Decreases in CD31 and CD47 Levels on the Cell Surface during Etoposide-Induced Jurkat Cell Apoptosis

Yutaro AZUMA,* Hideaki NAKAGAWA, Kanae DOTE, Koji HIGAI, and Kojiro MATSUMOTO

Department of Clinical Chemistry, Faculty of Pharmaceutical Sciences, Toho University; 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan. Received May 31, 2011; accepted September 9, 2011; published online September 14, 2011

Engulfment of apoptotic cells is regulated by ‘eat me’ and ‘don’t eat me’ signals on the cell surface. Alterations to the ‘eat me’ signals have been well described; however, very little is known about the ‘don’t eat me’ signals on the cell surface during apoptosis. In the present study, apoptosis of Jurkat cells was induced by treatment with topoisomerase II inhibitor etoposide, and then the CD31 and CD47 levels on the apoptotic cell surface and in microparticles were estimated by flow cytometry and immunoblotting methods in the presence of caspase, metalloproteinase, and Rho-associated coiled-coil containing protein kinase 1 (ROCK1) inhibitors. The CD31 and CD47 levels on the cell surface of apoptotic Jurkat cells had decreased after treatment with etoposide. These decreases in CD31 and CD47 levels on the apoptotic cell surface were almost completely suppressed by the caspase 3 inhibitor, Ac-DEVD-CHO, and partially suppressed by caspase 8 (Ac-IETD-CHO) and caspase 9 (Ac-LEHE-CHO) inhibitors but not by the metalloproteinase inhibitors GM6001 and TAPI-0. Microparticle counts in culture supernatants were higher during etoposide-induced apoptosis. The ROCK1 inhibitor, Y27632, suppressed blebbing formation and microparticle release. Moreover, flow cytometry and immunoblotting revealed CD31 and CD47 in the microparticles. These results indicate that CD31 and CD47 were released by the apoptotic Jurkat cells into the culture supernatant in microparticles, but not in soluble forms, resulting in decreased levels on the apoptotic cell surface.

Key words apoptosis; CD31; CD47; caspase; Rho-associated coiled-coil containing protein kinase 1; blebbing

The efficient removal of apoptotic cells by phagocytes is crucial for tissue homeostasis, the resolution of inflammation, the modulation of immune responses and cancer immunotherapy.^{1–3)} Apoptosis is a dynamic process, during which cell surface molecules are continuously changing. Enhanced ‘eat me’ signals and reduced ‘don’t eat me’ signals in apoptotic cells induce phagocytes to promptly and efficiently engulf apoptotic cells during the early and late stages of cell death, preventing them from releasing noxious intracellular contents. Direct ‘eat me’ signals include the newly expressed molecules phosphatidylserine (PS) and annexin I, modification of the surface molecules intercellular cell adhesion molecule (ICAM)-3 and platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), changes in the cell surface charge, and presence of extracellular bridging molecules such as milk-fat-globule-EGF-factor 8, the collectin family proteins, mannose-binding protein, complement C1q, and thrombospondin-1. However, very little is known about changes in the ‘don’t eat me’ signals during apoptosis.⁴⁾

CD47 (integrin-associated protein, IAP) is a 50 kDa integral membrane protein composed of an extracellular immunoglobulin variable (IgV) domain, five transmembrane domains, and a short C-terminal cytoplasmic tail with four alternatively spliced forms, and is a widely expressed member of the Ig superfamily. CD47 functions as a receptor for thrombospondin-1 and as a ligand for the transmembrane signal regulatory proteins (SIRP)- α and - γ , and also interacts with integrins such as the RGD receptor $\alpha v \beta 3$, the fibrinogen receptor $\alpha IIb \beta 3$, and the collagen receptor $\alpha 2 \beta 1$ to induce various cellular signaling.^{5,6)} Integrin $\alpha v \beta 3$ /CD47 ligation initiates a signal transduction cascade, dependent upon the $\beta 3$ cytoplasmic tail, which inhibits the phagocytic function of $\alpha 5 \beta 1$ at a step subsequent to modulation of integrin affinity.⁷⁾

