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Augmented Adriamycin Sensitivity in Cells Transduced with an Antisense Tumor Necrosis Factor Gene Is Mediated by Caspase-3 Downstream from Reactive Oxygen Species

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While transduction of an antisense tumor necrosis factor (*TNF*) gene sequence can augment the cytotoxicity of adriamycin (ADM) in human cancer cells, the specific effect of introducing this sequence on the signal transduction pathway leading to cell death remains unclear. In ADM-resistant pancreatic carcinoma (PANC-1) cells, both the antioxidant N-acetyl-L-cysteine (NAC) and the caspase-3 inhibitor acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde (Ac-DMQD-CHO) prevented ADM-induced cytotoxicity. NAC additionally inhibited caspase-3 activity induced by ADM treatment, while Ac-DMQD-CHO showed no suppressive effect on reactive oxygen species (ROS). Stable antisense-TNF transfectants showed higher ADM sensitivity and greater ADM-induced ROS production and caspase-3 activity than mock transfectant or parent cells. These results indicate that increased caspase-3 activity downstream from ROS production is among the mechanisms by which transduction of the antisense TNF sequence of augments ADM sensitivity of pancreatic carcinoma cells.

Key words: Tumor necrosis factor — Antisense — Reactive oxygen species — Caspase-3

Adriamycin (ADM) is widely used to treat solid tumors and malignant hematologic diseases. Numerous attempts have been made to overcome cancer cell resistance against antineoplastic drugs including ADM by inhibition of intracellular resistance factors.[1, 2] We previously have reported that endogenous tumor necrosis factor (enTNF) exerts a protective effect against ADM by enhancing the scavenging of intracellular reactive oxygen species (ROS) via induction of manganous superoxide dismutase (MnSOD).[3] We also found that measurement of enTNF expression could predict ADM sensitivity in tumor cells.[4] Thus, antisense *TNF* gene transduction could augment ADM-induced toxicity in human cancer cells by decreasing MnSOD activity.[4, 5] However, whether transduction of the antisense *TNF* gene actually affects the signal transduction pathway leading to ADM-induced cell death is unclear. We therefore investigated changes following antisense *TNF* gene transduction in expression of molecules leading to cell death in human pancreatic carcinoma (PANC-1) cells showing resistance to ADM. Our investigative group and others recently identified a signaling pathway including production of reactive oxygen species and activation of caspase-3 that leads to apoptosis in cells exposed to heat or ionizing radiation.[6, 7] We therefore examined whether and how antisense *TNF* gene transduction augmented this signaling pathway to result in increased ADM sensitivity. As the PANC-1 cells used in this study harbor a mutant-type p53, effects from several molecules that interact with caspase-3 in cells with wild-type p53 could be excluded.

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MATERIALS AND METHODS

Cell culture and gene transduction PANC-1 cells were cultured in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biological Industries, Kibbutzbeit Haemek, Israel). Transduction of antisense TNF mRNA expression vector (pLJ-antiTNF) to PANC-1 cells was performed according to the method described in our previous reports.[5] Briefly, PANC-1 cells were infected with viruses produced by A211 cells. After 48 h of cultivation, cells were selected in the presence of G418 (800 μg/ml) for 10 days, and G-418 resistant clones were isolated by limiting dilution. The G418 resistant clone transfected with pSV2-neo without antisense *TNF* gene was also selected, and used as a control.

Analysis of apoptosis The cells undergoing apoptosis were assessed by fluorogenic staining. Briefly, the cells in a 100 mm culture dish (Costar, Tokyo) were treated with 5 μM ADM with or without the specific caspase-3 inhibitor acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde (Ac-DMQD-CHO, Peptide Institute, Inc., Osaka) or the antioxidant N-acetyl-L-cysteine (NAC). Additional incubation was performed at the appropriate temperature for 48 h, and the cells were collected. Each sample was then stained with Hoechst 33258 (Sigma, St. Louis, MO), and apoptotic cells were identified morphologically

under a fluorescence microscope by counting 400 cells per sample.

