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Rapamycin Enhances Apoptosis and Increases Sensitivity to Cisplatin *in Vitro*[1]

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ABSTRACT

Apoptosis can be regulated in a number of different systems by the actions of cytokines. Rapamycin has been shown to exert its effects on growth factor-induced cell proliferation, at least in part, by blocking the activation of the p70 S6 kinase and thus preventing the downstream signaling process, such as the activation of the members of the cdk family. To determine whether this pathway plays a role in the regulation of apoptosis, we assessed the effect of rapamycin on apoptosis induced by interleukin 2 deprivation in murine T-cell lines, by T-cell receptor ligation in a murine T-cell hybridoma, by enforced c-*myc* expression in murine fibroblasts, and by corticosteroids in murine T-lymphoma cell lines. Although rapamycin did not induce apoptosis on its own, rapamycin augmented apoptosis in each of the cell lines used as indicated by increased genomic DNA fragmentation, decreased cell viability, and characteristic apoptotic changes in morphology. These results suggest that a signal transduction pathway(s) inhibited by rapamycin plays an important role in the susceptibility of cells to apoptosis. Many chemotherapeutic agents kill cancer cells through the induction of apoptosis. Strikingly, rapamycin increased the ability of the alkylating agent, cisplatin, to induce apoptosis in the human promyelocytic leukemia cell line HL-60 and the human ovarian cancer cell line SKOV3. These data suggest that a signal transduction pathway, likely related to p70 S6 kinase, inhibited by rapamycin may be an important component of the pathway which prevents cell death in many cell lineages and also indicate that rapamycin has the potential to augment the efficacy of selected anticancer therapies.

INTRODUCTION

The discovery of the immunosuppressive drugs CsA,[3] FK506, and rapamycin has revolutionized organ transplantation and the treatment of autoimmune diseases (1). These immunosuppressants exert their effects by binding to a class of intracellular proteins called immunophilins, specifically interfering with the signaling pathways leading to cytokine production or proliferation of T lymphocytes upon activation (2). CsA binds to an immunophilin called cyclophilin A, while FK-506 and rapamycin bind to FKBP (2). Both cyclophilin and FKBP are peptidyl-prolyl *cis-trans* isomerases, the enzymatic activity of which is inhibited by the immunosuppressants. However, inhibition of the peptidyl-prolyl *cis-trans* isomerase does not account for the demonstrated immunosuppressive activity (3). The complex of cyclophilin A and cyclosporin A, as well as the complex of FK506 and FKBP, binds to and inactivates calcineurin, an intracellular calcium/calmodulin-activated protein phosphatase (2); while the complex of rapamycin and FKBP exerts its effect, at least in part, on the p70 S6 kinase pathway (4). Studies of the mechanisms by which these immunosuppressive drugs act have not only demonstrated how they function in cells but also have proved that they are useful tools to dissect cell signaling pathways (5).

Unlike cyclosporin A and FK506, which suppress a calcium-dependent pathway in the early stages of T-cell activation, rapamycin does not alter the early events following the activation of T cells through the T-cell antigen receptor, although it binds to and competes with FK506 for the same protein, FKBP (6). Instead, rapamycin inhibits signal transduction from the IL-2, epidermal growth factor, and other cytokine receptors, thus blocking the G_1 to S phase transition required for cell cycle progression (6). In addition, rapamycin also inhibits the proliferation of 3T3 cells (7) and the hepatoma cell line H4 (8), presumably by blocking the effects of the growth factor-like activity of serum. The signal transduction pathway inhibited by the rapamycin-FKBP complex is not completely understood. Regardless of the mechanism, rapamycin blocks the activation of p70 S6 kinase by diverse agents. It has also been shown that rapamycin blocks the activation of p34cdc2 kinase in T cells and in the myogenic cell line BC3H1 (9), presumably because p34cdc2 is a downstream target of p70 S6 kinase. This, combined with the evidence that p34cdc2 deregulation is an obligatory component of the induction of apoptosis by natural killers in target cells (10), suggests that rapamycin could potentially alter the induction of apoptosis.

Apoptosis, a physiologically programmed event, is an active suicide process requiring energy-dependent participation of the dying cells (11). Apoptosis can be induced in T cells by activation, or in growth factor- and hormone-dependent cells by deprivation of the dependent factors, or in malignant cells by chemotherapeutic agents. Cyclosporin A and FK-506 have been shown to block activation-induced apoptosis in T-cell lines (12), while rapamycin does not (13). Herein, we report that rapamycin augments apoptosis in a number of different systems. This effect can be demonstrated in the murine T-cell line CTLL-2 induced by IL-2 withdrawal, in T-cell hybridomas induced by activation through the T-cell antigen receptor, in murine S49 cells treated with steroids, and in *myc*-transformed RAT-1 fibroblasts induced by culturing under low serum conditions. Strikingly, rapamycin also promotes apoptosis in the human promyelocytic line HL-60 cells and the human ovarian cancer SKOV3 cells induced by the chemotherapeutic drug, cisplatin, indicating potential clinical applications.