CD47 on red blood cells (RBCs) functions as a ‘don’t eat

me’ marker by sending a negative engulfment signal to splenic red pulp macrophages through SIRP α .⁸⁾ Most signaling through CD47-SIRP α interaction is mediated by the phosphatase Src homology 2 domain-containing protein tyrosine phosphatase (SHP-1).⁹⁾ Ligation of SIRP α on macrophages by CD47 on RBCs inhibits Fc γ R-mediated phagocytosis by promoting tyrosine phosphorylation of SIRP α and its interaction with SHP-1 and preventing SIRP α dephosphorylation in response to Fc γ R activation.¹⁰⁾ CD47 is altered and/or lost on apoptotic cells, and disruption of interactions between CD47 on apoptotic cells and SIRP α on the engulfing cells permits uptake of apoptotic cells in a calreticulin/LDL receptor-related protein (LRP)-dependent manner.¹¹⁾ Phagocytosis of opsonized RBCs by splenic macrophages from mice that express the mutant form of SIRP α (lacking most of its cytoplasmic region) is increased compared with wild-type macrophages.¹²⁾ However, macrophages require CD47 and PS on apoptotic cells for engulfment and CD47-SIRP α interaction works as a tethering step in the phagocytosis in lymphocytes.¹³⁾ CD47 on healthy cells and its engagement of the phagocytic receptor SIRP α appears to contribute a key ‘don’t eat me’ signal.¹⁴⁾

CD31 is a 130 kDa glycoprotein belonging to the Ig superfamily of cell adhesion molecules and is restrictedly expressed in the vascular system, platelets, monocytes, neutrophils, selected T-cells and endothelial cells. CD31 is composed of six extracellular Ig-domains, comprising homophilic interaction involving Ig domain 1 and heterophilic ligands integrin $\alpha v \beta 3$ and CD38 involving Ig-domains 1–3. CD31 signaling is mediated through phosphorylation of specific tyrosine residues located in two immunoregulatory tyrosine-based inhibition motif (ITIM) domains in its cytoplasmic tail and interactions with SHP-2 and SHP-1.^{15–17)} CD31 homophilic interaction induces cytoprotective signaling to inhibit Bax-mediated mitochondrial damage by SHP-2 inter-

* To whom correspondence should be addressed. e-mail: yutaro@phar.toho-u.ac.jp

actions with cytoplasmic ITIMs.¹⁸⁾ A homophilic CD31 interaction can discriminate between apoptotic and viable leukocytes by selectively imparting 'detachment' signals to viable cells under low shear, whereas such CD31-mediated detachment is disabled in apoptotic leukocytes, promoting tight binding and macrophage ingestion of dying cells.¹⁹⁾ Homophilic interactions of CD31 between macrophages and apoptotic bone marrow-derived B-cells treated with corticosteroid increase macrophage ingestion but heterophilic interactions between lymphocyte CD31 and macrophage $\alpha v \beta 3$ integrin does not.²⁰⁾ CD31 homophilic interaction between phagocytes and target cells leads to activation of phagocyte $\alpha 5 \beta 1$ integrin and the engulfment of apoptotic Jurkat T-lymphocytes *via* a fibronectin bridge.²¹⁾ CD31 deficiency on either the phagocytes or the target cells significantly reduces engulfment; however, serum opsonization masks the contribution of CD31.²²⁾

In a previous report,²³⁾ we demonstrated that ICAM-2 and ICAM-3 on the cell surface were decreased during etoposide-induced Jurkat cell apoptosis, and that the decrease in ICAM-2 was partially suppressed by metalloproteinase inhibitors GM6001 and TAPI-0 but not by caspase inhibitors, while the decrease in ICAM-3 was completely suppressed by caspase 3 and 8 inhibitors but not by metalloproteinase inhibitors. In the present study, we clarified that 'don't eat me' signal molecules CD31 and CD47 on the apoptotic cell surface are decreased during etoposide-induced Jurkat cell apoptosis, and that decreases in CD31 and CD47 are suppressed by caspase 3, 8, and 9 inhibitors but not by metalloproteinase inhibitors. The most commonly used criteria for distinguishing apoptosis from other physiological processes are dynamic membrane blebbing and the release of microparticles (cell debris), along with chromatin condensation and DNA laddering. During blebbing, Rho-associated coiled-coil containing protein kinase 1 (ROCK1) contributes to the cytoskeleton rearrangement.^{24–26)} In the present study, we found that microparticles containing CD31 and CD47 are released into culture medium during apoptosis and that this release is suppressed by the ROCK1 inhibitor, Y27632. These results indicate that CD31 and CD47 are released into culture supernatant in microparticles to decrease their levels on the apoptotic cell surface.