Assay for enTNF expression The expression of enTNF was measured by an indirect method using a fluorescein isothiocyanate (FITC)-labeled second antibody, according to a modification of the method described earlier.[4] Briefly, cells (1×10^4/100 μl) were added to the wells of a 96-well microculture plate (Costar) and incubated at 37°C in 5% CO_2 for 18 h. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with paraformaldehyde solution (4% v/v in PBS) for 20 min at room temperature. Next they were treated with Triton X-100 (0.2% v/v in PBS) to permeabilize the cell membrane. For blocking, 1% goat serum in PBS was added to each tube for 30 min at 37°C. As a first antibody, appropriately diluted rabbit anti-recombinant human TNF polyclonal antibody (Asahi Chemical Industry, Tokyo) was added to each tube. Following 1 h incubation at room temperature, the cells were washed with 1% bovine serum albumin (BSA, Seikagaku, Tokyo)-PBS 3 times, and then treated with appropriately diluted FITC-labeled goat anti-rabbit IgG (E.Y Laboratories, San Mateo, CA) as a second antibody. Following an additional 30 min incubation at room temperature, cells were washed 3 times with 1% BSA-PBS. Intensity of cell fluorescence was measured with a Spectrafluor (SLT Labinstruments GmbH, Salzburg, Austria; excitation 485 nm, emission 538 nm). Relative fluorescence intensity (FI) was determined compared to the FI of untransduced PANC-1 cells.

Measurement of ROS Endogenous amounts of ROS were measured by fluorometric assay using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Inc., Eugene, OR). Briefly, cells in a 100 mm dish (Costar) were incubated with 10 μM DCFH-DA in the medium at 37°C for 30 min, and then detached from the dish in 0.25% trypsin, 0.02% EDTA PBS. The cells were washed with PBS twice, and the FI of the resuspended cells was measured with a Spectrafluor (SLT Labinstruments; excitation 485 nm, emission 538 nm) in a 96-well microtiter plate (Costar). FI of each sample was corrected for viable cell number counted by trypan blue staining. The linearity of fluorescence intensity was confirmed in the range of cell number from 2×10^4 to 2×10^5 cells in a 96-well plate.

Measurement of caspase-3 activity Enzymatic activity of caspase-3 was measured by fluorometric assay according to the instruction manual for apoptosis assay published by Pharmingen (2nd Ed., December 1998, San Diego, CA). Cells were treated with lysis buffer (10 m*M* Tris-HCl, 10 m*M* $NaH_2PO_4/NaHPO_4$ pH 7.5, 130 m*M* NaCl, 1% Triton-X100, 10 m*M* sodium pyrophosphate) and the lysate was centrifuged at 12 000*g* for 15 min. The supernatant was collected and the protein concentration was measured by BioRad DC-protein assay. Next the lysate, a fluorogenic substrate acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-α (4-methylcoumaryl-7-amide) (Ac-DEVD-MCA, Peptide Institute, Inc.) with or without the caspase-3 inhibitor Ac-DMQD-CHO (Peptide Institute, Inc.), and reaction buffer (40 m*M* HEPES pH 7.5, 20% glycerol, 4 m*M* dithiothreitol (DTT)) were mixed in a 96-well microtiter plate (Costar). The cleavage of the substrate was monitored in terms of amino-4-methylcoumarin (AMC) liberation with a Spectrafluor (SLT Labinstruments; excitation 360 nm, emission 465 nm) at 37°C. The fluorescence due to produced AMC was measured every 1 min during a 60-min period, and the caspase-3 activity was calculated as fluorescence intensity/min/mg protein.

RESULTS

Involvement of ROS generation and caspase-3 activation in ADM-induced apoptosis We first examined the effects of an ROS inhibitor, NAC, and a caspase-3 inhibitor, Ac-DMQD-CHO, on ADM-induced apoptosis in PANC-1 cells. Both inhibitors prevented ADM-induced apoptosis (Fig. 1). We next examined the effects of each inhibitor on ROS generation and caspase-3 activation to clarify the signaling order between these two events. In cells treated with ADM for 24 h, ROS production was increased to approximately 1.5 times that in untreated cells. NAC prevented this ROS production, but Ac-DMQD-CHO had no inhibitory effect (Table I). We next examined directly whether caspase-3 activity was elevated in ADM-treated cells. Nearly complete inhibition of AMC

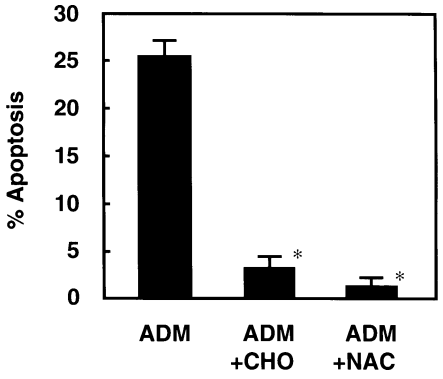

Fig. 1. Effect of a caspase-3 inhibitor and an antioxidant on apoptosis in PANC-1 cells. Ac-DMQD-CHO (100 μM) and NAC (10 m*M*) were added to cultures 3 h and 1 h before addition of 5 μM ADM, and incubation was continued at 37°C for 48 h. Apoptotic cells were detected by a fluorometric assay using Hoechst 33258. The data represent the mean±SD from three independent experiments. Statistical significance was determined by Student's *t* test, as compared to the value in PANC-1 cells without treatment, * $P<0.01$. ADM, adriamycin; CHO, Ac-DMQD-CHO, acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde; NAC, N-acetyl-L-cysteine.