MATERIALS AND METHODS

Reagents. MTT and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). Rapamycin was provided by the National Cancer Institute, NIH (Bethesda, MD). Recombinant human IL-2 was obtained from the former Cetus Corp. (La Jolla, CA). Cisplatin was from David Bull Laboratories Pty Ltd. (Mulgrave, Victoria, Australia). FK520 was a gift of Dr. N. H. Sigal (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ). Unless otherwise indicated, all other chemicals were the purest grade available and were obtained from Sigma.

Cell Lines. The T-cell hybridoma A1.1 was a gift of Dr. B. Singh (University of Western Ontario, London, Ontario, Canada; Ref. 14). They were recloned and selected for responsiveness to activation stimuli and T-cell receptor expression. A hamster anti-murine CD3 B cell hybridoma, 145–2c11 (15), was used as sources of antibodies to the T-cell receptor complex. The

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[3] The abbreviations used are: CsA, cyclosporin A; FKBP, FK-506/rapamycin binding protein; IL-2, interleukin 2; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

cell line HL-60, and human ovarian cancer cell line SKOV3 were obtained from American Type Culture Collection (Rockville, MD). The mouse lymphoma, S49, was kindly provided by Dr. G. T. Williams (University of Birmingham, Birmingham, United Kingdom). Rat-1 cells constitutively expressing c-*myc* under the control of the muLV retroviral promoter have been described previously (16). All cells were cultured at 37°C in humidified atmosphere containing 5% CO_2 in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 μM 2-mercaptoethanol, 5–10% heat-inactivated fetal bovine serum (Sigma), and 10 μM gentamicin (GIBCO).

Apoptosis Induction. The T-cell hybridoma A1.1 was activated by anti-CD3 coated on tissue culture plastic by incubating with 0.05 M Tris-HCl (pH 9.0) overnight at 4°C or for 1 h at 37°C. Plates were washed with PBS to remove unbound antibody before A1.1 cells were added. Cells were harvested for DNA fragmentation analysis after a 12-h incubation at 37°C in humidified 5% CO_2. Apoptosis in CTLL-2 cells was induced by IL-2 starvation, in S49 cells by dexamethasone treatment, in Rat-1-*myc* cells by low serum, and in HL-60 cells and SKOV3 cells by cisplatin treatment as indicated.

Genomic DNA Fragmentation Assay. Cells ($4–6 \times 10^5$) were harvested and resuspended in an Eppendorf tube in 30 μl PBS and lysed with 30 μl of lysis buffer [80 mM EDTA, 200 mM Tris (pH 8.0), 1.6% (w/v) sodium lauryl sarcosinate, and 5 mg proteinase K/ml]. The lysate was mixed and then incubated in a 50°C water bath for 1.5 h. After adding 0.2 mg/ml RNase A, the mixture was incubated in a 37°C water bath for an additional 30 min. The resulting DNA solution was analyzed on 1% agarose gels in TAE buffer (10 mM Tris and 1 mM EDTA).

Alternatively, genomic DNA fragmentation was quantitated by labeling actively dividing cells with ^{125}IUDR at the concentration of 10^6 cells/ml with 1 μCi/ml of ^{125}IUDR at 37°C for 8–10 h. Labeled cells were harvested and washed with cold media at least three times. Treatments were then carried out in 200 μl media in 96-well tissue culture plates; genomic DNA fragmentation was then assayed as follows. Cells were harvested in 1.5-ml Eppendorf tubes and lysed by adding 900 μl lysis buffer [5 mM Tris (pH 7.4), 2 mM EDTA, and 0.5% Triton X-100 (nonionic detergent); total volume, 1.1 ml]. The Eppendorf tubes were then vortexed vigorously to ensure complete lysis of cells. After incubating on ice for 20 min, the tubes were centrifuged at 14,000 cpm for 20 min in a microfuge. One ml of supernatant (containing fragmented DNA) was transferred to a new Eppendorf tube, leaving 100 μl supernatant with the pellet to ensure that the pellets were not cotransferred. The radioactivity of the supernatant and the pellet were measured with a gamma counter. The percentage of fragmentation was calculated by:

$$\% \text{ DNA fragmentation} = \frac{\text{Supernatant cpm} \times 1.1}{\text{Supernatant cpm} + \text{pellet cpm}} \times 100$$

