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Actin Cytoskeleton Is Required for Early Apoptosis Signaling Induced by Anti-Fas Antibody but Not Fas Ligand in Murine B Lymphoma A20 Cells

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Murine B lymphoma A20 cells are highly sensitive to Fas-mediated death signals induced by anti-Fas antibody Jo2 or cross-linked Fas ligand (FasL). We have found that the microfilament poison cytochalasin D blocks Fas-mediated apoptosis induced by Jo2 but not FasL in A20 cells. The induction of Fas-mediated apoptosis by Jo2 was antagonized by anti-Fcγ RII/RIII receptor (FcγR) antibody, and defective in FcγR-negative A20 cells. Since the induction of Jo2-mediated apoptosis in FcγR-negative A20 cells was reversed by the addition of wild type A20 cells or the cross-linking agent protein A, Fas-expressing bystander A20 cells seem to be killed by other A20 cells that capture and cross-link monomeric Jo2 via FcγR. Although cytochalasin D affected FcγR-mediated cross-linking of Jo2 molecules, the drug markedly inhibited the intracellular signaling pathway induced by Jo2. The blockade of Jo2-induced apoptosis by cytochalasin D occurred upstream of caspase-8 activation. Thus, these observations suggest that actin cytoskeleton is required for early apoptosis signaling induced by Jo2, but not physiological FasL.

Key Words: **actin; apoptosis; cytochalasin D; DISC; Fas; Fcγ receptor; Jo2.**

Fas, one of the death receptors, is widely expressed in diverse cell types and involved in the regulation of tissue homeostasis (1). Fas contains a cytoplasmic sequence known as "death domain" that is essential for transmitting apoptotic signals (2). Upon engagement with Fas ligand (FasL), Fas binds to the adaptor protein FADD via the death domain interaction. The initiator caspase, caspase-8 is subsequently recruited to Fas-FADD complexes via the homotypic interaction of death effector domain, allowing the formation of death-inducing signaling complex (DISC) (3). Immediately after its recruitment, caspase-8 is placed in close proximity in the DISC, and gets activated by self-cleavage, leading to activation of downstream substrates such as caspase-3.

In addition to physiological FasL, Fas-meditated death signals are induced by agonistic anti-Fas antibodies (4–6). To investigate the molecular basis of Fas-mediated signaling pathway, we screened for specific agents that block apoptosis induced by anti-Fas antibody or cross-linked FasL. We found that a microfilament-disrupting agent cytochalasin D inhibits apoptosis induced by anti-Fas antibody but not cross-linked FasL in murine B lymphoma A20 cells. In this paper, we demonstrate that actin cytoskeleton is required for early apoptosis signaling pathway induced by anti-Fas antibody, but not physiological FasL.

MATERIALS AND METHODS

Cells. Mouse B lymphoma A20 cells were maintained in RPMI 1640 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% (v/v) heat-inactivated fetal calf serum (JRH Bioscience, Lenexa, KS), 50 μM 2-mercaptoethanol, and penicillin-streptomycin-neomycin antibiotic mixture (Life Technologies, Grand Island, NY). A Fas-negative variant A20.FO was subcloned from the Fas-positive parent cell line A20.2J (7).

Reagents. Recombinant human soluble FasL (8) was kindly provided by Dr. J. Tschopp (Lausanne, Switzerland). Cytochalasin D and protein A were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Hamster anti-mouse Fas IgG antibody Jo2 was obtained from PharMingen (San Diego, CA). Rat anti-Fcγ RII/RIII (FcγR) IgG antibody 2.4G2 was prepared as culture supernatants of hybridoma cells, collected by ammonium sulfate precipitation, and dialyzed four times against PBS.

Abbreviations used: AMC, 7-amino-4-methyl-coumarin; DISC, death-inducing signaling complex; FasL, Fas ligand; FcγR, Fcγ RII/RIII receptors; MCA, 4-methyl-coumaryl-7-amide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylÅ@tetrazorium bromide; TdR, thymidine.

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Preparation of FcγR-negative A20 cells. Wild-type A20 cells (4–5 × 10^7 cells) were incubated with 2.4G2 on ice for 30 min, and washed with medium. The cells were incubated with rabbit anti-rat IgG antibody on ice for 30 min, and then washed with medium. The antibody-coated cells were incubated with rabbit serum complement in the high glucose medium at 37°C for 45 min (9). After dead cells were removed by using Lymphoprep (Nycomed, Oslo, Norway), remaining viable cells were allowed to expand for several days. The cytotoxic elimination of FcγR-expressing A20 cells was repeated three times. FcγR-negative A20 clones were established by limiting dilution.

