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Rosmarinic Acid Induces p56\(^{\text{ck}}\)-Dependent Apoptosis in Jurkat and Peripheral T Cells via Mitochondrial Pathway Independent from Fas/Fas Ligand Interaction

Yun-Gyoung Hur,* Yungdae Yun,† and Jonghwa Won²*

Apoptosis is one way of controlling immune responses, and a variety of immunosuppressive drugs suppress harmful immune responses by inducing apoptosis of lymphocytes. In this study we observed that rosmarinic acid, a secondary metabolite of herbal plants, induced apoptosis in an p56\(^{\text{ck}}\) (Lck)-dependent manner; Lck\(^{+}\) Jurkat T cells undergo apoptosis in response to rosmarinic acid (RosA) treatment, whereas Lck\(^{-}\) Jurkat subclone J.CaM1.6 cells do not. J.CaM1.6 cells with various Lck mutants indicated that Lck SH2 domain, but not Lck kinase activity, was required for RosA-induced apoptosis. RosA induced apoptosis in the absence of a TCR stimulus, and this was not prevented by interruption of the Fas/Fas ligand interaction. Instead, RosA-mediated apoptosis involved a mitochondrial pathway as indicated by cytochrome \(c\) release and the complete blockage of apoptosis by an inhibitor of mitochondrial membrane depolarization. Both caspase-3 and -8 were indispensable in RosA-induced apoptosis and work downstream of mitochondria and caspase-9 in the order of caspase-9/caspase-3/caspase-8. In freshly isolated human PBMC, RosA specifically induced apoptosis of Lck\(^{+}\) subsets such as T and NK cells, but not Lck-deficient cells, including B cells and monocytes. Moreover, RosA’s ability to kill T and NK cells was restricted to actively proliferating cells, but not to resting cells. In conclusion, Lck-dependent apoptotic activity may make RosA an attractive therapeutic tool for the treatment of diseases in which T cell apoptosis is beneficial. The Journal of Immunology, 2004, 172: 79–87.

A

poptosis, or cell suicide, is essential for normal lymphocyte development and homeostasis in the immune system (1). It involves a series of typical biochemical and morphological changes resulting in chromatin condensation and phagocytosis to ensure the safe, noninflammatory removal of cellular debris. The controlling mechanisms of cell death in the immune system can be classified into two categories. One is death by instruction resulting from death receptor-initiated signal transduction (Fas, TNF-\(\alpha\) receptor), and the other is death by neglect caused by the loss of extracellular survival signals (1). Receptor-mediated apoptosis is important during development, in negative selection of T lymphocytes (2), and in p53-mediated apoptosis (3). The death receptor-mediated apoptosis associates with the recruitment of adapter proteins and procaspase molecules to the membrane-proximal region, forming the complex called death-inducing signaling complex, where the aggregated procaspases are activated. Recent reports also demonstrated that death receptor-initiated apoptosis associates with mitochondrial dysfunction in several cases. Mitochondria play a critical role in the process of cell death induced by cytotoxic drugs and by depletion of extracellular survival signals. One of major events in mitochondrial dysfunction is the loss of mitochondrial transmembrane potential and the subsequent release of apoptosis-inducing factors such as cytochrome \(c\) and reactive oxygen species (ROS)\(^{\text{3}}\) (4). Cytochrome \(c\) binds to Apaf-1 and procaspase-9 and, by forming an apoptosome, induces caspase-9 activation and results in activation of downstream caspases, including caspase-2, -3, -6, -7, -8, and -10. Mitochondrial membrane depolarization is triggered by various apoptosis-inducing factors, such as calcium, ceramide, and saturated fatty acid palmitate, and by death signals transmitted from death receptors, including Fas and TNF-\(\alpha\) receptors (5). Proteins of the Bcl-2 family also affect the integrity of mitochondria (6). The Bcl-2 family consists of proapoptotic, apoptosis executioners (Bax, Bak), BH3-only proteins (Bid, Bim), and antiapoptotic proteins (Bcl-2, Bcl-\(\text{X}_L\)). Bax oligomerization triggers the disruption of mitochondrial membrane potential (MMP), resulting in the release of various apoptosis-inducing factors (7, 8). In contrast, Bcl-2 and Bcl-\(\text{X}_L\) bind to mitochondria and inhibit the release of cytochrome \(c\) (9). Bax oligomerization and MMP disruption were prevented by the overexpression of antiapoptotic proteins in the Bcl-2 family (10, 11).

Drug-induced apoptosis of lymphocytes to eliminate residual or undesired immune responses is of significant interest in pharmaceutical research in the area of allograft transplantation and autoimmune diseases. Frequently, more than one mechanism is involved in drug-induced immunosuppression, and apoptosis has recently been recognized as one of these mechanisms. Immunosuppressive tautomycin and FK506 were reported to induce apoptosis of lymphocytes as well as inhibit TCR-induced signaling and IL-2R-mediated signaling, respectively (12, 13). Several metabolic inhibitors, including methotrexate and mycophenolic acid of mycophenolate mofetil, also elicited apoptosis of lymphocytes (14, 15) and methotrexate-induced apoptosis of activated human PBL.