MTT Staining. Cell viability was assessed essentially as described by Mosmann (17). Briefly, cells were incubated in 100 μl media in 96-well plates with additions as indicated. Following incubation, 10 μl of MTT solution (5 mg MTT/ml in H_2O) were added and incubated at 37°C for 4 h. One hundred μl of acid-isopropanol (0.04 N HCl in isopropanol) were added to each culture and mixed by pipetting or shaking on a plate shaker to dissolve the reduced MTT crystals; the relative cell viability was obtained by scanning with an ELISA reader with a 570-nm filter.

Cell Proliferation Assay. Cells were incubated in 96-well plates with appropriate treatments. The proliferative response was determined by [^{3}H]thymidine incorporation in which 1 μCi of [^{3}H]thymidine was added to each well and incubated for 3 h. Cells were then harvested onto glass-fiber filter paper, and the rate of [^{3}H]thymidine uptake was quantitated by liquid scintillation counting.

RESULTS

The immunosuppressive effect of rapamycin is not through its effect on the signals from the T-cell antigen receptor but rather through the effects on the signals induced by IL-2 produced after T-cell activation (18). We tested the effect of rapamycin on IL-2-induced proliferation of a murine T-cell line, CTLL-2, by varying the concentrations of both IL-2 and rapamycin. As indicated by [^{3}H]thymidine incorporation shown in Fig. 1, IL-2 and rapamycin showed a

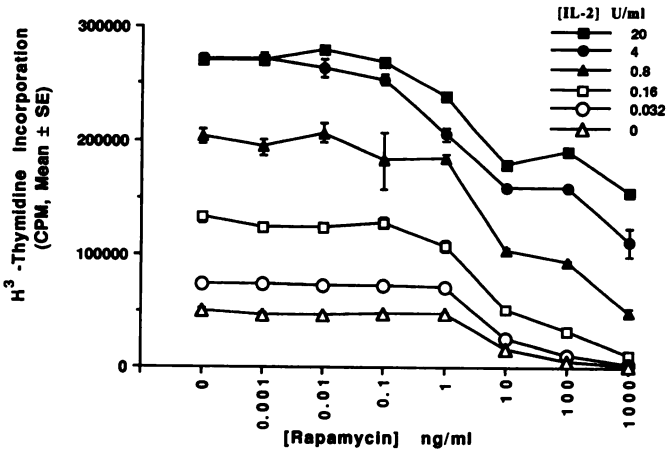

Fig. 1. Effect of rapamycin on IL-2-induced cell proliferation. CTLL-2 cells were plated in 96-well plates at 1×10^4 cells/well with different concentrations of rapamycin and recombinant human IL-2. After 20 h, 1 μCi of [^{3}H]thymidine was added to each well for 3 h. Cell proliferation was determined by measuring [^{3}H]thymidine incorporation by scintillation counting. Results represent the mean of six wells; *bars*, SE.

reciprocal effect; the higher the concentration of IL-2 in the culture medium, the higher the concentration of rapamycin required to suppress the proliferation of CTLL-2 cells to background levels. (The effect of rapamycin on IL-2-induced proliferation was analyzed by two-way ANOVA, $P < 0.0001$.) This suggests that the concentration of IL-2 determines the quantity or quality of the transmembrane signals and that rapamycin is able to completely block IL-2-induced proliferation only in the presence of relatively low concentrations of IL-2.

Since IL-2 induces proliferation in responsive cells, IL-2 must provide both mitogenic and cell survival signals upon the interaction with its receptor. It is conceivable that the effect of rapamycin on IL-2-induced proliferation of CTLL-2 cells might be due to the inhibition of cell survival signals rather than on the mitogenic signal induced by IL-2. We tested this hypothesis by using a model in which IL-2 deprivation induces apoptosis in CTLL-2 cells. As shown previously (19), 24 to 48 h after IL-2 withdrawal, the majority of CTLL-2 cells undergo apoptosis. When rapamycin was added, there was a significant increase of apoptosis in CTLL-2 cells as indicated by genomic DNA fragmentation assessed on agarose gels (Fig. 2) and by release of label from ^{125}IUDR-labeled cells (data not presented). However, this effect was only apparent when limiting concentrations of IL-2 were present. Indeed, in the presence of IL-2 (10 units/ml), rapamycin did not induce apoptosis at any concentration tested (Fig. 2 and data not shown). This is apparently discordant to Fig. 1, in which cell proliferation induced by as much as 20 units/ml of IL-2 can still be decreased by high concentrations of rapamycin. The most likely explanation for this discrepancy is that apoptosis only occurs when IL-2 signals are decreased below a critical threshold. The immunosuppressant FK-520, an analogue of FK506 which competes with rapamycin for FKBP, did not promote apoptosis, but rather reversed the effect of rapamycin when FK520 was present at a 20-fold excess (Fig. 3), indicating that the apoptosis-promoting effect of rapamycin depends on the binding of rapamycin to FKBP.