DNA fragmentation assay. A20 cells were labeled with 37 kBq of [^{3}H]thymidine (TdR) (ICN Biomedicals, Costa Mesa, CA) for 16 h, and washed three times with medium. A20 cells (5 × 10^4 cells, 100 μl) were cultured with Jo2 or cross-linked FasL for indicated time periods in 96-well microtiter plates, and then lysed by 1% Triton X-100. The cells were solubilized by pipetting and then centrifuged (600*g*, 5 min). Supernatants were removed and measured for radioactivity. DNA fragmentation (%) was calculated using the following formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

FACS analysis. Cells were treated with Jo2 (hamster IgG) or hamster IgG isotypic control (Cedarlane Laboratories Ltd., Ontario, Canada) or 2.4G 2 (rat IgG) on ice for 45 min. The cells were washed three times, and then stained with FITC-conjugated anti-hamster IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) or FITC-conjugated anti-rat IgG antibody (Jackson ImmunoResearch Laboratories) on ice for 30 min. After washing, the stained cells were analyzed under flow cytometer (FACScalibur, Becton–Dickinson, Mountain View, CA).

Western blotting analysis. Cells were collected by centrifugation and washed twice with ice-cold PBS. The cells were lysed in lysis buffer containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.4), 1 mM DTT, 0.5 mM EDTA and protease inhibitor cocktail (Complete, Boehringer Mannheim, Mannheim, Germany). After centrifugation at 10,000*g* for 5 min, supernatants were collected. Postnuclear lysates (30 μg/lane) were separated by SDS–polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech., Buckinghamshire, UK). The nitrocellulose membranes were probed with rabbit anti-mouse FLIP IgG antibody (R&D System Inc.) or rabbit anti-caspase-3 IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were treated with horseradish peroxidase-conjugated mouse anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories), and developed by the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech).

Measurement of caspase activity. Postnuclear lysates were mixed with Ac-DEVD-4-methyl-coumaryl-7-amide (MCA; Peptide Institute Inc., Osaka, Japan) for the caspase-3 substrate or Ac-IETD-MCA (Peptide Institute Inc.) for the caspase-8 substrate in the reaction buffer (50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.1% Chaps, 10% sucrose, 10 mM DTT) for 1 h. The release of 7-amino-4-methylcoumarin (AMC) was measured by CytoFluor multiwell plate reader series 4000 (Applied Biosystems, Foster City, CA) using an excitation filter (360 nm) and an emission filter (460 nm). To block caspase-8-independent cleavage of Ac-IETD-MCA, the proteasome inhibitor MG-132 (Peptide Institute Inc.) was included in the reaction mixture at the final concentration of 2.5 μM (10).

Sepharose CL-2B gel filtration. Jo2 (20 μg) was applied to Sepharose CL-2B column (15 × 600 mm) equilibrated with 10 mM Tris–HCl (pH 7.5) and eluted with the same buffer (1 ml/min). Each fraction was concentrated and analyzed by Western blotting with horseradish peroxidase-conjugated anti-hamster IgG antibody. Cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