\(^{3}\) Abbreviations used in this paper: ROS, reactive oxygen species; AICD, activation-induced cell death; CAPE, caffeic acid phenethyl ester; DHR123, dihydrorhodamine123; FasL, Fas ligand; MMP, mitochondrial membrane potential; PS, phosphatidyl serine; RosA, rosmarinic acid; PP2, 4-amino-5-(4-chlorophenyl)-7-(4-butylypyrazolo [3,4-d]pyramidine.

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occurred independently from Fas/Fas ligand (FasL) interaction. Several naturally occurring polyphenols, such as curcumin, caffeic acid phenethyl ester (CAPE), resveratrol, and the Chinese herbal medicine *Tripterygium wilfordii* Hook-F (TWHF, or a thunder god vine), have been reported to have apoptotic function, and their efficacy in the treatment of autoimmune diseases and leukemia has been demonstrated (16–20).

Rosmarinic acid (RosA), frequently found as a secondary metabolite in herbs and medicinal plants, has exhibited antimicrobial, antiviral, antioxidant, and anti-inflammatory activities (21–24). Previously, we demonstrated that RosA inhibited TCR-induced Ca²⁺ flux and IL-2 promoter activation by inhibiting inducible T cell kinase and phospholipase Cy-1 activity (25, 26). As 1) RosA was screened out as an Lck SH2 inhibitor in ELISA, and 2) RosA did not inhibit Lck kinase activity, we suggested that the inhibitory activity of RosA on TCR-induced signaling and subsequent proliferation is ascribed from its interaction with the Lck SH2 domain (25). Besides RosA-mediated inhibition of TCR signaling, RosA suppressed Jurkat T cell proliferation in the absence of TCR stimulus, and this prompted us to expect apoptosis to be another suppressive mechanism of T cell function. In this study we demonstrated that RosA induces apoptosis in an Lck-dependent manner. RosA-induced apoptosis involves the mitochondrial apoptotic pathway where caspases act as apoptosis executioners rather than as mitochondrial dysfunction inducers. Lck appears to work upstream of mitochondria, but not downstream of Fas and FasL interaction. These results indicate participation of Lck in RosA-induced apoptosis and potential connection of Lck with the mitochondrial pathway. Finally, RosA’s ability to induce Lck-dependent apoptosis may make RosA a good candidate for the treatment of T cell-mediated pathologic conditions such as rheumatoid arthritis and T cell leukemia.

**Materials and Methods**

**Cell cultures**

Jurkat (T lymphocyte; acute T cell leukemia) and JCaM1.6 (Jurkat-derived, Lck-deficient T cell line) were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and streptomycin (Life Technologies, Gaithersburg, MD), and 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO).

**FACS analysis of apoptotic cells and Fas/FasL expression**

To detect externalized phosphatidylserine (PS) as an early indication of apoptosis, cells (1 × 10⁶ cells/ml) were incubated with 30 μM RosA (Indofine, Somerville, NJ) for the indicated time at 37°C. Cells were then harvested, stained with Annexin V FITC according to the manufacturer’s protocol (Clontech Laboratories, Palo Alto, CA), and analyzed with FACStation (BD Biosciences, Mountain View, CA). Where required, a CD3 stimulus was given by incubating cells in the plates previously coated with anti-CD3ε Ab (10 μg/ml in PBS; BD Pharmingen, San Diego, CA). For the detection of Fas and Fasl expression, Jurkat cells were stained primarily with 1 μg of rabbit polyclonal anti-Fas or anti-Fasl Ab (Santa Cruz Biotechnology, Santa Cruz, CA), followed by staining with FITC-conjugated goat anti-rabbit Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and analyzed using a FACStation. For inhibition of the Fas/FasL interaction, Fas/Fc chimeric protein (200 ng/ml; R&D Systems, Minneapollis, MN) or neutralizing anti-Fas (ZB4) Ab (250 ng/ml; Upstate Biotechnology, Lake Placid, NY) was added to Jurkat cells before RosA treatment and incubated for the entire 48-h incubation period. Human rFasL and activating anti-Fas (CH11) Ab were obtained from R&D Systems and Upstate Biotechnology, respectively. To assess the involvement of caspases in RosA-induced apoptosis, Jurkat cells were incubated with caspase inhibitors (Z-VA-D-fmk, Z-DEVD-fmk, Z-IETD-fmk; Clontech Laboratories) for 3 h before and throughout the entire incubation period with RosA.

**Transfection**

JCaM1.6 cells (5 × 10⁵) were transfected with 10 μg of pcDNA3.1, pcDNA3.1-Lck, pcDNA3.1-LckK154, pcDNA3.1-LckR273, or pcDNA3.1-LckF505 using 80 μl of Lipofectamine according to the manufacturer’s description (Life Technologies). The transfected cells were cultured for ~10 passages in growth medium containing 400 μg/ml geneticin (Life Technologies). To determine the effect of Bcl-2 overexpression on blocking apoptosis, Jurkat cells were transfected with vector only or with pcDNA3.1-Lck or pcDNA3.1-Bcl-2 (obtained from Dr. P.-G. Sun; University of Science and Technology, Pohang, Korea) and analyzed for apoptosis as described above.