MATERIALS AND METHODS

Materials Topoisomerase II inhibitor etoposide, ROCK-1 inhibitor Y27632, and L-glutamate were purchased from Wako Pure Chemical Co. (Osaka, Japan). Culture medium RPMI 1640 was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan), and fetal bovine serum (FBS), protease inhibitor cocktail and propidium iodide (PI) were purchased from Sigma Aldrich Co. (St. Louis, MO, U.S.A.). Fluorescein isothiocyanate (FITC)-conjugated annexin V was purchased from Medical Biological Laboratories Co. (Nagoya, Japan). Caspase-Glo[®] was purchased from Promega Co. (Madison, WI, U.S.A.). The primary mouse monoclonal IgG antibodies used were anti-CD31 (9G11) from R & D Systems (Minneapolis, MN, U.S.A.) and anti-CD47 (B6H12) from Santa Cruz Biotechnology Co. (Santa Cruz, CA, U.S.A.). The secondary antibodies, Alexa Fluor[®] 488-conjugated anti-mouse IgG (H+L) and peroxidase (POD)-conjugated goat

anti-mouse IgG antibodies were purchased from Invitrogen Co. (Carlsbad, CA, U.S.A.) and GE Healthcare U.K. Ltd. (Little Chalfont, U.K.), respectively. Hoechst 33258 was purchased from Anaspec Inc. (San Jose, CA, U.S.A.). Metalloproteinase inhibitors GM 6001 from Calbiochem (San Diego, CA, U.S.A.) and TAPI-0 from Peptide Institute Inc. (Osaka, Japan) were used. Caspase inhibitors acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO, an inhibitor of caspase 3), acetyl-Ile-Glu-Thr-Asp-aldehyde (Ac-IETD-CHO, an inhibitor of caspase 8) and acetyl-Leu-Glu-His-Asp-aldehyde (Ac-LEHD-CHO, an inhibitor of caspase 9) were obtained from Peptide Institute Inc. All other chemicals were purchased from Sigma Aldrich Co. or Nacalai Tesque Co. (Kyoto, Japan).

Culture Conditions and Apoptosis Induction Human T-lymphoblastic Jurkat cells (Human Science Research Resource Cell Bank, Tokyo, Japan) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 0.3 mg/ml L-glutamate at 37 °C in a humidified atmosphere containing 5% CO₂.

Jurkat cells (5×10^5 cells/ml) were induced into apoptosis by treatment with 10 μ M topoisomerase II inhibitor etoposide in 10% FBS/RPMI for 0 to 9 h at 37 °C. For inhibition of metalloproteinases, a broad spectrum consisting of hydroxamate GM 6001 or tumor necrosis factor (TNF)- α protease inhibitor TAPI-0, was added to the culture medium at a final concentration of 50 μ M. For inhibition of caspases, Ac-DEVD-CHO, Ac-IETD-CHO or Ac-LEHD-CHO was added to the culture medium at a final concentration of 100 μ M. For inhibition of ROCK1, Y27632 was also added to the culture medium at a final concentration of 10 μ M.

Estimation of Apoptosis and Blebbing Cell viability was estimated by the trypan blue exclusion test. For measurement of phosphatidylserine (PS) on the cell surface, the cells were treated with 5 μ g/ml FITC-conjugated annexin V in binding buffer (10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂) at room temperature in the dark for 10 min, and then subjected to flow cytometry using a FAC-Scan flow cytometer (BD Biosciences, San Jose CA, U.S.A.). Analysis was performed using Flow Jo software (Tree Star, Ashland OR, U.S.A.). Caspase 3 activity was determined using Caspase-Glo[®] according to the manufacturer's instructions.

