Fas-mediated Signaling Enhances Sensitivity of Human Soft Tissue Sarcoma Cells to Anticancer Drugs by Activation of p38 Kinase[1]

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Abstract

Sensitivity of human soft tissue sarcoma (STS) cells to methotrexate, doxorubicin, and paclitaxel was examined after cells were pretreated with CH-11, an agonistic anti-Fas antibody. A subtoxic dose (6 ng/ml) of CH-11 sensitized STS cells but not normal fibroblast cells to these anticancer drugs. CH-11 increased cytochrome *c* release and consequent activation of caspase-9, independent of caspase-8 and increased p38 activation. Addition of SB203580, a specific inhibitor of p38, resulted in a decrease in activation of this kinase and abrogation of enhanced chemosensitivity (doxorubicin and paclitaxel) by CH-11. These results demonstrate that stimulation of the Fas pathway by a subtoxic dose of a Fas agonist can selectively enhance sensitivity of STS cells to certain chemotherapeutic agents through activation of p38.

Introduction

The poor response of STSs[3] to anticancer drug-induced cell death is a major obstacle to successful treatment. Understanding how to enhance drug-induced cell death through specific pathways may help to improve chemotherapy in this and other solid tumors. Cell death induced by anticancer drugs, regardless of distinct intracellular targets of drug action, may finally link to activation of mitochondrial caspase-9, a common death pathway (1, 2). Therefore, the pathways that potentate activation of mitochondrial caspase-9 may enhance drug-induced cell death or drug sensitivity. Among these pathways, the Fas/Fas-L system is considered to be an attractive candidate for this approach. Activation of the Fas receptor by its natural ligand or by agonistic antibodies generates a death-inducing signaling complex, which recruits caspase-8 through Fas-associated death domain (FADD)/MORT-1 and leads to activation of the protease cascade and finally to cell death (3, 4). More importantly, activation of the Fas receptor also triggers mitochondria apoptotic events through cleavage of Bid by caspase-8 (5, 6).

In addition, stimulation of the Fas receptor may involve activation of other signaling pathways such as the MAPK pathway through a caspase-dependent and -independent manner (7, 8). MAPK has been thus far identified to be comprised of three subfamilies: classical MAPK (ERK); stress-activated protein kinase (JNK); and p38 kinase. Activation of the MAPK pathway may result in different effects on the regulation of cell death depending on type of MAPK affected and cell conditions. For example, activation of ERK can protect tumor cells from Fas-induced apoptosis (9), whereas inhibition of the p38 may diminish DNA damaging agent-induced cell death (10, 11). Of interest, activation of the p38 kinase appears to trigger an apoptotic response to cellular stress in tumor cells but not in normal cells (12, 13).

Enhancement of anticancer drug sensitivity by Fas agonists has been reported (14). However a toxic dose of Fas-L agonist was used in this study, and furthermore, the mechanism of how Fas signaling potentiates drug sensitivity was not defined. Because a toxic dose of the Fas-L agonists, in general, will activate the caspase-8 cascade, thereby leading to a nonselective cell killing in both tumor and normal cells, we asked whether a nontoxic or subtoxic dose of the Fas-L agonist is also able to enhance drug sensitivity without activating caspase-8. If so, the selectivity of anticancer drugs would be enhanced. We also asked if the MAPK pathway, particularly p38, is involved in Fas-enhanced drug sensitivity as this kinase can be activated by low levels of cellular stress (15).

To answer these questions, we examined the expression of Fas/Fas-L and the effect of Fas signaling on sensitivity of STS cells to three anticancer drugs with different mechanisms of action. Our results demonstrate that STS cells express Fas/Fas-L, and stimulation of the Fas receptor by a low dose of a Fas-L agonist is able to enhance the sensitivity of STS cells to certain anticancer drugs associated with an increase in p38 activity.