release resulted from exposure to the specific caspase-3 inhibitor Ac-DMQD-CHO, indicating that AMC fluorescence detected in this assay truly represented caspase-3 enzymatic activity (Fig. 2). Caspase-3 activity was increased 2.8 times by 24 h of ADM treatment. Further, NAC exposure inhibited the increase in caspase-3 activity ordinarily produced by ADM, resulting in activity similar to that seen with no ADM treatment. Basal activity in the absence of ADM was not altered by NAC (Fig. 3).

Effect of antisense *TNF* gene transduction on ADM-induced apoptotic signaling We next examined the effect of antisense *TNF* gene transduction on apoptotic events via ROS production and caspase-3 activation. ADM-induced apoptosis was significantly augmented, being 1.5 to 2 times greater in cells with low enTNF as a result of transduction of antisense TNF mRNA expression vector.

Table I. Effect of Caspase-3 Inhibitor on ADM-induced ROS Production in PANC-1 Cells

Additives	Groups			
ADM (5 μM)	−	+	+	+
CHO (100 μM)[a]	−	−	−	+
NAC (10 mM)[b]	−	−	+	−
ROS production[c]	2680±126	4082±144[d]	2549±86	4213±221[d]

a) CHO, Ac-DMQD-CHO.
b) NAC, N-acetyl-L-cysteine.
c) ROS production was examined 30 min after DCFH-DA treatment and represented as the mean FI from three independent experiments.
d) Statistical significance was determined by Student's *t* test, as compared to the value in PANC-1 cells without treatment, $P<0.01$.

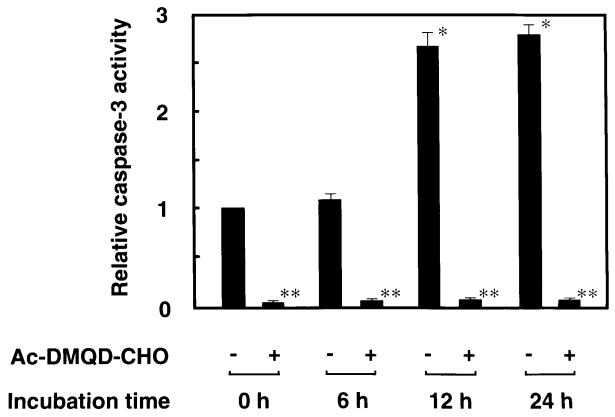

Fig. 2. Kinetic measurement of caspase-3 activity in cells treated with ADM. Cells were treated with 5 μM ADM for 24 h, after which cell lysates were assayed with or without the caspase-3 inhibitor Ac-DMQD-CHO. Cell lysates were incubated at 37°C for 1 h, and the amount of AMC released was measured by cleavage of the fluorogenic substrate Ac-DEVD-MCA (100 μM). The vertical axis represents relative values compared to untreated cells. The data represent the mean±SD of three independent experiments. Statistical significance was determined by Student's *t* test, as compared to the value in PANC-1 cells without treatment, * $P<0.05$, ** $P<0.02$. Ac-DMQD-CHO, acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde; ADM, adriamycin; AMC, amino-4-methylcoumarin; Ac-DEVD-MCA, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-α (4-methylcoumaryl-7-amide).

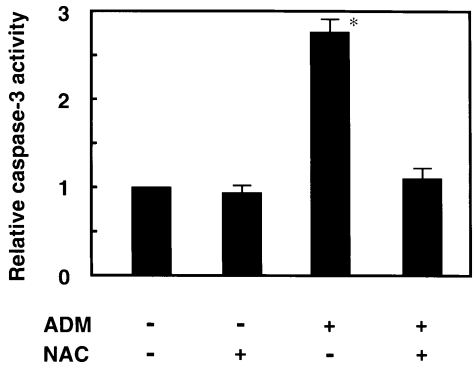

Fig. 3. The effect of NAC on caspase-3 activity in PANC-1 cells treated with ADM. Cells were treated with or without 10 mM NAC 1 h before the addition of 5 μM ADM followed by incubation at 37°C for 24 h. Cell lysates were incubated at 37°C for 1 h, and the amount of AMC released was measured by cleavage of the fluorogenic substrate Ac-DEVD-MCA (100 μM). The vertical axis represents relative values compared to untreated cells. The data represent the mean±SD of three independent experiments. Statistical significance was determined by Student's *t* test, as compared to the value in PANC-1 cells without treatment, * $P<0.02$. NAC, N-acetyl-L-cysteine; ADM, adriamycin; AMC, amino-4-methylcoumarin; Ac-DEVD-MCA, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-α (4-methylcoumaryl-7-amide).