**Measurement of ROS**

ROS production was measured by flow cytometry as described previously (27). Cells were washed with Dulbecco’s PBS, resuspended in 1 ml of Dulbecco’s PBS, and incubated with 1 μM dihydrothoridine 123 (DHR123; 5 mM stock in DMSO; Molecular Probes, Eugene, OR) for 1 h at 37°C. After thorough washing, Jurkat cells were resuspended in Dulbecco’s PBS. Rhodamine 123 fluorescence intensity resulting from oxidation of DHR123 was measured with a FACSscan flow cytometer with the detection wavelength between 515 and 550 nm after excitation at 488 nm.

**Subcellular fractionation**

The subcellular fractionation of Jurkat cells was performed as described previously (7, 8). After the incubation with RosA (30 μM) for 24 or 48 h at 37°C, cells (3 × 10⁶) were harvested by centrifugation at 1,500 × g for 5 min and washed once with PBS. The pellet was lysed in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES (pH 7.5), and protease inhibitor mixture (one tablet per 50 ml of buffer)) and spun at 2,500 × g for 10 min at 4°C. The supernatant was further fractionated by centrifugation at 15,000 × g for 15 min at 4°C. The pellet corresponding to the mitochondrial fraction was suspended in the mitochondrial buffer containing 4 mM MgCl₂, 0.5% Nonidet P-40, and 5 mM CaCl₂. The supernatant was spun at 100,000 × g for 1 h at 4°C, and the supernatant was collected as a cytosolic fraction. Protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA).

**SDS-PAGE and Western blot analysis**

For the detection of Bax and cytochrome c, 30 μg of protein from both the cytosolic and mitochondrial fractions (obtained as above) were run on 15% SDS-PAGE. To detect the expression of Bcl-2, Bcl-xL, caspase-3, caspase-8, caspase-9, Fas, and Fasl, Jurkat cells (3 × 10⁶) were lysed in RIPA B buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, and protease inhibitor mixture) and spun at 15,000 × g for 30 min at 4°C, and 50 μg of supernatants were loaded onto 12% SDS-PAGE. Proteins were transferred into the nitrocellulose membrane (Bio-Rad). Membranes were blocked with PBS with 0.05% Tween 20 (PBS-T) containing 5% skim milk overnight at 4°C and probed with corresponding Abs for 1 h at room temperature at the suggested concentration in the manufacturer’s protocol; rabbit polyclonal Bcl-2, Bcl-xL, and Bax Abs (Santa Cruz Biotechnology) were used at a 1/500 dilution; rabbit polyclonal caspase-3 and caspase-8 Ab (BD PharMingen) and caspase-9 Ab (Cell Signaling Technology, Beverly, MA) were used at a 1/1000 dilution, rabbit polyclonal Fas and Fasl Abs were used at a 1/500 dilution, and cytochrome c mAb (BD Pharmingen) was used at a 1/1000 dilution. The membranes were then washed with PBS-T and incubated with HRP-conjugated anti-rabbit (0.5 μg/ml) or anti-mouse Abs (0.5 μg/ml; Transduction Laboratories, Lexington, KY) for 1 h at room temperature. After washing with PBS-T, membranes were developed using the ECL system (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Where indicated, membranes were stripped by incubation in stripping solution (100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7)) at 60°C for 30 min with intermittent agitation and reprobed with anti-β-actin mAb (1/3000; Sigma-Aldrich) according to the general Western blot method described above.
Isolation, activation, and apoptosis analysis of human PBMC subsets

PBMCs were isolated from the peripheral blood of normal healthy donors by centrifugation over Ficoll-Paque Pluss (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and streptomycin, and 50 µM 2-ME. CD4⁺ or CD8⁺ T cells, CD56⁻ NK cells, CD19⁺ B cells, and CD14⁺ monocytes were isolated from human PBMC by automatic MACS (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were incubated with magnetic beads with corresponding Abs (Miltenyi Biotec) for 15 min at 4°C. After being washed, cells were isolated via positive selection by passing through the separation column in autoMACS. PBMC subpopulations were checked for purity by FACS analysis using PE-labeled anti-CD3, -CD56, or -CD14 or CyChrome-labeled CD19 Ab (BD Pharmingen). Consequent cells were of >95% purity for CD3⁺ (mixtures of CD4⁺ and CD8⁺ cells) or CD56⁻ cells, and 85% purity for CD19⁺ and CD14⁺ populations.

Total human PBMCs or purified subsets of PBMC were incubated with or without PHA (5 µg/ml), IL-2 (50 U/ml; Roche, Mannheim, Germany) and LPS (10 µg/ml) to stimulate T cells, NK cells, B cells, and monocytes, respectively (Sigma-Aldrich). Cells were incubated in the presence or the absence of RosA for 24–48 h. To analyze apoptosis, cells were stained with FITC-conjugated annexin V either with or without appropriately labeled anti-CD3 or -CD19 Ab (BD Pharmingen) for 15 min at room temperature. The cell suspension was washed once in PBS and then directly analyzed on a flow cytometer.