Materials and Methods

Chemicals and Antibodies. MTX was obtained from Lederle Laboratories (Pearl River, NY). DOX, PD98059, and DAPI were obtained from Sigma Chemical Co. (St. Louis, MI). Paclitaxel was obtained from Bristol-Myers Squibb Co. (Princeton, NJ). The monoclonal antibody to CH-11 was obtained from Immunotech Co. (Marseille, France). The monoclonal antibody to Fas was obtained from Transduction Laboratories (Lexington, KY) The monoclonal antibody to Fas-L and polyclonal antibody to caspase-9 were obtained from Pharmengen (San Diego, CA). Monoclonal antibodies to caspase-8, cytochrome *c*, and p38

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[3] The abbreviations used are: STS, soft tissue sarcoma; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MTX, methotrexate; DOX, doxorubicin; DAPI, 4′6-diamidino-2-phenylindole; PMSF, phenylmethylsulfonyl fluoride; FACS, fluorescence-activated cell sorter; SRB, sulforhodamine B.

were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to phospho-Erk1/2, phospho-p38, and phospo-stress-activated protein kinase/JNK were obtained from New England Biolabs (Beverly, MA). IETD-pna and IETD(Ome)-fmk were obtained from Enzyme System Product Co. (Livermore, CA). SB203580 was obtained from SmithKline Beecham Co.

Cell Lines and Culture Conditions. HT-1080, a human fibrosarcoma cell line, A204, a human rhabdomyosarcoma cell line, SaOs-2, a human osteosarcoma cell line, and WI-38, a human fibroblast cell line, were obtained from the American Type Culture Collection. HS-16, a mesenchymal chondrosarcoma cell line, HS-30, a malignant hemangiopericytoma cell line, HS-42, a mixed mesodermal sarcoma cell line, and M9110, a malignant fibrohistiosarcoma cell line were established in this laboratory (16). All cell lines were maintained as monolayer cultures in RPMI 1640 containing 10% fetal bovine serum.

Cytotoxicity Assay. Cytotoxicity to drugs was determined by the SRB cytotoxicity assay carried out in 96-well microtiter plates. Cells were plated in duplicate wells (5000 cells/well) and exposed to MTX, DOX, or paclitaxel and CH-11 at different concentrations for different time periods. Cells were then fixed with 25% trichloroacetic acid solution for 1 h, and 0.4% SRB (Sigma Chemical Co.) was added to each well. After 30 min of incubation, the plates were washed with 1% acetic acid and read at 570 nm on a Biowhitaker microplate reader 2001. The wells with cells containing no drug and with medium plus drug but no cells were used as positive and negative controls, respectively.

Cell Death Assay. Two different assays were used to determine cell death: (*a*) measurement of the DNA content of cells by FACS analysis after PI staining. The cells were harvested after treatment and then fixed with ice-cold 70% methanol for cytometric analysis. DNA content of the subdiploid peak (<2 N DNA content), which indicates dead cells, was determined by FACS analysis. (*b*) DAPI staining. In this assay, the cells were collected and fixed with 4% formaldehyde for 1 h. The fixed cells then washed with PBS and stained with 5 μg/ml of DAPI for 15 min. The cells containing condensed or fragmented nuclei were examined under a fluorescence microscope.

Cell Extracts and Western Blot Analysis. Whole cell extracts were prepared by trypsinizing and resuspending cells in an equal volume of lysis buffer containing 200 mM HEPES, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and protease inhibitors. Cytosolic extracts were prepared as described by Juin *et al.* (17). Cells were trypsinized and collected by centrifugation at 2000 × *g* and washed twice with PBS. The cell pellet was resuspended in sucrose-supplemented cell extract buffer, containing 300 mM sucrose, 10 mM HEPES at pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, aprotinin (1 μg/ml), and leupeptin (1 μg/ml). After 30 min of incubation on ice, cells were homogenized with a glass Dounce homogenizer (40 strokes). Cell homogenates were centrifuged at 12,000 × g for 10 min, and supernatants were removed and stored at −80°C until use. For Western blot analysis, cell extracts (50 μg/lane) were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with various primary antibodies, and protein was detected using an ECL detection kit (Amersham Corp, Little Chalfont, United Kingdom).