The distinction between cell survival and cell proliferation signals has been well established by genetic complementation of bcl-2 and *myc* (20, 21). The proto-oncogene c-*myc*, which plays an important role in cell proliferation and transformation, is also required for the induction of apoptosis in some cell lineages (22, 23). When cells constitutively expressing *myc* are cultured under reduced serum conditions, they undergo apoptosis (23). In this case, apoptosis can be suppressed by cell survival signals provided by the constitutive

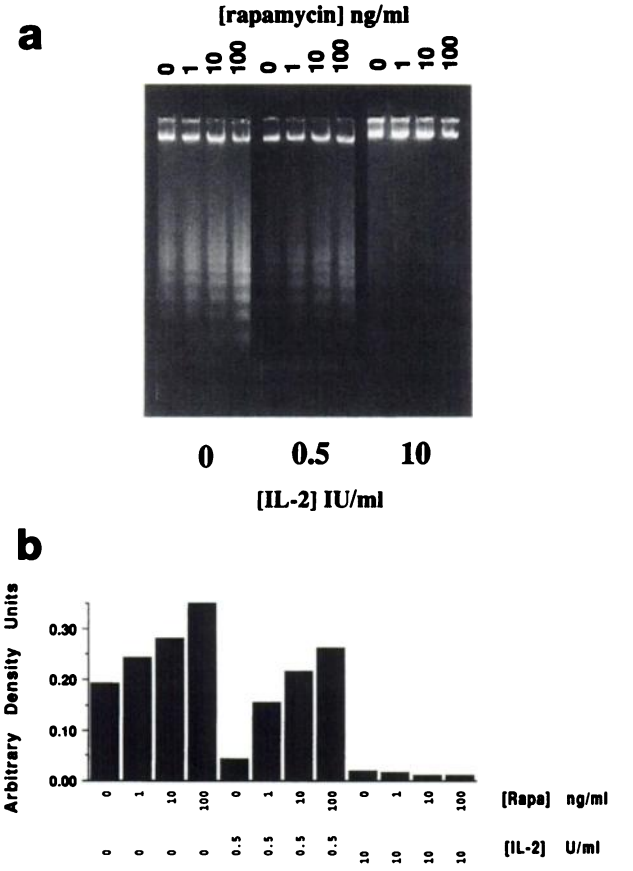

Fig. 2. Rapamycin increases IL-2 deprivation-induced genomic DNA fragmentation. CTLL-2 cells (1×10^6) were cultured without or with IL-2 at 0.5 or 10 units/ml, and rapamycin at 0, 1, 10, or 100 ng/ml. After 20 h, cells were harvested and lysed, and genomic DNA was extracted and analyzed by agarose gel electrophoresis (*a*). Densitometric analysis of the fragmented DNA of the second bands in the agarose gel is also presented (*b*).

expression of *bcl-2* or the presence of high concentrations of serum (20, 23). In accordance with previous studies, when Rat-1 cells constitutively expressing c-*myc* were cultured in media containing 0.5% serum, there was a dramatic decrease in cell viability as indicated by MTT reduction. This decrease in cell viability under low serum conditions was augmented by the addition of 10 ng/ml rapamycin (Fig. 4). Thus, rapamycin increases programmed cell death induced by the constitutive expression of c-*myc*, likely by interfering with cell survival signals mediated by the low concentration of serum present in the assays (one-way ANOVA, $P < 0.001$). As with CTLL-2 cells incubated with high concentrations of IL-2 (Figs. 1 and 2), rapamycin exerted little effect on apoptosis in RAT-1 cells in the presence of high concentrations of serum ($P < 0.05$).