Flow Cytometric Analysis of CD31 and CD47 For determination of the CD31 and CD47 levels on the cell surface, cells collected by 1000 *g* centrifugation for 3 min at 4 °C were treated with 50 μ l of each 10 μ g/ml primary antibody in 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS) for 30 min followed by incubation with 50 μ l of 10 μ g/ml anti-mouse IgG goat antibody conjugated with Alexa Fluor[®] 488 in 1% BSA/PBS for 30 min on ice. After washing twice with 1% BSA/PBS, the cells were further stained with 1 μ g/ml PI in 1% BSA/PBS for 5 min on ice. For determination of CD31 and CD47 content within the whole cells, cells washed twice by centrifugation at 1000 *g* for 3 min with PBS were treated with 4% *para*-formaldehyde for 15 min at 4 °C, and after washing twice with 1% BSA/PBS, the cells were then permeated with 0.1% Triton X-100 in 1% BSA/PBS for 5 min at 4 °C. The cells were again washed twice with 1% BSA/PBS, treated with the pri-

Fig. 1. Estimation of Etoposide-Induced Apoptosis of Jurkat Cells

Jurkat cells were treated with $10\ \mu\text{M}$ etoposide for 0 to 9 h and apoptosis was then estimated: (A) caspase 3 activity using Caspase-Glo® in control cells (closed circle) and etoposide-treated cells (open circle), (B) PS levels by FITC-conjugated annexin V binding, (C) cell viability (%) by trypan blue-staining. The results represent the mean $\pm$ S.D. ($n=3$) and an asterisk (*) denotes a significant difference ($p<0.05$).

mary and secondary antibodies described above and then analyzed by flow cytometry.

Flow Cytometry of Microparticles Jurkat cells (5×10^5 cells/ml) were cultured in flat-bottomed six-well plates (3 ml/well). The cells were treated with $10\ \mu\text{M}$ etoposide in the presence or absence of $10\ \mu\text{M}$ Y27632 for 0, 3, 6, and 9 h at 37°C . After centrifugation at $1000g$ for 3 min, the supernatant was collected and the microparticle numbers then determined by flow cytometry as counts per minute.

For determination of the CD31 and CD47 levels in microparticles, microparticles collected from the supernatant by centrifugation at $100000g$ for 30 min were incubated with $10\ \mu\text{g/ml}$ primary antibodies in 1% BSA/PBS for 30 min on ice. After centrifugation at $100000g$ for 30 min, the microparticles were further treated with $10\ \mu\text{g/ml}$ anti-mouse IgG goat antibody conjugated with Alexa Fluor® 488 in 1% BSA/PBS for 30 min on ice and subjected to flow cytometry. All media and buffers were first filtered with syringe filters (Minisart® $0.22\ \mu\text{m}$, Sartorius, Goettingen, Germany).

Immunoblotting for CD31 and CD47 Jurkat cells (5×10^5 cells/ml) were cultured in flat-bottomed six-well plates (3 ml/well). The cells were treated with $10\ \mu\text{M}$ etoposide for 24 h. After centrifugation at $1000g$ for 5 min, $500\ \mu\text{l}$ of supernatant with and without filtration was directly blotted onto Hybond-P® polyvinylidene difluoride (PVDF) membrane (GE Healthcare) and the membrane then blocked with Blocking One (Nacalai Tesque) and washed three times with 0.1% Tween 20 in PBS (PBS-T). The membrane was then incubated with anti-CD31 or anti-CD47 antibody (1:1000) in PBS-T at room temperature for 1 h. After washing three times with PBS-T, the membrane was incubated with POD-conjugated goat anti-mouse IgG antibody (1:1000) in PBS-T at room temperature for 1 h. The membrane was then washed three times with PBS-T, incubated with ECL plus (GE Healthcare) at room temperature for 5 min, and analyzed using a Bioimager, Storm™ 830 imager (GE Healthcare).

RESULTS

CD31 and CD47 Levels on the Cell Surface during Etoposide-Induced Jurkat Cell Apoptosis We treated Jurkat cells with $10\ \mu\text{M}$ etoposide for 0 to 9 h and estimated time-dependently the caspase 3 activity in the cells using Caspase-Glo®, the PS expression levels on the cell surface by binding of FITC-labeled annexin V, and cell viability by the Trypan blue exclusion test (Fig. 1). Etoposide rapidly induced increases in PS expression and caspase 3 activity 3 h

Fig. 2. CD31 and CD47 Levels on the Cell Surface and in Whole Cells of Etoposide-Treated Jurkat Cells

(A) CD31 and CD47 levels on the cell surface. Jurkat cells were treated with $10\ \mu\text{M}$ etoposide for 0 to 12 h and CD31 and CD47 levels were determined by flow cytometry using anti-CD31 and anti-CD47 antibodies followed by Alexa® 488-conjugated secondary antibody. (B) CD31 and CD47 levels in whole cells. Jurkat cells treated with etoposide for 9 h were immobilized with 4% *para*-formaldehyde for 15 min followed by permeation with 0.1% Triton X-100 for 5 min and protein levels were determined as described above. The results represent the mean $\pm$ S.D. ($n=3$) and an asterisk (*) denotes a significant difference ($p<0.05$).

after treatment (Figs. 1A, B), followed by decreases in cell viability 6 h after treatment (Fig. 1C).