Results

RosA induces apoptosis in Jurkat cells independent of CD3 stimulation

Cells undergoing apoptosis show characteristic changes such as nuclear condensation, DNA fragmentation, and translocation of PS to the outer leaflets of the plasma membrane (28). We measured apoptosis by annexin V staining of PS following RosA treatment. Jurkat cells were incubated with various concentrations of RosA (3–30 µM) for 24 h. RosA induced apoptosis in Jurkat cells at a concentration of 30 µM (Fig. 1A). Jurkat cells not exposed to RosA were incubated with the same amount of DMSO, but apoptotic changes (ascribed to DMSO treatment) were not detected. After 24 h of RosA treatment, ~30–50% of Jurkat cells displayed apoptosis (Fig. 1, A and B), and at least 48 h of incubation with RosA was required to induce apoptosis in almost 80–100% of the cells (Fig. 1B). To determine whether RosA-induced apoptosis is dependent on CD3 stimulation, Jurkat cells were incubated with RosA in the presence or the absence of anti-CD3 Ab. CD3 stimulation alone caused activation-induced cell death (AICD; Fig. 1C, upper panel, thin line). RosA-induced apoptosis in a similar portion of cells independent of CD3 stimulation (Fig. 1C, lower panel).

RosA-mediated apoptosis is Lck SH2 dependent, but is not dependent on Lck kinase activity

In our previous study we observed that RosA inhibits the proliferation of Jurkat cells, but not Lck-deficient J.CaM1.6 cells (25). As this Lck-dependent proliferation inhibition was observed in the absence of TCR stimulation, we assumed that another mechanism might be inhibiting Jurkat T cell proliferation besides blocking TCR-induced signaling (25, 26). We looked for the possible participation of Lck in RosA-induced apoptosis by comparing RosA-mediated apoptosis in Jurkat and J.CaM1.6 cells. RosA selectively induced time-dependent apoptosis in Jurkat cells, but not in J.CaM1.6 cells (Fig. 2A, left panel). Furthermore, Lck reconstitution made J.CaM1.6 cells vulnerable to RosA-induced cell death, whereas vector-transfected J.CaM1.6 cells are still resistant to RosA-induced apoptosis (Fig. 2A, right panel).

To determine the Lck domain of importance in RosA-induced apoptosis, J.CaM1.6 cells were transfected with various Lck mutants and determined for susceptibility to RosA-induced apoptosis. Reconstitution with the Lck kinase mutant LckR273 made J.CaM1.6 cells susceptible to RosA-induced apoptosis just like wild-type Lck, whereas Lck SH2 mutant LckK154 (unable to bind phosphotyrosine and, therefore, cannot make molecular interactions) did not (29) (Fig. 2B). This suggests that Lck kinase activity is dispensable in RosA-induced apoptosis, whereas Lck SH2 domain is indispensable. The same results were obtained in single J.CaM1.6 clones with stably expressed Lck mutants (data not shown). Moreover, an Src kinase-specific inhibitor, 4-amino-5-[(4-chlorophenyl)-7-(-t-butyl)pyrazolo[3,4-d]pyramidine (PP2), did not prevent RosA-induced apoptosis even at the concentration of 10 µM, suggesting that Lck kinase activity is not required (data not shown). PP2 selectively inhibits Lck kinase activity with a 50% inhibitory concentration of 4 nM by binding a deep, hydrophobic pocket adjacent to the ATP-binding site of the Lck kinase domain (30, 31).

As LckK154 itself did not induce apoptosis in the absence of RosA, it is not likely that RosA-induced apoptosis results from interrupting intermolecular interactions through the SH2 domain. Not only intermolecular interactions but also the Lck SH2 domain regulates Lck kinase activity by binding to its phosphotyrosine residue at 505 (Y505) (32). To eliminate the possibility that RosA-induced apoptosis involves breaking the intramolecular interactions, J.CaM1.6 cells were transfected with LckFS505. LckFS505, with Y505 substitution to F, cannot make intramolecular interaction and therefore has constitutively active kinase activity. Reconstitution with LckFS505 did not lead J.CaM1.6 cells to apoptosis in the absence of RosA and did not inhibit or augment RosA-induced apoptosis (Fig. 2B). These data further confirms that RosA-induced apoptosis result neither from activated Lck kinase activity nor from interruption of intra- and intermolecular interactions through the Lck SH2 domain. Instead, these data suggest that direct molecular interaction of RosA with Lck SH2 domain itself is pivotal in RosA-induced apoptosis. More study is required to unveil the detailed mechanism.

An Lck-dependent apoptosis was not observed in other polyphenolic compounds, such as curcumin, resveratrol, and CAPE (17, 18, 33), at the range of 12.5–30 µM (Fig. 2D; data not shown).