Activation-induced apoptosis in T-cell hybridoma cells has been used as a model system to explore the mechanism regulating negative selection during T-cell development in the thymus (24). Cyclosporin A and FK-506 can completely block activation-induced apoptosis in T-cell hybridoma A1.1 cells (12, 13) and other cells (data not shown). However, when the effect of rapamycin was tested, it was found that, unlike CsA or FK506, rapamycin does not block activation-induced apoptosis (13). We sought to determine whether rapamycin would promote apoptosis in this system following activation of A1.1 cells with varying concentrations of anti-CD3. At an optimal dose of anti-CD3 (2 μg/ml), rapamycin did not alter activation-induced apoptosis in A1.1 cells, likely because the majority of the cells were committed to undergo apoptosis by anti-CD3 alone (25). However, at lower doses of anti-CD3 (0.5 μg/ml or lower), rapamycin dramatically promoted anti-CD3-induced apoptosis in A1.1 cells as indicated by genomic DNA fragmentation detected by agarose gel electrophoresis (Fig. 5, *a* and *b*) and by the release of ^{125}IUDR from labeled cells (Fig. 5*c*). Rapamycin alone did not induce apoptosis in the T-cell hybridoma cells (Fig. 5).

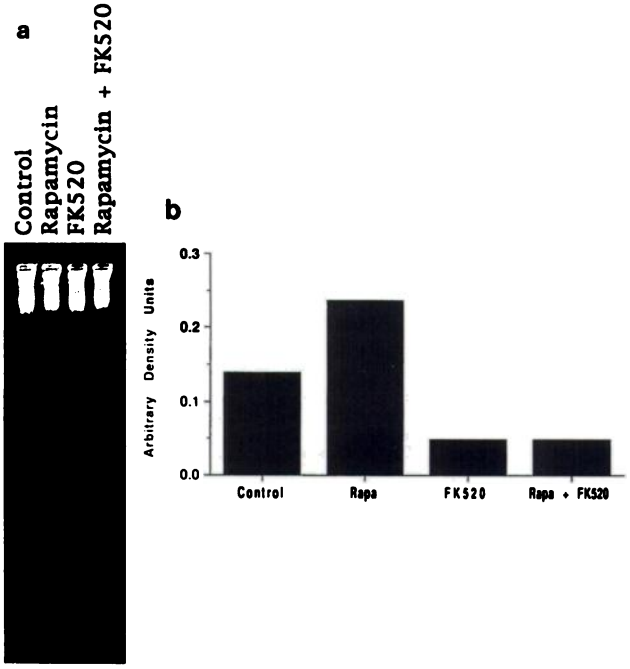

Fig. 3. FK520 reverses the effect of rapamycin on genomic DNA fragmentation induced by IL-2 deprivation. CTLL-2 cells (1×10^6) were cultured with or without rapamycin at 1 ng/ml in the presence or absence of FK520 at 20 ng/ml. Cells were harvested and assessed for genomic DNA fragmentation on an agarose gel following a 20-h incubation (*a*). Densitometric analysis of the second bands in the gel is presented in *b*.

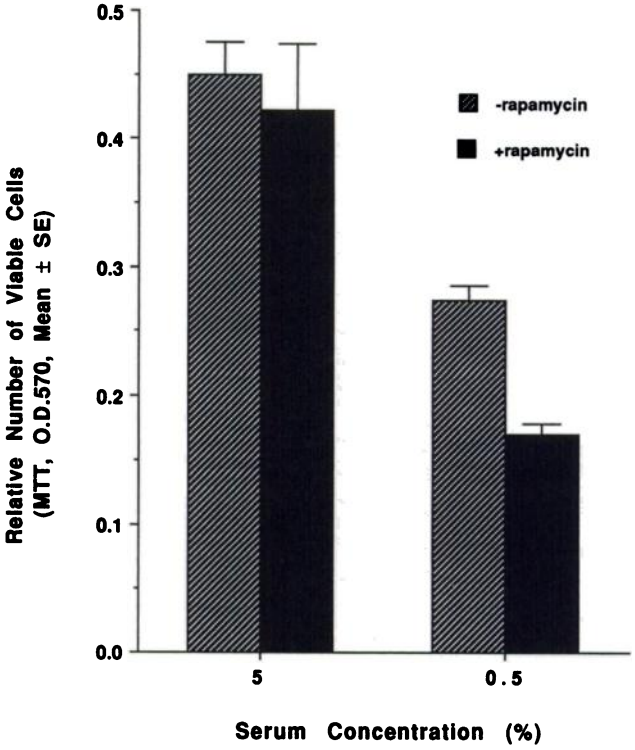

Fig. 4. Rapamycin enhances programmed cell death in *myc*-transformed fibroblasts induced by serum deprivation. *myc*-transformed Rat-1 cells were cultured in RPMI supplemented with 5 or 0.5% fetal bovine serum for 60 h with or without rapamycin at 10