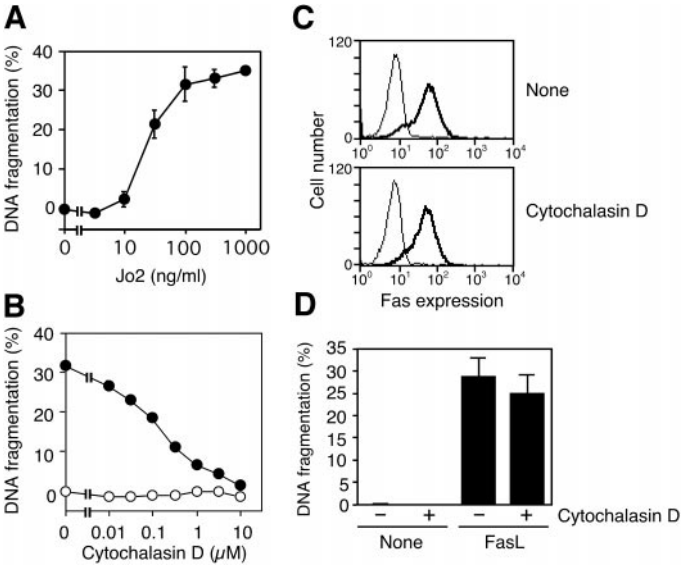

FIG. 1. The microfilament poison cytochalasin D inhibits apoptosis induced by anti-Fas antibody Jo2 but not FasL. (A) [^{3}H]TdR-labeled A20 cells were incubated with serially diluted concentrations of Jo2 for 4 h. Radioactivity of fragmented DNA was measured. The results represent the mean ± SD of triplicate cultures. (B) [^{3}H]TdR-labeled A20 cells were pretreated with various concentrations of cytochalasin D for 1 h and then incubated with (closed circles) or without (open circles) 200 ng/ml of Jo2 for 4 h. Radioactivity of fragmented DNA was measured. The results represent the mean ± SD of triplicate cultures. (C) A20 cells were treated with or without 3 μM cytochalasin D for 1 h. Cell surface expression of Fas (bold lines) and background staining (thin lines) were detected by FACS analysis. (D) [^{3}H]TdR-labeled A20 cells were preincubated with (+) or without (−) 3 μM cytochalasin D for 1 h and then incubated with 50 ng/ml of cross-linked FasL for 4 h. Radioactivity of fragmented DNA was measured. The results represent the mean ± SD of triplicate cultures.

RESULTS

Cytochalasin D Inhibits Apoptosis Induced by Anti-Fas Antibody Jo2 but Not FasL in A20 Cells

Mouse B lymphoma A20 cells expressed a large amount of Fas and were induced to exhibit DNA fragmentation characteristic of apoptosis by hamster anti-mouse Fas IgG antibody Jo2 in a dose-dependent manner (Fig. 1A). We extensively screened for specific agents that inhibit apoptosis induced by Jo2, and found that the microfilament poison cytochalasin D inhibits Jo2-induced apoptosis in a dose-dependent manner, whereas cytochalasin D alone did not affect cell viability (Fig. 1B; data not shown). Structural derivatives of cytochalasin D that disrupt microfilaments such as chaetoglobosins A and F (11) prevented Jo2-induced apoptosis, whereas microtubule inhibitors (e.g., colchicine and Taxol) were totally inactive (data not shown). The surface expression of Fas was unaffected by cytochalasin D (Fig. 1C). As previously observed (8), in contrast to soluble trimeric FasL, cross-linked FasL

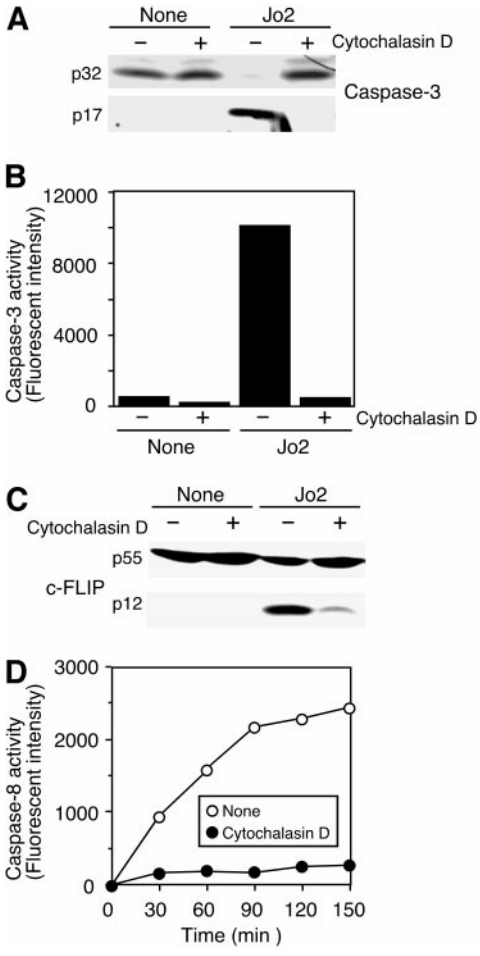

FIG. 2. Cytochalasin D inhibits activation of caspase-3 and caspase-8. (A) A20 cells were pretreated with (+) or without (−) 3 μM cytochalasin D for 1 h, and then incubated with 1 μg/ml of Jo2 for 2 h. Caspase-3 processing was analyzed by Western blotting. (B) A20 cells were pretreated with (+) or without (−) 3 μM cytochalasin D for 1 h and then incubated with 1 μg/ml of Jo2 for 2 h. Caspase-3 activity was measured by hydrolysis of Ac-DEVD-MCA. (C) Mouse c-FLIP-transfected A20 clone was pretreated with (+) or without (−) 3 μM cytochalasin D for 1 h, and then incubated with 200 ng/ml of Jo2 for 1 h. c-FLIP cleavage was analyzed by Western blotting. (D) A20 cells were pretreated with (open circles) or without (closed circles) 3 μM cytochalasin D and then incubated with 200 ng/ml of Jo2 for indicated time periods. Caspase-8 activity was measured by hydrolysis of Ac-IETD-MCA.

was highly cytotoxic to A20 cells and induced DNA fragmentation (data not shown). However, cytochalasin D failed to block apoptosis induced by cross-linked FasL (Fig. 1D).