Table II. ADM-induced Apoptosis in PANC-1 Cells Transduced with Antisense TNF Sequence

Strain	enTNF expression (relative FI)[a]	%Apoptosis[b]
PANC-1	1.00	26.5±2.5
pSV2neo	1.03	26.9±4.2
PAT 3	0.70	40.2±2.4[c]
PAT 17	0.66	52.1±4.5[c]

a) Constitutive protein expression of enTNF was expressed as relative FI compared with that of parent PANC-1 cells.
b) Data are represented by the mean±SD from three independent experiments.
c) Statistical significance was determined by Student's *t* test, as compared to the value in parent PANC-1 cells, $P<0.02$.

Table III. ROS Production by ADM Treatment in PANC-1 Cells Transduced with Antisense TNF Sequence

Strain	ADM treatment	ROS production[a)]
PANC-1	–	2680±126
	+	4082±144
pSV2neo	–	2796±180
	+	4290±204
PAT 3	–	3308±167
	+	7980±198[b)]
PAT 17	–	3686±187
	+	8250±167[b)]

a) Data represent the mean±SD from three independent experiments.
b) Statistical significance was determined by Student's *t* test, as compared to the value in parent cells treated with ADM, $P < 0.02$.

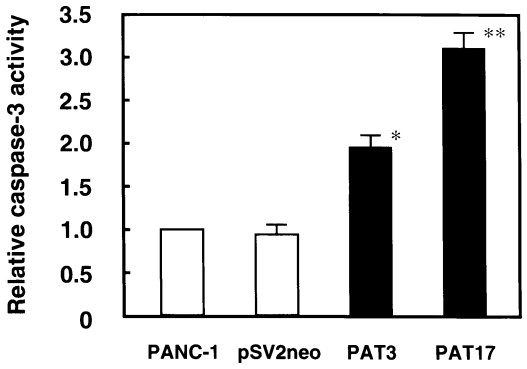

Fig. 4. Effect of antisense *TNF* gene transduction on caspase-3 activity in ADM-treated PANC-1 cells. Cells were treated with 5 μM ADM followed by incubation at 37°C for 24 h. The vertical axis represents relative values compared to parent cells (PANC-1). The data represent the mean±SD of three independent experiments. Statistical significance was determined by Student's *t* test, as compared to the value in PANC-1 cells, * $P<0.05$, ** $P<0.02$. pSV2neo, PANC-1 cells transduced with pSV2neo alone; PAT, PANC-1 cells transduced with antisense TNF mRNA expression vector; TNF, tumor necrosis factor; ADM, adriamycin.

No effect was seen in cells transduced by pSV2neo alone (Table II). We confirmed that MnSOD activity in cells transduced with the antisense *TNF* gene sequence was reduced to approximately 60% of that in mock transfectants (data not shown). Next, ROS production was assessed in transduced clones. In untransduced cells, ADM treatment increased ROS production as described above. Antisense *TNF* gene transduction resulted in an apparent augmentation of ROS production (Table III). Furthermore, caspase-3 activity in transduced cells treated with ADM was higher than that in ADM-treated parent cells or cells transduced by pSV2neo (Fig. 4).

DISCUSSION

Using cells transduced with nonsecretory-type TNF expression vector, we previously found that endogenous TNF exerts its protective effect against cytotoxicity of ADM by inducing MnSOD to scavenge ROS.[4, 5)] In the present study we hypothesized that antisense *TNF* gene sequence transduction can lead to augmentation of ADM-induced apoptosis not only via enhancement of ROS production, but also by activation of caspase-3, a pivotally important enzyme leading to apoptosis in response to various genotoxic stresses.[8)] We first showed that ADM-induced apoptosis occurs via activation of caspase-3 downstream of ROS production in PANC-1 cells. Previous studies had indicated that loss of mitochondrial membrane potential and ROS production are involved in ADM-induced cell damage.[9, 10)] This mitochondrial event was thought to occur upstream of other apoptosis-inducing signals such as caspase-3 activation. However, the order of signaling between ROS production and caspase-3 activation has become a subject of considerable controversy.