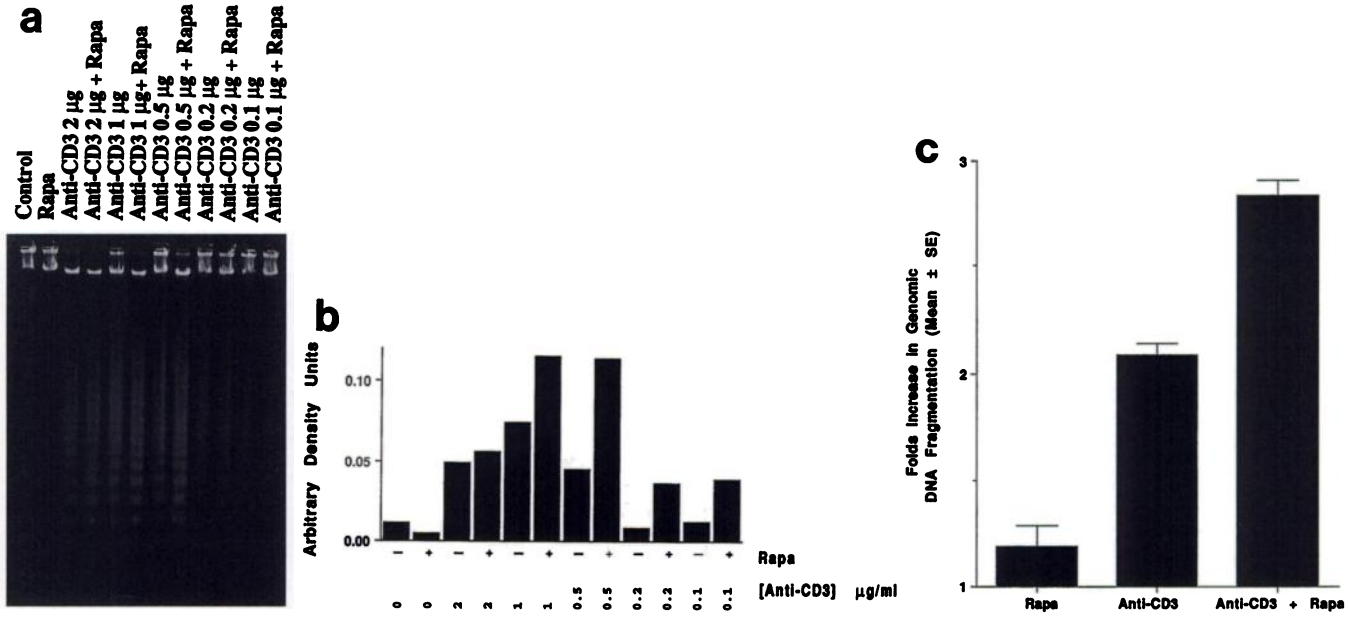

Fig. 5. Rapamycin augments activation-induced genomic DNA fragmentation in mouse T-cell hybridoma cells. T-cell hybridoma A1.1 cells were stimulated with different concentrations of anti-CD3 (145-2c11) coated on tissue culture plastic with or without rapamycin at 10 ng/ml. Cells were harvested 12 h after culture and assessed for genomic DNA fragmentation by agarose gel electrophoresis (*a*). Densitometric analysis of the fragmented genomic DNA is presented in *b*. A1.1 cells were also labeled with ^{125}IUDR at 1 μCi/1 × 10^6 cells/ml. After 10 h, cells were washed and cultured with 0.5 μg/ml anti-CD3 for 12 h. The percentage of genomic DNA fragmentation was assessed by the release of ^{125}IUDR-labeled fragmented DNA (*c*); *bars*, SE.

Corticosteroid-induced apoptosis in T cells and thymocytes is the best characterized model system for studying programmed cell death (26). The corticosteroid-sensitive mouse lymphoma cell line S49 undergoes characteristic apoptosis upon treatment with dexamethasone. When ^{125}IUDR-labeled S49 cells were incubated with a suboptimal concentration of dexamethasone (10^{-7} M), rapamycin significantly increased genomic DNA fragmentation (Fig. 6; one-way ANOVA; dexamethasone *versus* dexamethasone plus rapamycin; $P < 0.001$).