Thus, Lck-dependent apoptosis is not a common phenomenon of polyphenols; this is a unique characteristic that discriminates RosA from other polyphenolic compounds.
RosA induces Fas and FasL expression, but Fas/FasL interaction is not required for RosA-mediated apoptosis

RosA induced Fas and FasL expression, as indicated by FACS and Western blot analysis (Fig. 3, A and B). To determine whether the Fas/FasL interaction is required for RosA-mediated apoptosis, Jurkat cells were treated with RosA in the presence of various Fas/FasL interaction inhibitors. Fas:Fc is a chimeric extracellular domain of Fas fused to the Fc portion of the Ab (34), and ZB4 is a Fas-neutralizing Ab (35). Interruption of the Fas/FasL interaction by Fas:Fc or ZB4 did not inhibit RosA-mediated apoptosis, suggesting that the Fas/FasL interaction is dispensable in RosA-mediated apoptosis (Fig. 3C). Fas oligomerization induced by either FasL or the Fas-cross-linking Ab CH11 normally elicited apoptosis, which was completely blocked by the chimeric Fas:Fc protein or the Fas-neutralizing Ab ZB4 (Fig. 3D). Therefore, both Fas:Fc and ZB4 function properly at the concentrations used in this study, and their inability to block RosA-mediated apoptosis is not ascribed from malfunction.

RosA induces apoptosis through the mitochondrial pathway

The mitochondrial pathway has been implicated in apoptosis elicited by a variety of sources, including oxidative stress, drugs, and death receptor oligomerization (36, 37). To determine the involvement of mitochondria in RosA-induced apoptosis, Jurkat cells were incubated with RosA, and mitochondrial membrane depolarization was monitored with JC-1 dye (38, 39). In the absence of RosA, Jurkat cells displayed red fluorescence, but RosA shifted cells to diffuse green fluorescence area, indicating that mitochondrial membrane depolarization occurred. Incubation of Jurkat cells with bongkrekic acid, a mitochondrial membrane depolarization inhibitor, prevented RosA-induced shifts of Jurkat cells to green fluorescence area (Fig. 4A, left panel). Furthermore, bongkrekic acid also inhibited RosA-induced apoptosis, as marked by the lack of annexin V staining (Fig. 4A, right panel). This indicates that RosA induces MMP disruption, and this is critical for RosA-induced apoptosis.

Next, we examined downstream events of MMP disruption. Mitochondria were fractionated out of the cytosol, and cytochrome c expression was monitored. Cytochrome c began to release to cytosol at 24 h and almost completely released after 48-h incubation with RosA (Fig. 4B). In addition, procaspase-9 was cleaved to its active forms in a time-dependent manner (Fig. 4C). As procaspase-9 is activated mainly by the Apaf-1/cytochrome c complex (40), this finding supports the idea that RosA-mediated apoptosis involves the mitochondrial apoptotic pathway. ROS is one of the

FIGURE 2. RosA-induced apoptosis is dependent on the Lck SH2 domain, but not its kinase activity, a genuine character only observed in RosA among polyphenolic compounds. A, Jurkat and J.CaM1.6 cells transfected with or without vector or Lck were cultured with RosA (30 μM) for 48 h. Cells were stained with Annexin VFITC and analyzed by flow cytometry. B, J.CaM1.6 cells were transfected with Lck wild type or mutants (K154, R273, F505). Then the cells were cultured with RosA (30 μM) for 48 h. Cells were stained with Annexin VFITC and analyzed by flow cytometry. C, Cell lysates (1 × 10⁶) were measured for Lck expression by Western blot analysis using anti-Lck Ab. D, Jurkat and J.CaM1.6 cells were incubated with curcumin (30 μM), resveratrol (30 μM), or CAPE (30 μM) for 48 h and stained with Annexin V FITC. The thin line represents untreated cells, and the thick line represents cells incubated with the various phenolic compounds.

FIGURE 3. RosA induces Fas/FasL expression, but the Fas/FasL interaction is dispensable in RosA-induced apoptosis. A, Jurkat cells were stained for Fas and FasL expression using FITC-labeled Abs. Jurkat cells with or without RosA treatment are indicated as thick or thin lines, respectively. B, Jurkat cells were lysed in RIPA B buffer and spun, and supernatants (50 μg) were used for SDS-PAGE and Western blot analysis for Fas or FasL expression. The membranes were stripped and reprobed with anti-β-actin Ab. C, Jurkat cells were incubated with RosA (30 μM) in the presence or the absence of the Fas/FasL interaction inhibitors, soluble Fas/Fc chimeric protein (200 ng/ml) or Fas neutralizing Ab, ZB4 (250 ng/ml) for 48 h and were analyzed for apoptosis by staining with FITC-labeled annexin V. The thin and thick lines represent untreated and RosA-treated Jurkat cells, respectively. D, Jurkat cells were preincubated with or without RosA, FasL interaction inhibitors (Fas:Fc chimeric protein or ZB4) and incubated with human rFasL (100 ng/ml) or with Fas-cross-linking Ab CH11 (100 ng/ml) for 48 h. Jurkat cells were stained with Annexin VFITC for apoptosis.
How p56Lck works upstream of caspase-3 and caspase-8 in RosA-mediated apoptosis

How p56Lck contributes to various Lck-dependent apoptosis has not been determined. Lck has been reported to work upstream of apoptosis-inducing factors released from mitochondria. ROS can elicit mitochondrial membrane depolarization, but also can be released from mitochondria and participate in the apoptotic degradation phase downstream of the mitochondrial apoptotic pathway (37). To determine whether ROS is generated and involved in RosA-mediated apoptosis, we measured the oxidation level with DHR123. Colorless DHR123 is readily oxidized to fluorescent rhodamine123 by ROS and is therefore frequently used to detect ROS generation (37). Over 95% of DHR123 was oxidized after RosA treatment, indicating that ROS is generated in response to RosA (Fig. 4D).