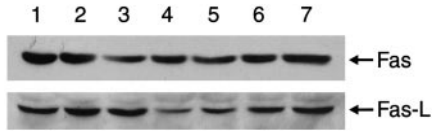

Fig. 1. Fas and Fas-L expression. 100 μg of cellular extract was used for Western blotting analysis as described in "Materials and Methods." *Lane 1:* SaOs-2; *Lane 2:* HT-1080; *Lane 3:* HS-16; *Lane 4:* HS-30; *Lane 5:* HS-42; *Lane 6:* A204; and *Lane 7:* M9110.

Flow Cytometric Analysis. Exponentially growing cells untreated or treated with drugs for different time periods were collected and then fixed with ice-cold 70% methanol. DNA was stained with propidium iodide (Calbiochem, San Diego, CA) as described previously (18). Ten thousand cells were analyzed on a Becton Dickinson FACS for each time point.

Caspase-8 Activity Assay. A modified method described by Wang *et al.* (19) was used to examine caspase-8 activity. CH-11 treated and untreated cells were lysed in hypotonic buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 μM PMSF, aprotinin (2 μg/ml), pepstatin (2 μg/ml), and leupeptin (2 μg/ml). The supernatants were collected and incubated with 100 μM IETD-pna as the substrate at 37°C. The reaction was measured by the change in absorbance at 405 nm using a plate reader.

Results

Human Sarcoma Cells Express Fas and Fas-L. The expression of Fas and Fas-L protein was examined in six human STS cell lines. SaOs-2, an osteosarcoma cell line, which is known to express Fas and Fas-L, was used as a positive control (20). As seen in the Western blot shown in Fig. 1, Fas proteins were detected in all STS cell lines. A single band of M_r 38,000–40,000 Fas-L was also recognized with anti-Fas-L antibody in all of the STS cell lines. To demonstrate specificity of the Fas-L antibody, RPMI-1788, a human lymphoblastoid cell line, was used as a negative control in which the expression of Fas-L is not detectable (data not shown).

Stimulation of the Fas Receptor by CH-11 Sensitizes Sarcoma Cells to Anticancer Drugs. Because drug-induced cell death was not mediated by the Fas pathway in these STS cell lines (unpublished data), we investigated the possibility that activation of the Fas pathway is able to sensitize STS cells to anticancer drugs. HT-1080 and HS-16 cell lines, which are sensitive to CH-11, an agonistic Fas antibody, were used to test this possibility. IC_{50}s of CH-11 for HT-1080 and HS-16 cells were 10.5 and 12.8 ng/ml, respectively. Treatment with MTX, DOX, or paclitaxel for 72 h resulted in cell killing with IC_{50}s of 387, 165, and 10.8 nM, respectively in HT-1080 cells and 960, 245, and 33.4 nM, respectively, in HS-16 cells. When cells were pretreated with a subtoxic dose (6 ng/ml, IC_{10}) of CH-11 for 1 h followed by MTX, DOX, or paclitaxel at different concentrations for 72 h, IC_{50}s for MTX, DOX, and paclitaxel were markedly reduced and were 13-, 54-, and 23-fold lower, respectively, in HT-