Cytochalasin D Inhibits Activation of Caspase-3 and Caspase-8 upon Stimulation with Jo2

Jo2 induced a complete cleavage of pro-caspase-3 (Fig. 2A) and a significant activation of caspase-3 (Fig. 2B) within 2 h. We found that cytochalasin D markedly inhibits Jo2-induced caspase-3 activation. However, in agreement with DNA fragmentation assay (Fig. 1D), FasL-induced caspase-3 activation was not prevented by cytochalasin D (data not shown). To address if cytochalasin D targets signaling events upstream of caspase-8 activation, we analyzed the cleavage of c-FLIP, an intracellular protein specifically cleaved by caspase-8 (12) and the bulk hydrolysis of the caspase-8 substrate Ac-IETD-MCA. In A20 cells treated with Jo2, cytochalasin D profoundly inhibited c-FLIP cleavage (Fig. 2C) as well as hydrolysis of Ac-IETD-MCA (Fig. 2D).

Anti-FcγR Antibody Neutralizes Apoptosis Induced by Jo2 in A20 Cells

Addition of cross-linking agents such as protein A or anti-hamster IgG antibody had no obvious effects on Jo2-induced apoptosis in A20 cells (data not shown). Since A20 cells expressed FcγR that bind to Fc regions of IgG (Fig. 4A), we addressed whether FcγR is neces-

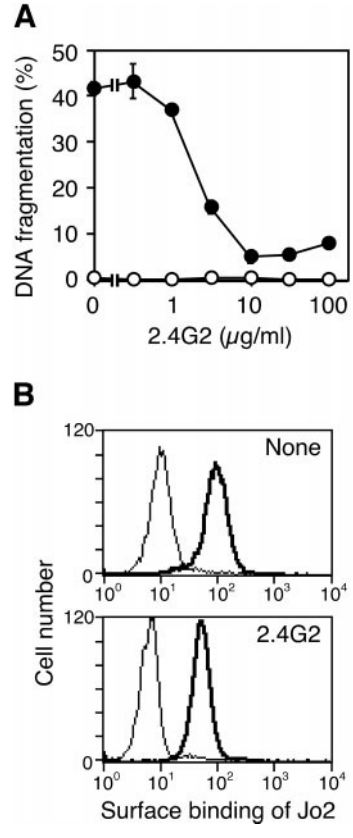

FIG. 3. Anti-FcγR antibody 2.4G2 inhibits apoptosis induced by Jo2. (A) [^{3}H]TdR-labeled A20 cells were preincubated with various concentrations of 2.4G2 for 1 h and then incubated with (closed circles) or without (open circles) 200 ng/ml of Jo2 for 4 h. Radioactivity of fragmented DNA was measured. The results represent the mean ± SD of triplicate cultures. (B) A20 cells were pretreated with or without 2.4G2 and then reacted with Jo2 (bold lines) or hamster IgG (thin lines) followed by FITC-conjugated anti-hamster IgG antibody. The surface binding of Jo2 was detected by FACS analysis.

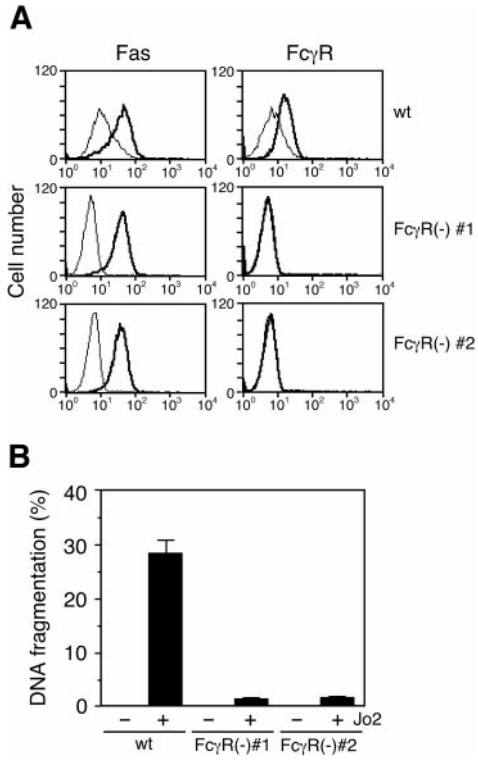

FIG. 4. FcγR-negative A20 clones are insensitive to Jo2-induced apoptosis. (A) Cell-surface expression of Fas and FcγR (bold lines; left and right panels, respectively) and background staining (thin lines) in wild-type A20 cells (wt) and two independently isolated FcγR-negative A20 clones designated FcγR(−) #1 and FcγR(−) #2 was detected by FACS analysis. (B) [^{3}H]TdR-labeled wild-type A20 cells (wt) and two FcγR-negative clones (FcγR(−) #1 and FcγR(−) #2) were incubated in the presence (+) or the absence (−) of 200 ng/ml of Jo2 for 4 h. Radioactivity of fragmented DNA was measured. The results represent the mean ± SD of triplicate cultures.