Mitochondrial membrane permeability is regulated by the equilibrium state between proapoptotic and antiapoptotic members of the Bcl-2 family. Mitochondrial apoptotic pathways can be negatively regulated by the overexpression of antiapoptotic proteins such as Bcl-2 and Bcl-xL (9, 41). In contrast, Bax oligomers translocate to the mitochondrial membrane and induce cytochrome c release, presumably by forming large channels (42, 43). We determined the possibility that RosA elicits MMP disruption by regulating the expression of Bcl-2 family members. The Bcl-2 expression level decreased starting 24 h after RosA treatment and was completely abrogated at 48 h, whereas Bcl-xL did not change significantly (Fig. 5, A and B). Overexpression of Bcl-2 in Jurkat cells prevented RosA-induced apoptosis (Fig. 5C), suggesting that RosA induces apoptosis by disrupting MMP. However, we cannot conclude whether RosA induces mitochondrial dysfunction by down-regulating Bcl-2 expression or through another signaling pathway. In the absence of RosA, Bax was distributed equally in cytoplasm and mitochondrial membrane. A 48-h exposure to RosA resulted in greatly reduced cytoplasmic Bax levels, whereas Bax levels in the mitochondrial membrane did not change or increased slightly (Fig. 5D). This preferential disappearance of cytoplasmic Bax was previously observed in staurosporine-treated HeLa cells (7).

Caspases work downstream of mitochondria, and both caspase-3 and caspase-8 are required in RosA-induced apoptosis

Generally, caspase-8 was known as an apical caspase in death receptor-mediated apoptosis. However, recent reports demonstrated that caspase-8 could also work as an apoptosis executor (44). In contrast, caspase-3 usually acts as an apoptosis executor either along with or without the mitochondrial pathway (36). To determine the hierarchy of caspases and mitochondria, mitochondrial membrane depolarization was measured in the presence or the absence of the broad-spectrum caspase inhibitor. Z-VAD-fmk prevented RosA-induced apoptosis, whereas it failed to inhibit mitochondrial membrane depolarization (Fig. 6A). This means that caspases do not induce MMP disruption, but are activated downstream of mitochondria.

To assess the involvement of caspases in RosA-induced cell death, Jurkat cells were preincubated with caspase inhibitors, followed by incubation with RosA for 48 h. Z-DEVD-fmk and Z-IETD-fmk are caspase-3- and caspase-8-specific inhibitors, respectively. As both Z-DEVD-fmk and Z-IETD-fmk inhibited RosA-induced apoptosis, we conclude that both caspase-3 and -8 are absolutely required for RosA-induced apoptosis (Fig. 6B). In hierarchy analysis, a caspase-3-specific inhibitor prevented cleavage of both caspase-3 and -8, whereas the caspase-8-specific inhibitor blocked the cleavage of caspase-8 only (data not shown). Both caspase-3 and -8 inhibitors failed to block the cleavage of caspase-9 (data not shown). Thus, caspase-3 apparently works upstream of caspase-8, and both caspase-3 and -8 are activated downstream of caspase-9 in RosA-induced apoptosis.
CD4− subsets of PBMC, including T cells (mixtures of persistently, purified apoptotic population is T cells, not B cells (data not shown). Conclusively, dual staining with FITC-labeled annexin V and PE-labeled anti-CD19 Ab suggested that the major population was T cells, not B cells (Fig. 8A)). FACS analysis after A viral infection with RosA for 3 h and incubated with RosA (30 μM) for 48 h. Thin and thick lines indicate Jurkat cells without and with RosA, respectively. The apoptotic cells were stained with Annexin VFITC and analyzed by FACS.

caspase-8 in ionizing radiation-induced apoptosis (45). Alternatively, Src family protein tyrosine kinases have also been reported to function downstream of caspase-3 and -8. Fyn and Lyn are cleaved in their unique N-terminal regions by caspase-3, and the resultant uncontrolled kinase activity ultimately leads to cell death (46). To determine whether Lck works upstream or downstream of caspase-3 and -8, we examined the cleavage patterns of pre-caspase-3 and -8 in the Lck-deficient J.CaM1.6 cells after treatment with RosA. We assumed that if Lck works downstream of caspases, caspase-3 would be cleaved regardless of Lck expression. As shown in Fig. 7A, RosA induced the cleavage of pre-caspase-3 and -8 to generate the active processed forms between 24 and 48 h after RosA treatment in Jurkat cells. In Lck-deficient J.CaM 1.6 cells, however, pre-caspase-3 and pre-caspase-8 were not cleaved (Fig. 7B); therefore, we conclude that Lck works upstream of caspase-3 and -8 and possibly of mitochondria.