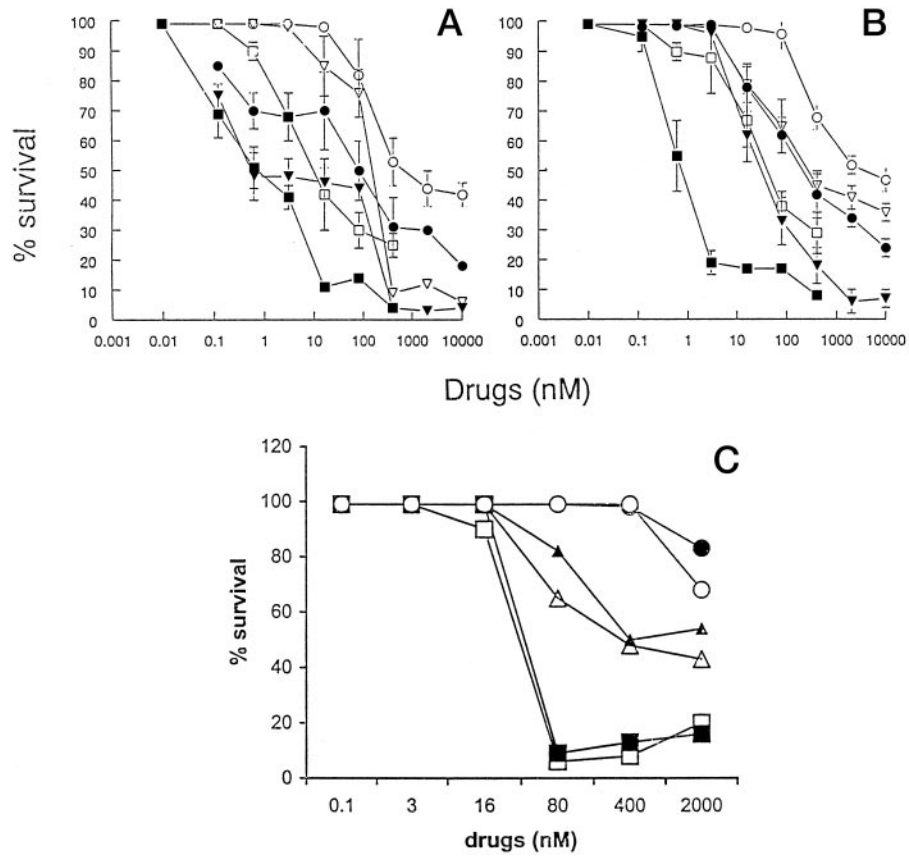

Fig. 2. Effect of CH-11 antibody on cytotoxicity-induced by anticancer drugs. Cells were exposed to anticancer drugs at different concentrations for 3 days or pretreated with CH-11 (6 ng/ml) for 1 h followed by anticancer drugs for 3 days. Cytotoxicity of drugs was determined using the SRB assay. *A*, HT-1080; *B*, HS-16; and *C*, WI-38. (○), MTX; (●), MTX + CH-11; (△), DOX; (▲), DOX + CH-11; (□), paclitaxel; and (■), paclitaxel + CH-11.

1080 cells and 7-, 21-, and 20-fold lower, respectively, in HS-16 cells (Fig. 2). To understand if a subtoxic dose of CH-11 also enhances sensitivity to these anticancer drugs in normal cells, WI-38, a human fibroblast cell line that expresses little Fas or Fas-L (unpublished observation; Ref. 21), was also pretreated with CH-11 (6 ng/ml) followed by MTX, DOX, or paclitaxel. As shown in Fig. 2*C*, sensitivity of WI-38 cells to these anticancer drugs was not significantly increased by CH-11. To additionally understand if enhanced drug sensitivity by CH-11 is attributable to increased cell death (apoptotic or nonapoptotic), subdiploid or apoptotic cells were examined after cells were treated with anticancer drugs alone or drugs combined with CH-11. As shown in Fig. 3, addition of CH-11 significantly increased DOX- or paclitaxel-induced subdiploid cells. This result was further confirmed by DAPI staining (data not shown).

Fas-mediated Signaling Increases Cytochrome *c* Release and Activation of Caspase-9 that Bypasses Caspase-8. To understand the mechanism of the enhanced drug sensitivity related to stimulation of the Fas receptor, caspase-8 expression and activity were determined in HT-1080 cells because caspase-8 is able to activate the mitochondrial caspase-9 pathway via Bid cleavage. As shown in Fig. 4, no significant difference of procaspase-8 expression and activity was observed between CH-11-treated and -untreated cells up to 24 h. However, there was a substantial increase in procaspase-9 activation after exposure to CH-11 for 24 h (decrease in protein level). In agreement with this,