It has been demonstrated that chemotherapeutic reagents can induce apoptosis in target cells (27). Rapamycin has already been tested clinically as an immunosuppressant in patients and is relatively nontoxic in short-term administration (28). If rapamycin can augment apoptosis induced by chemotherapeutic reagents, then the addition of rapamycin to chemotherapy protocols could potentially increase the efficacy of chemotherapy. Cisplatin, an effective chemotherapy agent, has been shown to induce apoptosis in a number of cell lineages (29). A 24-h incubation with cisplatin at 30 μM or more induced DNA fragmentation in the human promyelocytic leukemia cell line HL-60, and as predicted, rapamycin augmented cisplatin-induced DNA fragmentation (Fig. 7*a*). The densitometric analysis of the gel is presented in Fig. 7*b*. However, as before, rapamycin did not augment the effect of cisplatin at doses which already induced maximal apoptosis (*i.e.*, 100 μM cisplatin). When HL-60 cells were incubated with cisplatin for 96 h, there was a dose-dependent reduction of cell viability, as determined by MTT reduction, which was detectable at 1 μM and reached maximal effects at 5 to 10 μM (Fig. 7*c*; $R^2 = 0.995$ from 0.625 μM to 5 μM; note: the different concentrations of cisplatin in the experiments in Fig. 7, *a* and *b*, reflect the different periods of incubation). We selected 2.5 μM cisplatin as a suboptimal dose and determined the effect of rapamycin. In this system, rapamycin induced a dose-dependent augmentation of cisplatin-induced reduction of cell viability (Fig. 7*d*; two-way ANOVA, $P < 0.001$ and $R^2 = 0.982$ for rapamycin from 0.01 nM to 10 nM). In contrast, rapamycin alone did not significantly alter HL-60 viability at several concentrations tested (Fig. 7). The effect of rapamycin on cell death induced by suboptimal doses of cisplatin was also readily observed by changes in morphology at the microscopic level (Fig. 7*e*). Cisplatin at 2.5 μM induced morphological changes consistent with apoptosis in only a small proportion of cells; rapamycin alone had no effect. However, when cells were coincu-

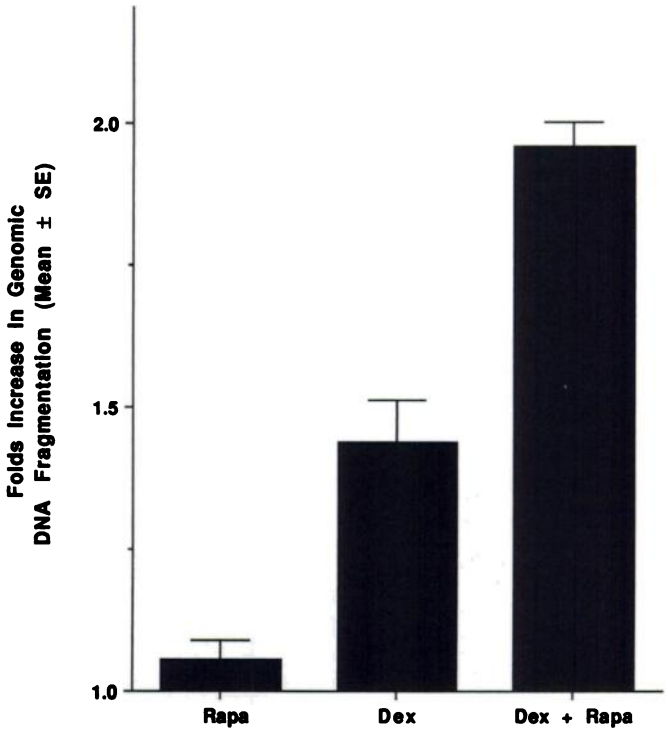

Fig. 6. Rapamycin increases genomic DNA fragmentation in steroid-sensitive cells. S49 cells were labeled with ^{125}IUDR at 1 μCi/1 × 10^6 cells/ml. After 10 h, cells were washed and cultured with dexamethasone at 10^{-7} M for 36 h. Percentage of genomic DNA fragmentation was assessed by the release of ^{125}IUDR-labeled fragmented DNA. *Bars*, SE.