To clarify whether the 'don't eat me' signal molecules CD31 and CD47 on the apoptotic cell surface are decreased during apoptosis, we treated Jurkat cells with $10\ \mu\text{M}$ etoposide for 0 to 12 h and estimated CD31 and CD47 levels on the apoptotic cell surface using anti-CD31 and anti-CD47 antibodies, Alexa® 488-conjugated secondary antibody and flow cytometry (Fig. 2A). The CD31 and CD47 levels on the cell surface were significantly decreased 6 h after etoposide treatment to reach plateau levels at 9 h. The CD31 and CD47 content in cells permeabilized with 4% *para*-formaldehyde followed by 0.1% Triton X-100 were similarly decreased by etoposide treatment (Fig. 2B), indicating that the decreases in the CD31 and CD47 levels on the cell surface during etoposide-induced apoptosis were caused by their release into the medium by the apoptotic cells and not by internalization of these molecules into the apoptotic cells.

CD31 and CD47 Levels on the Cell Surface during

Etoposide-Induced Apoptosis of Jurkat Cells in the Presence of Caspase and Metalloproteinase Inhibitors To clarify the mechanism of the decrease in the CD31 and CD47 levels on the cell surface, we treated Jurkat cells with 10 μM etoposide for 9 h in the presence of the caspase 3, 8, and 9 inhibitors Ac-DEVD-CHO, Ac-IETD-CHO, and Ac-LEHD-CHO, respectively, and the metalloproteinase inhibitors GM6001 and TAPI-0 and estimated CD31 and CD47 levels on the cell surface by flow cytometry (Fig. 3). The decreases in the CD31 and CD47 levels on the apoptotic cell surface were mostly suppressed in the presence of caspase 3 inhibitor Ac-DEVD-CHO and partially suppressed by the caspase 8 (Ac-IETD-CHO) and caspase 9 (Ac-LEHD-CHO) inhibitors. However, the metalloproteinase inhibitors GM6001 and TAPI-0 did not affect the decreases in the CD31 and CD47 levels on the cell surface of etoposide-treated Jurkat cells. These results suggest that the decreases in the CD31 and CD47 levels on the apoptotic cell surface are caused by caspase-dependent intracellular events but not by cleavage of extracellular regions of these molecules.

Release of Microparticles into Culture Supernatant

To clarify the release of microparticles from blebbed cells, we treated Jurkat cells with 10 μM etoposide for 0 to 9 h in the presence of ROCK1 inhibitor, Y27632, and estimated blebbing formation and microparticle counts in the culture supernatant (Fig. 4). Blebbing cells (%) rapidly increased 6 h after etoposide treatment and Y27632 completely prevented blebbing formation (Fig. 4A). After removal of apoptotic

cells by centrifugation at 1000 g for 5 min, microparticles were collected by centrifugation at 100000 g for 30 min and counted by flow cytometry (Fig. 4B). Microparticle counts in culture supernatants gradually increased after 3 h etoposide treatment. We then treated Jurkat cells with 10 μM etoposide for 9 h in the presence of Y27632 and counted microparticles by flow cytometry (Fig. 4C). The previous increase in microparticle counts was significantly suppressed in the presence of the ROCK1 inhibitor, Y27632. These results suggest that the microparticles in culture supernatant were released from blebbed membrane formed during early apoptosis of the Jurkat cells.