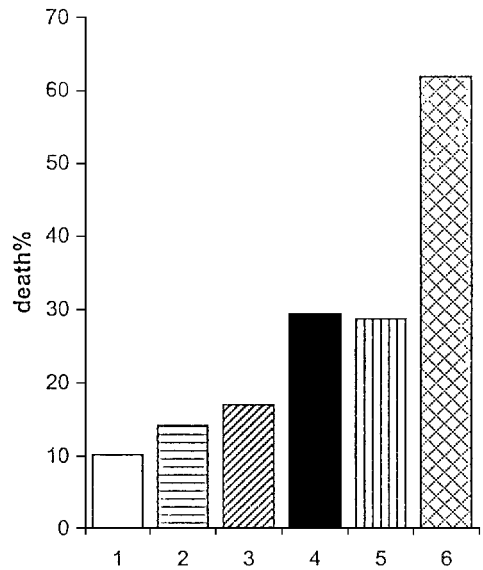

Fig. 3. Effect of CH-11 on induction of cell death by DOX and paclitaxel in HT-1080 cells. Cells were pretreated with CH-11 (6 ng/ml) for 1 h followed by DOX (0.5 μM) or paclitaxel (0.1 μM) for 24 h. Subdiploid cells were measured by FACS analysis as described in "Materials and Methods." (□), untreated control; (▤), CH-11; (▨), DOX; (■), DOX + CH-11; (▥), paclitaxel; and (▩), paclitaxel + CH-11. The figure is the representative of two similar experiments.

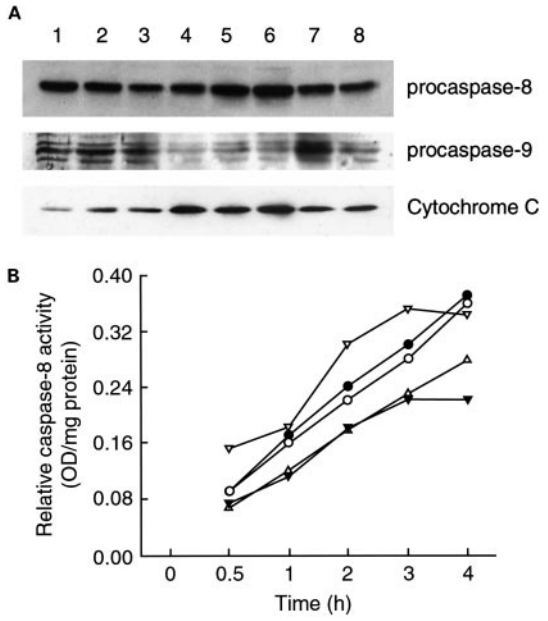

Fig. 4. Effect of CH-11 antibody on the expression of caspase-8 and caspase-9 and cytochrome *c* and activation of caspase-8. HT-1080 cells were treated with CH-11(6 ng/ml) or pretreated with SB203580 (10 μM) or z-IETD (20 μM), respectively, for 16 h followed by CH-11. Whole cell extracts (for caspase-8 and caspase-9) or cytosolic extracts (for cytochrome *c*) were prepared and used for Western blotting analysis. For caspase-8 activation assay, cells were lysed in hypotonic buffer as described in " Materials and Methods." *A*, expression of caspase-8 and caspase-9 and cytochrome *c*. *Lane 1:* untreated control; *Lane 2:* CH-11, 4 h; *Lane 3:* CH-11, 8 h; *Lane 4:* CH-11, 24 h; *Lane 5:* IETD(Ome)-fmk + CH-11, 8 h; *Lane 6:* IETD(Ome)-fmk + CH-11, 24 h; *Lane 7:* SB203580 + CH-11, 8 h; and *Lane 8:* SB203580 + CH-11, 24 h. *B*, activation of caspase-8. (○), untreated control; (●), CH-11, 1 h; (△), CH-11, 4 h; (▽), CH-11, 8 h; and (▼), CH-11, 24 h.