sary for Jo2-induced apoptosis. Anti-FcγR antibody 2.4G2 completely blocked DNA fragmentation induced by Jo2 in A20 cells (Fig. 3A). FACS analysis revealed that 2.4G2 substantially decreased surface binding of Jo2, indicating that a portion of Jo2 is captured by FcγR on the cell surface (Fig. 3B).

Jo2 Does Not Induce Apoptosis in FcγR-Negative A20 Cells

To obtain additional evidence that FcγR is required for Jo2-induced apoptosis, FcγR-negative A20 cells were established by cytotoxic elimination using anti-FcγR antibody. Two independently isolated clones expressed a comparable amount of Fas as parental A20 cells but they did not express FcγR at all (Fig. 4A). In contrast to wild-type cells, Jo2 failed to induce DNA fragmentation in both FcγR-negative A20 clones (Fig. 4B). These results indicate that FcγR is essential for Jo2-induced apoptosis in A20 cells.

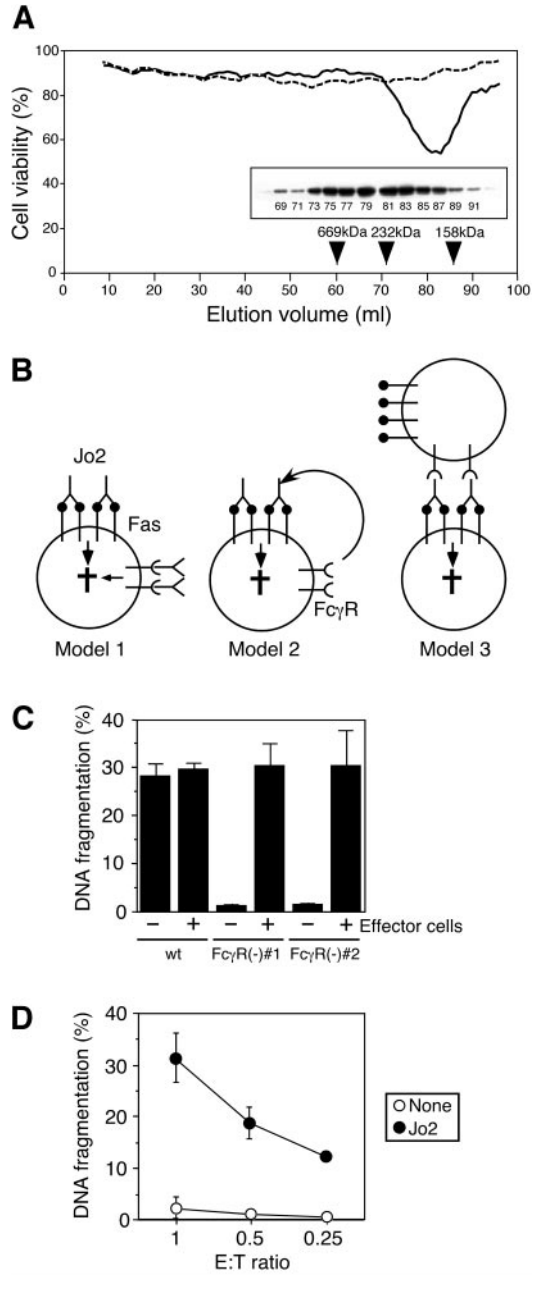

FIG. 5. Fas-expressing bystander A20 cells are killed by other A20 cells that capture and cross-link monomeric Jo2 via FcγR. (A) Jo2 was fractionated by Sepharose CL-2B column chromatography. Heavy chains of Jo2 were detected by Western blotting (inset). A20 cells were incubated with each fraction in the presence (dotted lines) or the absence (solid lines) of 1 μM cytochalasin D for 4 h. Cell viability (%) was measured by MTT assay. (B) Three models of Jo2-induced apoptosis in A20 cells. See text for detail. (C) [^{3}H]TdR-labeled wild-type A20 cells (wt) or FcγR-negative A20 clones (FcγR(−) #1 and FcγR(−) #2) were mixed with (+) or without (−) wild-type A20 cells (E:T ratio = 1) and then incubated in the presence of 200 ng/ml of Jo2 for 4 h. (D) [^{3}H]TdR-labeled FcγR-negative A20 clone FcγR(−) #1 was mixed with wild-type A20 cells (E:T ratio = 1, 0.5, 0.25) in the presence (closed circles) or the absence (open circles) of 200 ng/ml of Jo2 for 4 h. Radioactivity of fragmented DNA was measured. The results represent the mean ± SD of triplicate cultures.