Identification of CD31 and CD47 on Microparticles

To clarify whether CD31 and CD47 were concomitant in the microparticles, we treated Jurkat cells with 10 μM etoposide for 24 h and estimated the CD31 and CD47 levels in microparticles using flow cytometry and dot blotting methods (Fig. 5). After apoptotic cells were removed by centrifugation at 1000 g for 5 min, we collected microparticles from the supernatant by centrifugation at 100000 g for 30 min. Microparticles were treated with anti-CD31 and anti-CD47 antibodies followed by Alexa[®]-488-conjugated secondary antibody and subjected to flow cytometry (Fig. 5A). The results indicated that CD31 and CD47 were concomitant in the microparticles. Subsequently, after centrifugal removal of apoptotic cells, we directly blotted the supernatant to a PVDF membrane and stained with anti-CD31 and anti-CD47 antibodies followed by POD-conjugated secondary antibody

Fig. 3. CD31 and CD47 Levels on the Apoptotic Cells in the Presence of Caspase and Metalloproteinase Inhibitors

Jurkat cells were treated with 10 μM etoposide for 9 h in the presence or absence of caspase 3, 8, and 9 inhibitors (each 100 μM), and metalloproteinase inhibitors GM6001 and TAPI-0 (each 50 μM). (A) CD31 and (B) CD47 levels on the cell surface were determined as described in Fig. 2. The results represent the mean $\pm$ S.D. ($n=3$) and an asterisk (*) denotes a significant difference ($p<0.05$).

Fig. 4. Effect of ROCK1 Inhibitor on Blebbing Formation and Release of Microparticles

Jurkat cells were treated with 10 μM etoposide for 0 to 9 h in the presence (open circle) or absence (closed circle) of ROCK1 inhibitor Y27632 (10 μM). (A) Blebbing cells (%) estimated by microscopic examination. (B) Microparticle counts collected by centrifugation at 100000 g for 30 min after removal of apoptotic cells by centrifugation at 1000 g for 5 min and measured by flow cytometry. (C) Microparticle counts after 9 h etoposide treatment in the presence of 10 μM Y27632. The results represent the mean $\pm$ S.D. ($n=3$) and an asterisk (*) denotes a significant difference ($p<0.05$).

Fig. 5. CD31 and CD47 in the Microparticles

Jurkat cells were treated with 10 μ M etoposide for 24 h and centrifuged at 1000 g for 5 min. (A) CD31 and CD47 levels in the microparticles were analyzed by flow cytometry. The open histograms show the binding of anti-CD31 or anti-CD47, and the filled histograms show the binding of control mouse IgG. (B) CD31 and CD47 in the culture supernatant were directly blotted onto PVDF membrane. The culture supernatant was filtered with (open bar) or without (gray bar) a syringe filter ($<0.22 \mu$ m) and blotted onto PVDF membrane. CD31 and CD47 were stained with anti-CD31 and anti-CD47 followed by POD-conjugated secondary antibody. The values determined by densitometry were normalized to those of control (without the syringe filter) levels. The results represent the mean $\pm$ S.D. ($n=3$) and an asterisk (*) denotes a significant difference ($p<0.05$).

(Fig. 5B). The CD31 and CD47 levels in the supernatant of 24 h etoposide-treated cells were about four-fold higher than those of non-treated cells. We further filtered the culture supernatant through a syringe filter ($<0.22 \mu$ m) and then blotted and analyzed non-filtered and filtered supernatant (Fig. 5B). The CD31 and CD47 levels in the non-filtered supernatant increased following 24 h etoposide treatment; however, the CD31 and CD47 levels in the filtered supernatant from etoposide treatment were similar to those of the controls. These results indicate that CD31 and CD47 released into the culture medium were $>0.22 \mu$ m particle-bound forms but not soluble forms.

DISCUSSION

Apoptosis is a dynamic process, during which cell surface molecules are continuously changing. These changes play important roles in the phagocytosis of apoptotic cells, by working as 'eat me' or 'don't eat me' signals. We investigated the altered expression levels of CD31 and CD47 molecules, known as 'don't eat me' signals, on the cell surface and in whole cells undergoing etoposide-induced apoptosis. The expression levels of CD31 and CD47 on the cell surface and in permeabilized cells were markedly decreased by etoposide treatment, indicating that CD31 and CD47 are removed from the cell surface during etoposide-induced apoptosis. These results suggest that these decreases in the CD31 and CD47 levels can contribute to reduced 'don't eat me' signals on apoptotic cells and provide an environment

for facilitated interaction between apoptotic and phagocytic cells.