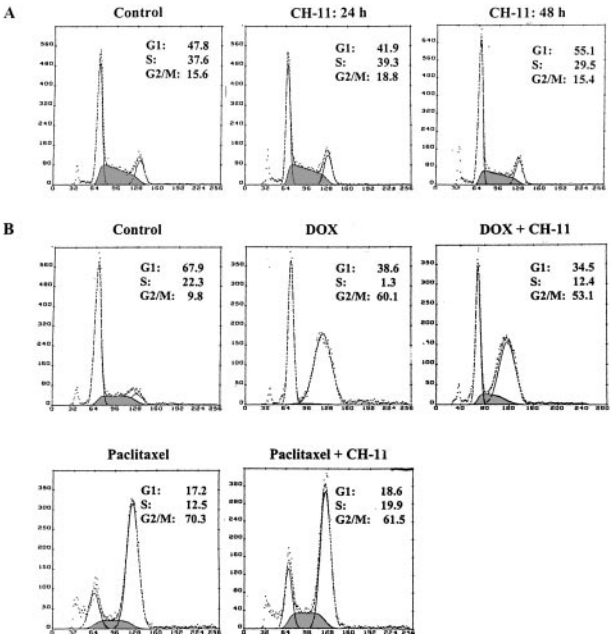

Fig. 5. Effect of CH-11 on cell cycle distribution in response to anticancer drugs in HT-1080 cells. *A*, cells were incubated with CH-11 (6 ng/ml) for 24 or 48 h. *B*, cells were exposed to DOX (20 nM) or paclitaxel (2 nM) or to CH-11 (6 ng/ml) for 1 h followed by DOX (20 nM) or paclitaxel (2 nM) for 24 h. Cells were then harvested and subjected to FACS analysis.

increased cytochrome *c* release was also detected after cells were treated with CH-11. Increased caspase-9 cleavage and cytochrome *c* release were not altered by IETD(Ome)-fmk, a specific inhibitor of caspase-8, but attenuated by SB203580, a specific inhibitor of p38 (Fig. 4*A*). To exclude the possibility that different expression of the proteins was because of unequal loading, a nonspecific band in the same membrane was examined, and it indicated that the loading was equal.

A Subtoxic Dose of CH-11 Does Not Drive Cell Cycle Progression. Because cell cycle position also plays an important role in determining sensitivity of cells to anticancer drugs and Fas can drive cell cycle progression (22), we further examined whether CH-11 could drive cell cycle progression or alter cell cycle distribution in response to anticancer drugs in the STS cells. As shown in Fig. 5*A*, treatment of HT-1080 cells with CH-11 for 24 h did not cause an increase in S-phase entry. A longer (48 h) exposure to CH-11 also did not appreciably change cell cycle distribution in HT-1080 cells. Treatment with DOX and paclitaxel led to a G_2-M block in HT-1080 cells. The addition of CH-11 led to an increase in the S-phase fraction but did not increase cell cycle progression-induced by anticancer drugs because the G_1 fraction was still unchanged (Fig. 5*B*).

Fas-enhanced Drug Sensitivity Is Linked to Elevated p38 MAPK Activity. To understand how Fas-mediated signaling increased cytochrome *c* release independently of caspase-8, thereby leading to enhancement of drug sensitivity, the activation of MAPK was examined by analyzing the phosphorylation of ERK, JNK, and p38 after cells were treated with CH-11 because stimulation of the Fas receptor is able to activate MAPK, and MAPK is involved in the regulation of cytochrome *c* release (23, 24). As shown in Fig. 6*A*, p38 phosphorylation was significantly enhanced after cells were exposed to CH-11 for as early as 30 min and persisted for at least 4 h. Phosphorylation of ERK but not JNK (data not shown) was detectable after cells were treated with CH-11 for up to 4 h. ERK and p38 but not JNK were also moderately activated by MTX, DOX, and paclitaxel. To confirm whether activation of p38 by CH-11 led to increased cytochrome *c* release and consequently enhanced drug-induced cell death, cells were pretreated with SB203580, a specific inhibitor of p38 for 16 h before addition of CH-11. As shown in Fig. 6*B*, SB203580 decreased the phosphorylation of p38 kinase, and as shown in Fig. 3, cytochrome *c* release. In contrast, this inhibitor markedly elevated the phosphorylation of ERK. Furthermore, SB203580 effectively abrogated the augmenting effect of CH-11 on DOX- and paclitaxel-induced cell death. In contrast, IETD(Ome)-fmk, a caspase-8 inhibitor did not significantly abolish the augmenting effect of CH-11 on drug-induced cell death (Fig. 6*C*). PD98059, a specific ERK inhibitor, increased further the enhanced effect of CH-11 combined with MTX or DOX or paclitaxel (data not shown). Similar results were obtained in HS-16 cells (Fig. 6*D*).