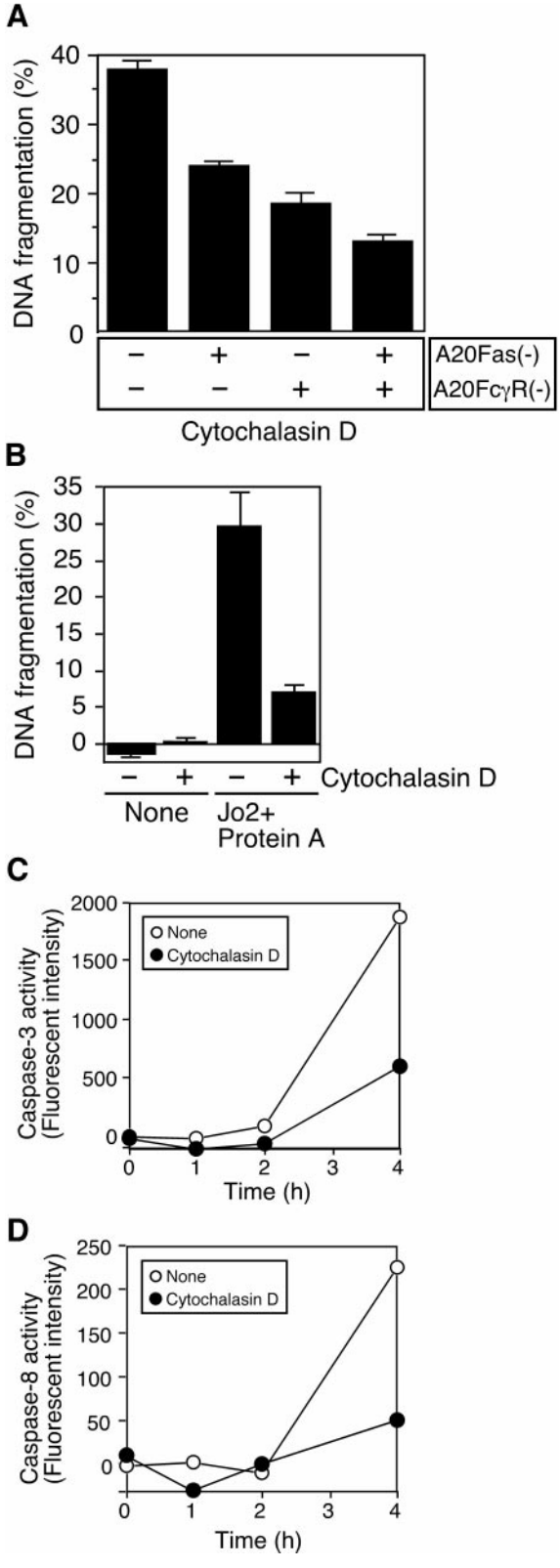

FIG. 6. Cytochalasin D inhibits intracellular Fas signaling pathway in Jo2-induced apoptosis. (A) Fas-negative A20 clone (A20.FO) or [^{3}H]TdR-labeled FcγR-negative A20 clone FcγR(−) #1 was pretreated with (+) or without (−) 10 μM cytochalasin D for 1 h and then washed three times with medium. A20.FO (effector cells) and FcγR(−) #1 (target cells) were mixed (E:T ratio = 1) and incubated with 200 ng/ml of Jo2 for 4 h. (B) FcγR-negative A20 clone FcγR(−) #1 was pretreated with (+) or without (−) 3 μM cytochalasin D for 1 h and then incubated with or without 200 ng/ml of Jo2 plus 200 ng/ml of protein A for 8 h. Radioactivity of fragmented DNA was measured. The results represent the mean ± SD of triplicate cultures. (C and D) FcγR-negative A20 clone FcγR(−) #1 was pretreated with (+) or without (−) 3 μM cytochalasin D for 1 h and then incubated with 100 ng/ml of Jo2 in the presence of 200 ng/ml of protein A for the indicated time periods. Caspase-3 activity (C) and caspase-8 activity (D) were measured by hydrolysis of Ac-DEVD-MCA and Ac-IETD-MCA, respectively. Data points are shown after the subtraction of spontaneous activity.

