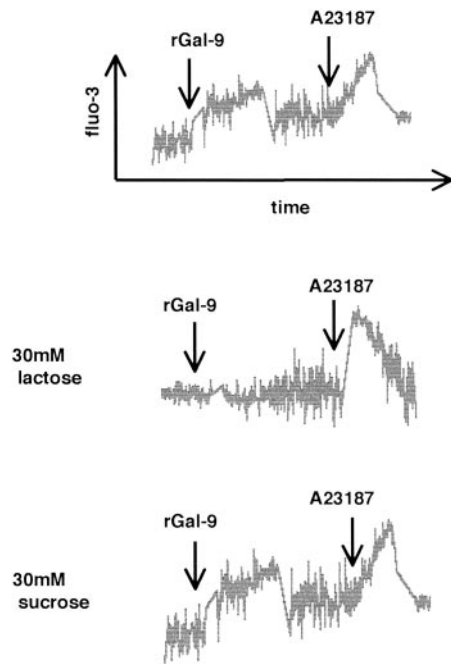
**FIGURE 5.** Calpain inhibitor inhibits Gal-9 induced apoptosis. Cells were incubated with calpain inhibitor (Z-LLY-FMK) or the control (Z-FA-FMK) at the indicated concentration for 1 h, followed by 1 μM rGal-9. Results were shown as percent inhibition of Gal-9-induced apoptosis. Data show the mean ± SEM of three experiments. \*,  $p < 0.05$ .

both BAPTA-AM and 2-APB at least partially suppressed Gal-9-induced apoptosis. In contrast, little suppression was induced by EGTA (Fig. 7). Thus, intracellular, but not extracellular,  $Ca^{2+}$  flux may be involved in Gal-9-mediated apoptosis.

## Discussion

Numerous reports concerning galectin-induced apoptosis have been published after the description of Gal-1-induced T cell apoptosis (24). To date, other galectins, such as Gal-7 (28–30), Gal-8 (46), and Gal-12 (47), have been shown to be proapoptotic, whereas Gal-3 functions as anti-apoptotic factor (48). Like other galectins, Gal-9 can induce the apoptosis of thymocytes and melanoma cells (12, 15). However, little is known about the pathway involved in Gal-9-induced apoptosis. We first assessed the proapoptotic activity of Gal-9 on various types of cells. The results showed that Gal-9 induced the apoptosis of all immune cells tested, including MOLT-4 and Jurkat cells (Figs. 1 and 3), indicating that Gal-9 functions as a proapoptotic factor for many cell types.

With respect to other stimuli, such as GC, anti-Fas Ab, and oxidative stress, the apoptotic pathways induced by respective stimulus have already been analyzed. For instance, anti-Fas Ab binds to Fas ligand and induces apoptosis through caspase-8 activation (19). Etoposide causes DNA damage and induces mitochondrial cytochrome *c* release, caspase-9 activation, and finally apoptosis (47). GC activates  $Ca^{2+}$ -dependent endonuclease and caspase-1 to induce cell death (16–18). Current studies have suggested that GC-induced apoptosis primarily involves caspase-9 initiation (49–51). In the present studies we confirmed that inhibitors

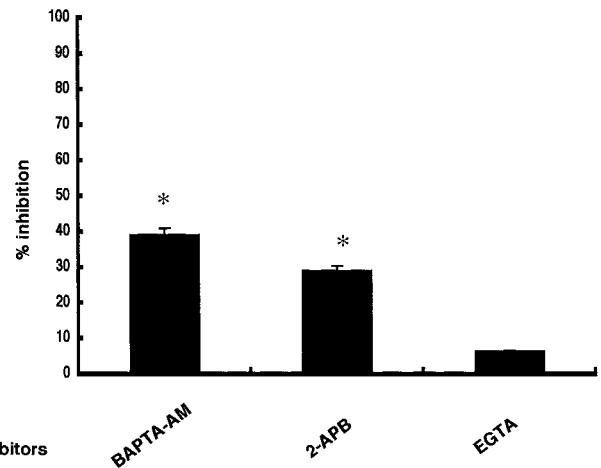


**FIGURE 6.** Gal-9-induced  $\text{Ca}^{2+}$  mobilization. Intracellular  $\text{Ca}^{2+}$  levels were measured in Fluo-3-loaded cells. Arrows indicate the time of stimulation with rGal-9 (6  $\mu\text{M}$ ) and A23187 as the positive control. For lactose inhibition assay, cells suspended in 30 mM lactose were stimulated with rGal-9 (6  $\mu\text{M}$ ) and A23187. Cells were also suspended in 30 mM sucrose as a control. Representative data from four experiments are presented.

for both caspase-1 and caspase-9 suppress DEX-mediated apoptosis (data not shown).