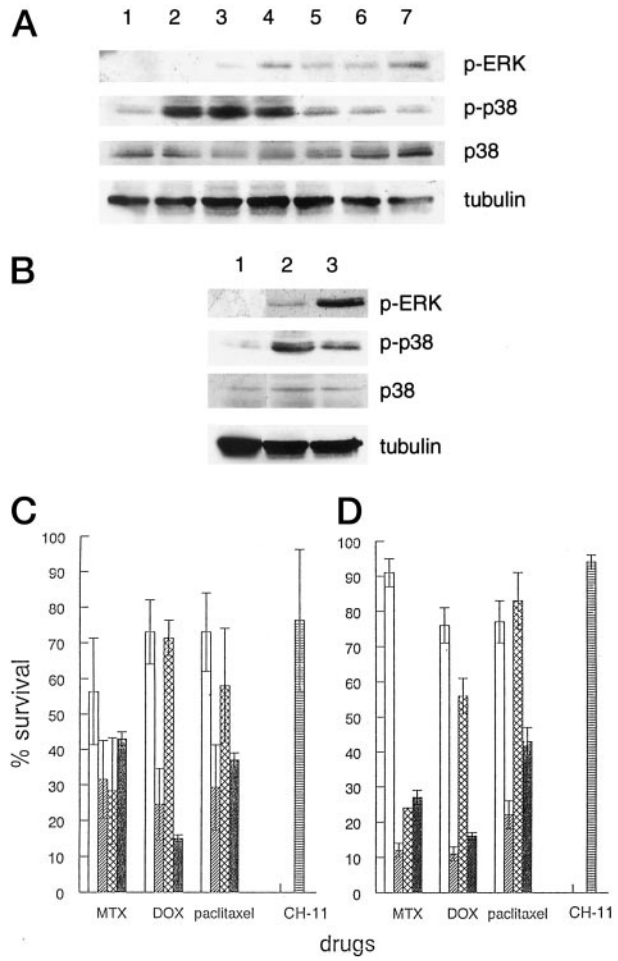

Fig. 6. Activation of MAPKs by CH-11 and anticancer drugs and the effect of SB203580 on CH-11-induced activation of MAPKs and cytotoxicity of CH-11 plus anticancer drugs. *A*, activation of MAPKs by CH-11 and anticancer drugs. HT-1080 cells were exposed to CH-11(6 ng/ml), MTX (200 nM), DOX (20 nM), or paclitaxel (2 nM) at the indicated times, whole cell extracts were prepared for Western blotting analysis. *Lane 1:* untreated control; *Lane 2:* CH-11, 30 min; *Lane 3:* CH-11, 1 h; *Lane 4:* CH-11, 4 h; *Lane 5:* MTX, 1 h; *Lane 6:* DOX, 1 h; and *Lane 7:* paclitaxel, 1 h. *B*, the effect of SB203580 on CH-11-induced activation of MAPKs. HT-1080 cells were pretreated with SB203580 (10 μM) for 16 h and followed by CH-11(6 ng/ml) for an additional 4 h. Whole cell extracts were prepared for Western blotting analysis. *Lane 1:* untreated control; *Lane 2:* CH-11 alone; and *Lane 3:* CH-11 + SB203580. *C* and *D*, SB203580 inhibits the enhanced effect of CH-11 combined with anticancer drugs. Cells were pretreated with SB203580 (10 μM) or IETD(Ome)-fmk (20 μM) for 16 h then treated with CH-11 (6 ng/ml) for 1 h followed by MTX or DOX or paclitaxel for 3 days. Cell killing was determined using a SRB assay. (□), drug alone; (▨), drug + CH-11; (), drug + CH-11 and SB203580; (■), drug + CH-11 and IETD(Ome)-fmk; and (▤), CH-11 alone. Drug concentration used in HT-1080 cells. (*C*): MTX, 200 nM; DOX, 20 nM; paclitaxel, 2 nM; and in HS-16 cells. (*D*): MTX, 500 nM; DOX, 100 nM; and paclitaxel, 10 nM.