RosA induces apoptosis of actively proliferating T and NK cells, but not Lck-deficient cells including B cells and monocytes

To verify that Lck-dependent apoptosis also occurs in freshly isolated human immune cells, we tested both total and purified single subsets of human PBMC. Total PBMCs were either activated or not with PHA, incubated with RosA (30 μM) for 48 h, and examined for apoptosis. RosA induced apoptosis up to 40−80% of PHA-activated PBMC at a concentration of 12.5 μM during 24−48 h, but not those in the resting state (Fig. 8A). FACS analysis after dual staining with FITC-labeled annexin V and PE-labeled anti-CD3 or CyChrome-labeled anti-CD19 Ab suggested that the major apoptotic population is T cells, not B cells (data not shown). Consistently, purified subsets of PBMC, including T cells (mixtures of CD4+ and CD8+ cells) and CD56+ NK cells, were susceptible to RosA-induced apoptosis only when they were activated with PHA and IL-2, respectively (Fig. 8B, upper panels). In contrast, RosA did not induce apoptosis of Lck-deficient PBMC subsets including CD19+ B cells and CD14+ monocytes regardless of activation state (Fig. 8B, lower panels). Among Src family protein tyrosine
kinases, Src is ubiquitously expressed. Others are tissue-restricted for expression; Lyn, c-Fgr, and Blk are expressed preferentially in B cells, and Lyn and c-Fgr are also expressed in myeloid cells (neutrophils, monocytes, and macrophages), whereas Lck is restricted to T and NK cells (47). Considering this distribution pattern, these results suggest that RosA acts specifically through Lck, but not through other Src family protein tyrosine kinases such as Src and Lyn.

**Discussion**

We first observed that RosA induces apoptosis in Jurkat T cells. Residual T cell activity can be controlled by apoptosis, and T cell apoptosis inducers have great potential for the treatment of autoimmune diseases and allograft rejection (14, 19, 48–50). Many drugs currently used for rheumatoid arthritis and transplantation rejection kill leukocytes, including T cells, macrophages, and monocytes (14, 19, 48). In our study RosA selectively killed Jurkat T cells in an Lck-dependent manner. This was clearly demonstrated by comparing the apoptotic activity of RosA in Jurkat cells and Lck-deficient J. CaM1.6 cells (Fig. 2A, left panel). Furthermore, Lck reconstitution made J.CaM1.6 cells vulnerable to RosA-induced apoptosis (Fig. 2A, right panel). As Lck is mostly restricted to T and NK cells (51), RosA is expected to have selectivity toward these cells. In agreement with this, RosA-induced apoptosis of activated T and NK cells, but not Lck-deficient subsets of human PBMC, including B cells and monocytes (Fig. 8). These results strongly suggest that RosA’s target is Lck, and RosA does not work via other Src family kinases, such as Src or Lyn. Moreover, up to 500 μM RosA did not induce apoptosis in Lck-deficient cells, including Chang liver, COS, and Lewis lung carcinoma cells (data not shown). This selectivity of RosA toward Lck+ Jurkat, and peripheral T and NK cells implicates that the observed apoptotic ability of RosA is not ascribed from general toxicity. Lck-dependent apoptosis was not observed in other structurally similar polyphenolic compounds, including curcumin, resveratrol, and CAPE, which are under intensive study due to their apoptotic activity (Fig. 2D). Generally these polyphenols induce apoptosis in a wide variety of tumor cells, including colon carcinoma, breast carcinoma, and B and T cell leukemia (52–55). Functional moieties of RosA responsible for the Lck-dependent apoptotic activity are therefore of interest and are under study in our laboratory.

Lck, an Src family protein tyrosine kinase, is mostly expressed in T and NK cells and plays a pivotal role in TCR-induced signal transduction, leading to Ca2+ mobilization and IL-2 promoter activation. Aside from its involvement in the TCR-induced activation process, Lck is also known to participate in AICD in T cells, possibly by inducing FasL expression, but not by participating in Fas-mediated apoptosis itself (56–58). In AICD, it was reported that Lck kinase activity, but not the Lck SH2 domain, is critical (56, 57). Although RosA-induced apoptosis requires Lck, it is not dependent on Lck kinase activity. This was demonstrated by the failure of Src kinase-specific inhibitor PP2 in blocking RosA-induced apoptosis (data not shown). Moreover, reconstitution of J.CaM1.6 cells with the kinase-defective Lck mutant (LckR273) restored RosA-induced apoptosis, indicating that Lck kinase activity is dispensable in RosA-induced apoptosis (Fig. 2B). In contrast, reconstitution of J.CaM1.6 cells with the Lck SH2 mutant LckK154 failed to elicit RosA-induced apoptosis, suggesting that molecular interaction of RosA through the Lck SH2 domain is pivotal in RosA-induced apoptosis (Fig. 2B). Previously we reported that RosA inhibits Lck SH2-pYEEI interaction and does not inhibit Lck kinase activity (25, 26). Therefore, our current data are consistent with our previous reports, and RosA is not expected to inhibit AICD. RosA-induced apoptosis does not occur through AICD either. Although RosA induced Fas/FasL expression, RosA did not require either Fas/FasL interaction or TCR stimulus to induce cell death (Fig. 3). Therefore, although Lck is involved in RosA-mediated apoptosis, the mechanism seems quite different from that in AICD. Along with Lck dependency, RosA’s ability to kill T cells independently from AICD and Fas/FasL interaction suggests the potential use of RosA for inducing apoptosis of T cells unusually resistant to AICD or Fas/FasL-induced apoptosis that are frequently found in T cells of rheumatoid arthritis patients (59, 60).