Bystander A20 Cells Are Killed by Other A20 Cells That Capture Cross-Linked Jo2 via FcγR

Gel-filtration analysis revealed that Jo2 molecules are mostly recovered from fractions corresponding to monomeric antibodies (180 kDa), and their apoptosis inducing activity was found in the same fractions (Fig. 5A). It is well established that Fas receptors should be extensively oligomerized to transmit death signals (8, 13). We thought that FcγR is utilized to cross-link Jo2 molecules to convert active forms or that FcγR signals render cells susceptible to Fas-mediated apoptosis as reported previously (14). Accordingly, three models of FcγR-dependent Jo2-induced apoptosis in A20 cells were initially hypothesized (Fig. 5B). In Model 1, two independent intracellular signals via Fas and FcγR are required for the induction of Fas-mediated apoptosis. In Model 2, Jo2 monomers bind to Fas and these complexes are oligomerized via FcγR to induce apoptosis in a single cell. In Model 3, Jo2 monomers are cross-linked by FcγR on one cell, and Jo2 oligomers kill Fas-expressing bystander cells. To address if Fas and FcγR should be placed on a single cell, FcγR-negative A20 cells ([^{3}H]TdR-labeled target cells) were mixed with FcγR-expressing A20 cells as effector cells in the presence of Jo2 (Figs. 5C and 5D). FcγR-negative A20 clones became susceptible to Jo2 as comparable as wild type A20 cells in the presence of FcγR-expressing parental cells. Therefore, as shown in Model 3, bystander A20 cells seem to be killed by other A20 cells that capture cross-linked Jo2 via FcγR.

Cytochalasin D Inhibits Early Signaling Events Upstream of Caspase-8 Activation in Apoptosis Induced by Jo2

We further addressed if cytochalasin D either affects FcγR-mediated cross-linking of Jo2 molecules or intracellular apoptosis signals after Fas engagement. As shown in Fig. 6A, DNA fragmentation was strongly inhibited when either A20.FO (effector cells) or A20 FcγR(−) clone (target cells) was pretreated with cytochalasin D. In the presence of the cross-linking agent protein A, Jo2 induced a significant induction of

apoptosis even in the FcγR-negative A20 clone (Fig. 6B). Under the same experimental conditions, cytochalasin D markedly inhibited the induction of apoptosis (Fig. 6B). These observations clearly indicate that cytochalasin D blocks intracellular apoptotic signals after Fas engagement, although the process of FcγR-mediated Jo2 cross-linking was affected by the drug. We further analyzed the effect of cytochalasin D on the intracellular signal transduction upon treatment with cross-linked Jo2 by using FcγR-negative A20 cells. Cytochalasin D significantly inhibited caspase-3 activation (Fig. 6C) as well as caspase-8 activation (Fig. 6D), suggesting that cytochalasin D targets early signaling events upstream of caspase-8 activation in Jo2-induced apoptosis.

DISCUSSION

Trimeric FasL is insufficient to induce apoptosis, whereas membrane-bound or cross-linked soluble FasL triggers aggregation of Fas trimers prerequisite for apoptosis induction (8, 13). Agonistic anti-Fas antibodies were believed to mimic physiological FasL to induce endogenous signaling pathway. It was reported that two types of cells exhibit different death signaling pathways upon treatment with anti-Fas antibodies (15). In type I cells, anti-Fas antibodies induce abundant recruitment of FADD and caspase-8 (DISC formation), resulting in apoptosis insensitive to Bcl-2. In type II cells, anti-Fas antibodies induce barely detectable DISC formation, and Fas-mediated apoptosis is blocked by Bcl-2. However, the physiological significance of these two Fas signaling pathways is controversial, since it was recently reported that Bcl-2 does not block apoptosis induced by physiological FasL in type II cells (16). In the present work, we have demonstrated that, in contrast to cross-linked FasL, anti-Fas antibody Jo2 induces apoptotic signals that depend on intact actin cytoskeleton in murine B lymphoma A20 cells. Thus, our study provides additional evidence that anti-Fas antibodies seem to transmit intracellular apoptotic signals functionally distinct from physiological FasL.

It was reported that ezrin is localized in the plasma membrane, and this protein links Fas to actin cytoskeleton and is essential for Fas-mediated apoptosis (17). In different cell types, actin cytoskeleton is involved in the regulation of susceptibility to apoptosis induced by anti-Fas antibodies (17, 18). In this work, we have shown that cytochalasin D inhibits apoptosis induced by anti-Fas antibody Jo2 but not FasL, and targets endogenous signaling events upstream of caspase-8 activation. Thus, in contrast to physiological FasL, anti-Fas antibodies seem to induce Fas-mediated signals that exclusively depend on actin cytoskeleton. The three-dimensional model of the Fas molecules clearly demonstrates that anti-Fas antibodies do not identically mimic physiological FasL to induce Fas clusters (19). Thus, it is likely that actin cytoskeleton interacts with the Fas molecules and facilitates aggregation and stabilization of the Fas clusters essential for the recruitment of FADD and caspase-8 upon treatment with anti-Fas antibodies.