Discussion

These studies show that activation of the Fas signaling pathway by a low dose of the Fas-L agonist, CH-11, sensitizes STS cells but not normal fibroblast cells to anticancer drugs. It is likely that a low dose of CH-11 could not activate the Fas pathway in normal fibroblast cells as they express little Fas/Fas-L. The combination of CH-11/MTX, CH-11/DOX, or CH-11/paclitaxel resulted in enhanced cell killing of STS cells rather than a simple additive effect. A mechanism to explain this effect could be activation of the caspase-8 cascade. However, activation of this caspase by the low dose of CH-11 used in this study was not observed in agreement with other investigators who also showed that a subtoxic dose of CH-11 was not able to activate caspase-8 (25). Furthermore, IETD(Ome)-fmk, a caspase-8 inhibitor, did not block CH-11-enhanced sensitivity of cells to DOX and paclitaxel. Therefore, activation of the caspase-8 cascade appears not to play a key role in Fas-enhanced drug sensitivity when a subtoxic concentration of CH-11 is used.

Another mechanism for investigation considered was Fas-triggered caspase-independent activation of MAPKs. Fas has been shown to activate the MAPKs through FADD/NIK (Fas-associated death domain/NF-κB-inducing kinase) or the Daxx pathway in different cell lines (8). Indeed, we observed that the stimulation of the Fas receptor by a low dose of CH-11 increased the active form (phosphorylation) of p38 in STS cells. As reported, p38 could then phosphorylate pRb, thereby allowing increased release of free E2F (26). Also, p38 is able to cause a G_2-M arrest (15, 27). A p38-mediated increase in cytochrome *c* release was also reported (23). Therefore, p38 activation could contribute to the susceptibility of tumor cells to DNA damaging agents through driving cell cycle progression and/or increasing cytochrome *c* release. Although Fas-driven cell cycle progression was not observed in these experiments, cytochrome *c* release was significantly increased by Fas stimulation. Thus, enhanced chemosensitivity noted with a low dose of CH-11 is likely mediated through the p38 kinase-cytochrome *c* pathway because SB203580, a specific inhibitor of p38 kinase that is able to block or reduce phosphorylation of p38 in addition to blocking its kinase activation (28, 29), decreased CH-11-induced activation of p38 and release of cytochrome *c*. Although CH-11 also increased the activation of ERK, this may not have contributed to sensitization of STS cells to anticancer drugs because inhibition of this kinase by PD98059 caused an additional increase in drug sensitivity. An unexpected observation was that Fas-enhanced sensitization of STS cells to MTX seems not to involve the p38 pathway because SB203580 did not abolish the augmenting effects of CH-11 on MTX-induced cell death. The mechanism for this needs additional exploration.

The finding that activation of the Fas-mediated signaling pathway is able to sensitize STS cells to anticancer drugs through an increase in activation of p38 and consequent cytochrome *c* release may have implications for treatment of patients with STS and other malignances. Despite the ability of agonistic Fas-antibodies, Fas-L, and even tumor necrosis factor-related apoptosis-inducing ligand to kill tumor cells at toxic doses, severe side effects on normal cells also are likely occur, thereby limiting their systemic use as single agents (30, 31). In addition, activation of p38 in response to various cellular stresses, including DNA damage, protects normal cells, including granulocytes, skeletal muscle cells, cardiomyocytes, and fibroblasts (32–34). Therefore, the use of subtoxic doses of a Fas-L agonist such as CH-11 to sensitize tumor cells but not normal cells to anticancer drugs through activation of p38 may increase selective tumor cell kill. These results provide a rational

basis for the use of a combination of low dose of a Fas agonist with anticancer drugs in the clinic.

Acknowledgments

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