Recently, two teams investigating the relationship between ROS and caspase-3 have suggested that signaling leading to apoptosis via activation of caspase-3 occurs upstream from the mitochondrial event.[11, 12)] Shimizu *et al.* examined the relationship between hydrogen peroxide (H_2O_2) production and activation of caspase-3-like proteases using human small cell lung carcinoma cells (Ms-1) and human lymphocytic leukemia cells (Jurkat). These investigators demonstrated that H_2O_2 production was inhibited by Ac-DEVD-CHO, an inhibitor of caspase-3 like proteases, in Ms-1 cells treated with ADM or camptothecin and in Jurkat cells treated with camptothecin. In addition, caspase-3 activity enhanced by the treatment with camptothecin was not inhibited by the antioxidant NAC or by diphenyleneiodonium chloride in Jurkat cells. These authors concluded that activation of caspase-3-like proteases by anticancer drugs causes generation of H_2O_2, which in turn induces apoptosis. Their result appears to differ from our findings with respect to the signaling order of ROS production and caspase-3 activation, but because of their experimental design they actually did not establish that caspase-3 activation occurs upstream from ROS production. Specifically, the effect of NAC on caspase-3 activity in cells treated with ADM was not examined, and Ac-DEVD-CHO is an inhibitor of both caspase-3 and -7. In contrast, we showed that the specific caspase-3 inhibitor Ac-DMQD-CHO could not eliminate ROS production, while NAC inhibited caspase-3 activity. Further, other studies clarified that ADM causes apoptosis via Fas-mediated signal transduction that includes activation of caspase-3.[13)] Thus, signaling via caspase-3 downstream of ROS production also may be an important pathway in ADM-induced apoptosis in pancreatic carcinoma cells.

The other group that placed caspase-3 signaling upstream to ROS examined the relationship between loss of mitochondrial membrane potential and activation of several caspases in Jurkat cells.[12] These investigators demonstrated that Z-VAD-fmk, which is general inhibitor of caspases except for caspase-2 to -4 and -6 to -8, prevented ADM-induced apoptosis and loss of mitochondrial membrane potential, even though several caspases including caspase-3 were activated by ADM. They suggested that the key mediator of ADM-induced apoptosis could be a Z-VAD-sensitive caspase "caspase-X" that caused a loss of mitochondrial membrane potential and release of apoptogenic factors from mitochondria. Considering the latter group's finding together with our results, ADM-induced apoptosis might occur via two main pathways, via ROS production upstream from activation of caspase-3, and also via loss of mitochondrial membrane potential induced by Z-VAD-sensitive caspase.

A recent study demonstrated that peroxynitrite, a potent inducer of apoptosis, directly induces caspase-3 activity.[14] The specific radical species that induces caspase-3 in ADM-treated cells remains to be determined among possibilities including superoxide, hydroxyl radicals, and peroxynitrite.

Some previous studies have investigated whether inhibition of antiapoptotic molecules such as bcl-2 and survivin is important in augmentation of apoptosis by anticancer drugs.[15, 16] These investigators found that apoptosis was augmented by enhancement of a signaling pathway involving mitochondrial dysfunction and activation of caspases, although ROS production was not mentioned. Our present findings represent the first evidence that an increase in ROS production resulting from inhibition of enTNF expression actually sensitizes pancreatic cancer cells to ADM via elevation of caspase-3 activity. Other studies have indicated that Fas-mediated apoptosis is also caused by ROS production and activation of caspase-3,[17] but neither Fas expression, caspase-3 activity induced by agonistic anti-Fas antibody (CH-11), nor Fas-mediated apoptosis was augmented in the cells that we transduced with the antisense *TNF* gene sequence (data not shown). Some recent studies suggest that while each Fas-induced apoptotic pathway contributes to drug-induced apoptosis, none is essential.[18] Accordingly, the effect of an antisense TNF sequence on apoptosis may differ between systems with respect to signaling order of molecules leading to apoptosis, and this issue required further investigation.

Consideration of protective factors against various cellular stresses may be informative. We previously found that enTNF can induce heat shock protein (HSP) 72 by enhancing binding of heat shock factor 1 to the heat shock element.[19, 20] In addition, a recent study clarified that HSP exerts its protective function against several apoptotic stimuli such as TNF and staurosporine downstream of caspase-3-like proteases.[21] Down-regulation of HSP72 may be one of the events that augment apoptosis in antisense *TNF* gene transduction. Taken together with those of others, our findings suggest that inhibition of endogenous TNF expression can augment the therapeutic effect of anticancer drugs that act via activation of caspase-3 downstream from ROS production.

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