Previously, we have reported that metalloproteinases and caspases or the caspases-mediated signaling cascade contribute to the decreases in ICAM-2 and ICAM-3 on the cell surface during etoposide-induced Jurkat cell apoptosis.²³⁾ In the present study, to clarify whether the decreases in CD31 and CD47 are accompanied by shedding and/or cleavage from the apoptotic cells by metalloproteinases and/or caspases, we studied the effects of caspase inhibitors and metalloproteinase inhibitors on the decreases in CD31 and CD47 expression levels. We found that the decreases in both CD31 and CD47 were strongly prevented by the caspase inhibitors but not by the metalloproteinase inhibitors, suggesting that these decreases are caused by activation of the caspases but not by the activation of the metalloproteinases.

Although CD31 and CD47 prevent engulfment by acting as 'don't eat me' signals, their structures and localization on the cell membrane are different. CD31 links to the actin cytoskeleton²⁷⁾ through phosphorylated ERM (ezrin/moesin/radixin), while CD47 localizes in cholesterol and sphingolipids-enriched membrane rafts.²⁸⁾ This suggested that the reduced expression of CD31 and CD47 on the cell surface may be induced by caspases and the caspase-induced cytoskeleton rearrangement.

During apoptosis, microparticle release may be related to blebbing, a process which causes the formation of small bubble-like extrusions on the membrane in association with the translocation of both nuclear and cytoplasmic compo-

nents.^{29–31)} In addition to microparticle release through blebbing, the formation of apoptotic bodies during apoptosis may produce cell fragments that phenotypically can also be characterized as microparticles. In this study, we collected microparticles by centrifugation or syringe filtration and could not make the distinction between blebs and apoptotic bodies. Further investigation is required to characterize microparticles released from Jurkat cells treated with etoposide.

One isoform of ROCK, ROCK1, is a direct cleavage substrate of activated caspase 3.³²⁾ ROCK1 is cleaved at a consensus caspase cleavage site to remove the inhibitory domain and create a constitutively active kinase,^{33,34)} which induces blebbing through phosphorylation of the light chain of myosin II.³⁵⁾ During blebbing formation, ROCK1 induces cytoskeleton rearrangement.^{24–26)} ROCK1 also induces phosphorylation of ezrin/radixin/moesin (ERM), membrane-cytoskeleton linkers, and regulates their linker function by the phosphorylation of a conserved Thr residue in the C-terminal domain of each ERM protein.^{36,37)} In the present study, the decreases in CD31 and CD47 were inhibited by caspase 3 inhibitor, and blebbing formation and microparticle release were prevented by ROCK1 inhibitor Y27632, suggesting that the decreases in CD31 and CD47 were due to microparticle release from blebs through caspase-induced ROCK-1 activation. In addition to ROCK1, phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphorylation on Ser/Thr residues by other kinases, including protein kinase C, can participate in activation of ERM. Further study is needed to clarify whether these kinases contribute to the decreases in CD31 and CD47 levels.

CD31 molecules on viable cells homophilically interact with their identical counterparts on phagocytes, promoting detachment and preventing engulfment of the viable cells by an active and temperature-dependent mechanism.¹⁹⁾ Decreased CD31 on apoptotic cells disrupts this repelling interaction to allow prompt ingestion by phagocytes. Another negative regulation of dying cell clearance is induced by disruption of the interaction between CD47 on the target cell and SIRP α on the engulfing cell.¹¹⁾ CD47 interacts with thrombospondin-1 (bridging molecule in cell–cell interactions), calreticulin (chaperone of the endoplasmic reticulum (ER)), integrins and CD91 (low density lipoprotein-related protein) to regulate T-cell motility.³⁸⁾ On apoptotic cells, altered and/or lost CD47 provides an environment where ‘eat me’ signals including calreticulin and PS are congregated for easy engulfment.^{11,39)} The localization of CD31 and CD47 on the cell surface and other factors, such as extracellular bridging molecules, may play an important role in engulfment, in addition to their surface expression levels. Further investigation concerning translocation of the other related factors is required to clarify the function of CD31 and CD47 responsible for phagocytosis.

In summary, we have demonstrated that decreased expression of CD31 and CD47 on the cell surface is mediated by the release of CD31 and CD47-containing microparticles, and that the caspases and ROCK1 play important roles in this process. These decreases in the ‘don’t eat me’ signals CD31 and CD47 on the apoptotic cell surface are crucial for recognition and removal of apoptotic cells. Further investigation is in progress to clarify the localization of molecules on the apoptotic cell surface associated with engulfment by phago-

cytic cells.

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