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REFERENCES

1. Nagata, S. (1997) Apoptosis by death factor. *Cell* **88,** 355–365.
2. Ashkenazi, A., and Dixit, V. M. (1998) Death receptors: Signaling and modulation. *Science* **281,** 1305–1308.
3. Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995) Cytotoxicity-dependent Apo-1 (Fas/CD95)-associated proteins from a death-inducing signaling complex. *EMBO J.* **14,** 5579–5588.
4. Yonehara, S., Ishii, A., and Yonehara, M. (1989) A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* **169,** 1747–1756.
5. Trauth, B. C., Klas, C., Peters, A. M. J., Matzku, S., Moller, P., Falk, W., Debatin, K.-M., and Krammer, P. H. (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* **245,** 301–305.
6. Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., and Nagata, S. (1993) Lethal effect of the anti-Fas antibody in mice. *Nature* **364,** 806–809
7. Kataoka, T., Shinohara, N., Takayama, H., Takaku, K., Kondo, S., Yonehara, S., and Nagai, K. (1996) Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J. Immunol.* **156,** 3678–3686.
8. Schneider, P., Holler, N., Bodmer, J. L., Hahne, M., Frei, K., Fontana, A., and Tschopp, J. (1998) Conversion of membrane-bound Fas (CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J. Exp. Med.* **187,** 1205–1213.
9. Park, S. Y., Kojima, M., Suzuki, H., and Shinohara, N. (1992) Effective blocking of natural cytotoxicity of young rabbit serum on murine thymocytes by high concentrations of glucose in complement-dependent cytotoxicity method. *J. Immunol. Methods* **154,** 109–119.
10. Stennicke, H. R., and Salvesen, G. S. (2000) Caspase assays. *Methods Enzymol.* **322,** 91–100.
11. Sekita, S., Yoshikawa, K., Natori, S., Udagawa, S., Sakabe, F., Kurata, H., Umeda, M. (1982) Chaetoglobosins, cytotoxic 10-(indol-3-yl)-[13]cytochalasins form *Chaetomium* spp. I. Production, isolation and some cytological effects of chaetoglobosins A–J. *Chem. Pharm. Bull. (Tokyo)* **5,** 1609–1617.
12. Scaffidi, C., Schmidt, I., Krammer, P. H., and Peter, M. E. (1999) The role of c-FLIP in modulation of CD95-induced apoptosis. *J. Biol. Chem.* **274,** 1541–1548.

13. Tanaka, M., Itai, T., Adachi, M., and Nagata, S. (1998) Down-regulation of Fas ligand by shedding. *Nat. Med.* **4,** 31–36.

14. Jodo, S., Hohlbaum, A. M., Xial, S., Chan, D., Strehlow, D., Sherr, D. H., Marshak-Rothstein, A., and Ju, S.-T. (2000) CD95 (Fas) Ligand-expressing vesicles display antibody-mediated, FcR-dependent enhancement of cytotoxicity. *J. Immunol.* **165,** 5487–5494.

15. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K.-M., Krammer, P. H., and Peter, M. E. (1998) Two CD95 (Apo-1/Fas) signaling pathway. *EMBO J.* **17.** 1675–1687.

16. Huang, D. C., Hahne, M., Schroeter, M., Frei, K., Fontana, A., Villunger, A., Newton, K., Tschopp, J., and Strasser, A. (1999) Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-X_L. *Proc. Natl. Acad. Sci. USA* **96,** 14871–14876.

17. Parlato, S., Giammarioli, A. M., Logozzi, M., Lozupone, F., Matarrese, P., Luciani, F., Falchi, M., Malorni, W., and Fais, S. (2000) CD95 (APO-1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: A novel regulatory mechanism of the CD95 apoptotic pathway. *EMBO J.* **19,** 5123–5134.

18. Varadhachary, A. S., Edidin, M., Hanlon, A. M., Peter, M. E., Krammer, P. H., and Salgame, P. (2001) Phosphatidylinositol 3′-kinase blocks CD95 aggregation and caspase-8 cleavage at the death-inducing signaling complex by modulating lateral diffusion of CD95. *J. Immunol.* **166,** 6564–6569.

19. Fadeel, B., Lindgerg, J., Achour, A., and Chiodi, F. (1998) A three-dimensional model of the Fas/APO-1 molecule: Cross-reactivity of anti-Fas antibodies explained by structural mimicry of antigenic sites. *Int. Immunol.* **10,** 131–140.