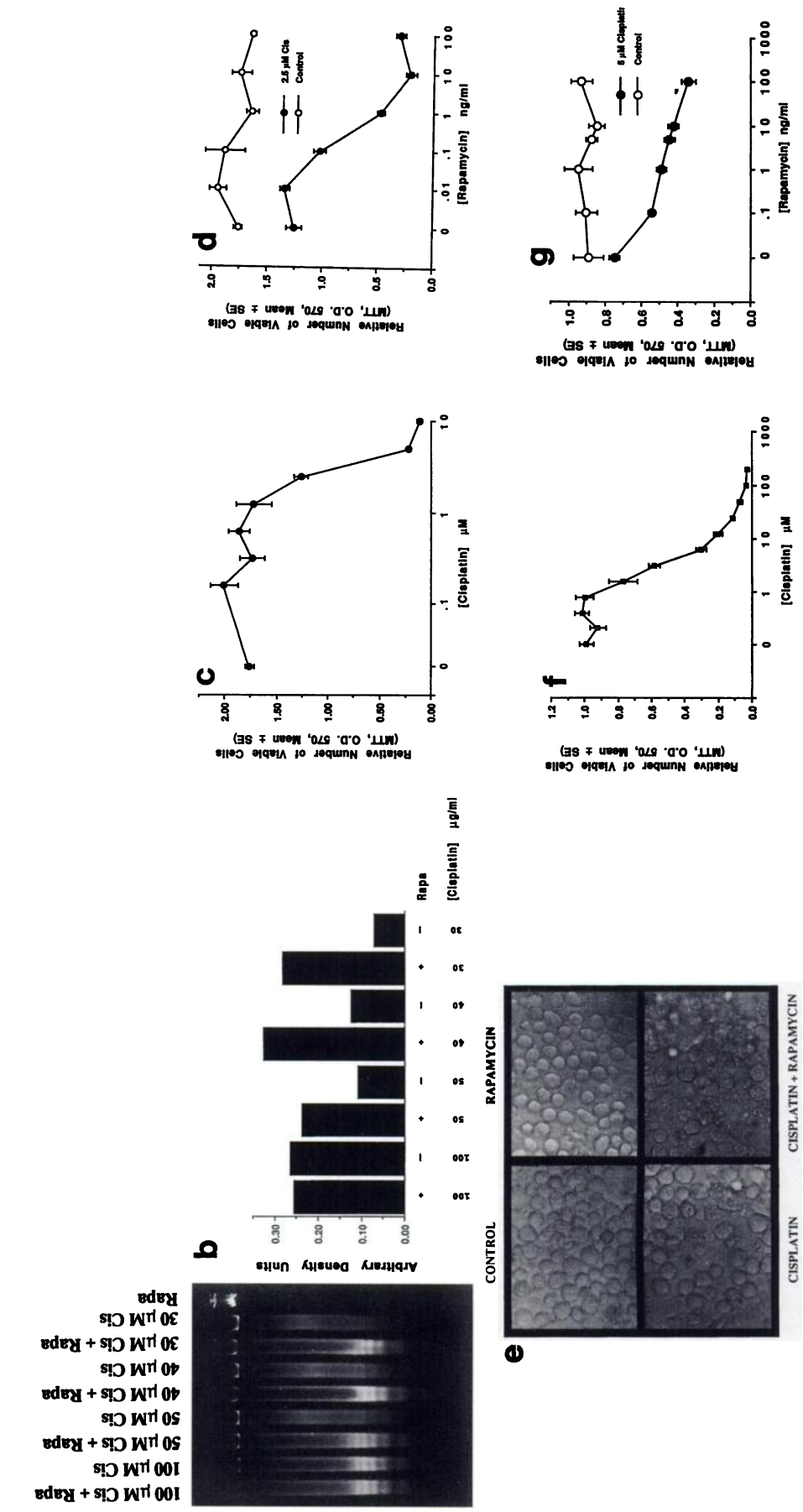

Fig. 7. Rapamycin augments cisplatin-induced apoptosis in human cancer cells. *a*, human promyelocytic leukemia HL-60 cells were incubated with different concentrations of cisplatin with or without rapamycin at 10 ng/ml for 24 h. Genomic DNA was extracted and analyzed by agarose gel electrophoresis. *b*, the density of the second bands of the fragmented genomic DNA in *a* is analyzed by densitometry. *c*, dose-response of the effect of cisplatin on HL-60 viability. HL-60 cells were cultured with different concentrations of cisplatin for 72 h, and cell viability was determined by the reduction of MTT; *bars*, SE. *d*, rapamycin augments cell viability reduction induced by cisplatin. HL-60 cells were cultured with or without 2.5 μM cisplatin in the presence or absence of different concentrations of rapamycin. Cell viability was assessed at 72 h by reduction of MTT; *bars*, SE. *e*, rapamycin enhances cisplatin-induced morphological changes. HL-60 cells were cultured with or without rapamycin at 1 ng/ml in the presence or absence of 2.5 μM cisplatin. Morphological changes were assessed 72 h after the initiation of culture. *f*, the dose-response of the effect of cisplatin on the viability of the human ovarian cancer cell line SKOV3 cells. MTT assay was carried out at 72 h after treatment; *bars*, SE. *g*, the effect of rapamycin on cisplatin-induced cell viability reduction in SKOV3 cells. Cells were treated with or without 5 μM cisplatin in the presence or absence of different concentrations of rapamycin. Cell viability was assessed by MTT staining at 72 h; *bars*, SE.

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