The present study found that Gal-9 induces apoptosis following  $\text{Ca}^{2+}$  mobilization (Fig. 6) and calpain (Fig. 5) and caspase-1 activation (Fig. 4), indicating that Gal-9 uses the  $\text{Ca}^{2+}$ -calpain-caspase-1 pathway to induce apoptosis. Furthermore, Gal-9-induced apoptosis was inhibited by either the intracellular  $\text{Ca}^{2+}$  chelator, BAPTA-AM, or the  $\text{IP}_3$  inhibitor, 2-APB, but not by the extracellular  $\text{Ca}^{2+}$  chelator, EGTA (Fig. 7). These results suggest that the pathway of Gal-9-mediated apoptosis differs from that induced by other stimuli, such as GC, anti-Fas, and etoposide.

It is important to compare Gal-9-mediated apoptosis with that mediated by other galectins. The Gal-1 gene is overexpressed during GC-induced cell death (52). Gal-1-induced apoptosis does not require an increase in intracellular  $\text{Ca}^{2+}$  (28), and Gal-1 fails to induce the apoptosis of Jurkat cells (24), although it induces  $\text{Ca}^{2+}$  mobilization in Jurkat cells (53). The apoptotic pathway induced by Gal-9 may differ from that induced by Gal-1, because Gal-9 induces the apoptosis of many types of cells, including Jurkat cells (Fig. 3A), and  $\text{Ca}^{2+}$  influx is required for Gal-9-mediated apoptosis (Fig. 7). Gal-1-mediated apoptosis occurs very rapidly; it is detected after 30 min of Gal-1 binding (24, 54), indicating that no de novo protein synthesis may be required, although Rabinovich and co-workers (55) have suggested that transcription is required for Gal-1-induced apoptosis. In contrast, Gal-9 may require a longer binding period and de novo protein synthesis to exhibit proapoptotic activity differing from Gal-1-mediated apoptosis (Fig. 2, A and B). However, the present data may not absolutely support the requirement of protein synthesis for Gal-9-mediated apoptosis, since the complete suppression was not induced at cycloheximide levels that do not increase background death (Fig. 2B). Further studies are therefore required to ascertain the requirement of protein synthesis for Gal-9-mediated cell death, but it is at least clear that death occurs with the machinery present in the cell.



**FIGURE 7.** Suppression of Gal-9-induced apoptosis by inhibition of  $\text{Ca}^{2+}$  mobilization. Cells were incubated with or without 30  $\mu\text{M}$  BAPTA-AM or 10  $\mu\text{M}$  2-APB, then apoptosis was induced with 1  $\mu\text{M}$  rGal-9. For the  $\text{Ca}^{2+}$ -free medium assay, cells were suspended in 1.8 mM  $\text{Ca}^{2+}$ -containing medium or in 4 mM EGTA in  $\text{Ca}^{2+}$ -free medium. Results were shown as the percent inhibition of Gal-9-induced apoptosis. Data represent the mean  $\pm$  SEM of three experiments. \*,  $p < 0.05$ .

Gal-7 also appears to be a proapoptotic galectin that functions intracellularly upstream of c-Jun N-terminal kinase activation and cytochrome *c* release (29, 30). Mitochondrial events may not be involved in Gal-9-induced apoptosis, because an inhibitor for caspase-9 essentially required for mitochondria-associated apoptosis (21) did not suppress Gal-9-mediated apoptosis (Fig. 4A). Further signal transduction studies are required to compare precisely Gal-9-mediated cell death and those mediated by other stimuli.

We have found that the release of Gal-9 from Jurkat cells is induced by PMA stimulation (56). Furthermore, Jurkat cells release negligible Gal-9 unless stimulated by PMA, whereas they express Gal-9 on their surface (56). It thus becomes important to clarify the reason why Jurkat cells expressing Gal-9 on the cell surface can survive. Two possibilities are that exogenously added Gal-9 or released Gal-9, but not surface-bound Gal-9, is involved in the apoptosis, or that exogenously added Gal-9 accesses different cell surface receptors than endogenous Gal-9.

It is well known that activated T cells undergo apoptosis more easily than resting T cells (57). Indeed, we have found that Gal-9 induces apoptosis in activated  $\text{CD4}^+$  and  $\text{CD8}^+$  T cells in human peripheral blood cells, whereas it does not induce apoptosis of resting T cells (Fig. 3B). These results suggest that cell surface Gal-9 binding molecules responsible for apoptosis are expressed during stimulation with anti-CD3. Tuchiya et al. (58) have shown that Gal-9 induces the selective apoptosis of activated  $\text{CD8}^+$ , but not  $\text{CD4}^+$ , splenic T cells in nephritic rats. However, Gal-9 induces much more apoptosis in activated  $\text{CD4}^+$  T cells than in  $\text{CD8}^+$  T cells in human peripheral blood cells (Fig. 3B). This discrepancy may be ascribed to the differences in tissue or animal, although further studies are required to ascertain the reason for this.

From the results of the present experiments, human Gal-9 induces the apoptosis of various immune cells, including activated  $\text{CD4}^+$  and  $\text{CD8}^+$  T cells, through the  $\text{Ca}^{2+}$ -calpain-caspase-1 pathway, suggesting that Gal-9 plays a role not only in thymocyte maturation, but also in immunomodulation, by inducing the apoptosis of those cells.

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