Compared with the inhibition of TCR-induced signaling pathway that occurs very rapidly within 2 min, RosA-induced apoptosis is a very slow process, requiring at least 24 h (26). Therefore, it is not likely that RosA-induced apoptosis causes inhibition of TCR signaling, which occurs 24–48 h before apoptosis starts. As RosA-induced apoptosis occurred in the absence of a TCR stimulus in Jurkat cells, blockage of TCR-induced signaling does not seem to elicit apoptosis (Fig. 1). In a collagen-induced arthritis model, RosA alleviated signs and progression of rheumatoid arthritis, as judged by reduced arthritis index and inflammation (61). Like other antiinflammatory drugs, it is believed that multiple properties of RosA, including TCR-induced signaling inhibition and apoptosis induction, contribute to T cell suppression and subsequent immunosuppression.

Several protein tyrosine kinases appear to be important in apoptosis downstream of caspases. Luciano et al. (46) reported that Fyn and Lyn are cleaved by caspases in their unique N-terminal domains during apoptosis. The resulting up-regulation of these kinases may lead to uncontrolled phosphorylation and apoptosis. Caspase-3 has also been reported to cleave Etk/Bmx, a member of the Btk/Tec kinase family. Cleaved Etk exhibits increased kinase activity and sensitizes cells toward apoptosis (62). Lck was also implicated as a key regulator in radiation-, ceramide-, and HIV-tat-induced apoptosis; however, the exact mechanism has not been fully clarified (45, 63, 64). Recently, mitochondrial dysfunction has been observed in radiation-induced apoptosis. As this mitochondrial apoptosis was not observed in Lck-deficient J.CaM1.6 cells, the authors concluded that Lck probably works in early phases in radiation-induced mitochondrial alterations (45, 65). Similar conclusions were made in RosA-induced apoptosis: 1) Lck is indispensable for RosA-induced MMP disruption and subsequent apoptosis; and 2) Lck-deficient J.CaM1.6 cells failed to show activated caspase-3 and -8 in response to RosA, suggesting that Lck works upstream of caspase-3 and -8.

RosA induced cytochrome c release and procaspase-9 activation as well as mitochondrial membrane depolarization (Fig. 4), indicating that RosA-induced apoptosis involves the mitochondrial pathway. Blockage of RosA-induced apoptosis by bongkrekic acid indicates that the mitochondrial pathway is the main route by which RosA induces apoptosis. As Bcl-2 has been reported to prevent cytochrome c release (9, 66) as well as oligomerization and translocation of Bax to the mitochondrial membrane (67, 68), RosA-mediated decrease of Bcl-2 expression may be responsible for the observed disruption of MMP (Fig. 5A). Prevention of RosA-induced apoptosis by Bcl-2 overexpression further supports this idea. However, as any MMP disruption, whether it is induced by shortage of Bcl-2 or not, can be prevented by Bcl-2 overexpression, we cannot exclude possibility that RosA elicits MMP disruption through another mechanism.

Caspase-3 and -8 were absolutely required for RosA-induced apoptosis (Fig. 6B). Caspase-8 is known as an apical protease acting proximal to death receptor-mediated apoptosis, whereas it is also known to act downstream of mitochondria as an apoptosis...
executioner (36). In our study general caspase inhibitor failed to block MMP disruption, whereas it prevented apoptosis in response to RosA. This means that most caspses inhibited by Z-VAD-fmk are activated after MMP disruption. Hierarchical analysis using various caspase inhibitors indicated that caspase-3 works upstream of caspase-8, and both caspase-3 and -8 are activated downstream of caspase-9 in RosA-induced apoptosis (data not shown). Various anticancer drugs induce apoptosis via the mitochondrial pathway, and caspase-8 has been reported to be one of the caspses activated by caspase-9 in the cytochrome c-initiated caspase cascade (18, 46–71).

In the proposed model, RosA induces apoptosis through the mitochondrial pathway in an Lck-dependent manner. The MMP disruption results in cytochrome c release, and a complex consisting of cytochrome c and Apaf-1 cleaves procaspase-9. Activated caspase-9 triggers caspase activation cascade in an order of caspase-3 and -8. ROS seems to be generated in response to mitochondrial dysfunction. However, we cannot exclude the possibility that ROS might be generated upstream of mitochondria and act as an MMP disrupter. Although it is obvious that Lck works upstream of mitochondria in RosA-induced apoptosis, how Lck participates in MMP disruption is not clear. Lck might control MMP by regulating the expression or translocation of Bcl-2 family proteins. It is also possible that Lck somehow regulates mitochondrial integrity through other routes. In conclusion, RosA induces apoptosis by eliciting mitochondrial dysfunction in a Fas-independent manner. Along with radiation-induced apoptosis, our data show a potential role of Lck in the mitochondrial apoptotic pathway.

References


