Doxorubicin and Selenium Cooperatively Induce Fas Signaling in the Absence of Fas/Fas Ligand Interaction

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Abstract. *Background: The synergistic effect of doxorubicin and selenium in apoptosis induction in MCF-7 breast cancer cells has been previously reported. Mitochondrial activation of caspase-9 is in part responsible for the synergy. The present study aimed at examining the death receptor pathway in activating caspase-8 by the two-drug combination. Materials and Methods: We determined the expression of TRAIL and FasL signaling molecules and monitored activated caspase-8 in response to neutralizing/blocking antibodies against ligands/receptors. Results: Our data suggest that TRAIL signaling might not play a role. With respect to the Fas pathway, it was found that doxorubicin enhanced Fas oligomerization (i.e. activation) independent of FasL-Fas interaction. Selenium, on the other hand, increased the expression of FADD, a key adaptor molecule responsible for recruitment of caspase-8 to the Fas oligomer. The significance of the above changes was confirmed by the detection of considerably more caspase-8 in both the Fas or FADD immunoprecipitate obtained from cells treated with the doxorubicin/selenium combination. Conclusion: Doxorubicin and selenium cooperatively activate Fas signaling by targeting key regulatory steps.*

Doxorubicin and other anthracycline drugs are widely used in the treatment of breast cancer. The benefits in response rate and overall survival, however, are often associated with myelosuppression and cardiomyopathies (1). Thus it is desirable to develop new modalities that can enhance anthracycline therapeutic efficacy. Recent research in our laboratory showed that doxorubicin-induced apoptosis was markedly enhanced by co-treatment with selenium in the MCF-7 breast cancer cells (2). Methylseleninic acid (MSA) was the active selenium metabolite used in these experiments. We found that the synergy between doxorubicin and MSA is achieved in part *via* activation of the mitochondrial apoptotic pathway. Doxorubicin and MSA modulate different molecules associated with the regulation of mitochondrial membrane permeability, leading to an increased activation of caspase-9. The death receptor pathway is known to play an equally important role in cell killing by many cytotoxic drugs (3-6). To further investigate the molecular mechanism by which doxorubicin and MSA might work cooperatively through death receptor signaling, we turned our attention to this area in the present study.

Death receptor signaling starts with the binding of ligands (*e.g.* FasL and TRAIL) to their cognate receptors (Fas for FasL, DR4 and DR5 for TRAIL). Upon oligomerization of the receptor, Fas-associated death domain protein (FADD) is recruited to the receptor, followed by the recruitment of procaspase-8 to form the death inducing signal complex or DISC (7, 8). Procaspase-8 then undergoes self-cleavage to an intermediate form. FADD is crucial to Fas signaling since the latter can be suppressed by dominant negative or antisense FADD (9-11). In fact, numerous studies have shown that Fas can be activated in a ligand-independent, but FADD-dependent, manner (9-15). Death receptor signaling is also controlled by negative regulatory molecules, as exemplified by FADD-like inhibitory protein (FLIP), which blocks the recruitment of procaspase-8 to DISC (16). A high expression of FLIP is found in many cancer cells and has been correlated with resistance to FasL or TRAIL treatment (17, 18).

Doxorubicin has been shown to induce the expression of death receptors, DR4 and DR5, in breast cancer cells, thus sensitizing them to TRAIL therapy (19). On the other hand, selenium has been reported to induce FasL in squamous cell

Abbreviations: DEF, death effector filament; DISC, death inducing signaling complex; DR, death receptor; FADD, Fas-associated death domain; FasL, Fas ligand; FLIP, FADD-like inhibitory protein; MSA, methylseleninic acid; TNF, tumor necrosis factor; TRAIL, TNF-associated apoptosis inducing ligand.

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carcinomas (20) and sensitize prostate cancer cells to TRAIL by down-regulating FLIP (21). Both FasL and TRAIL are known transcriptional targets of FOXO proteins (22, 23), and we recently documented a significant increase of FOXO transactivation by MSA in MCF-7 cells (2). Collectively, the above observations support the idea that doxorubicin and MSA may act in a concerted fashion to activate the death receptor pathway. The present objectives were to (i) examine the involvement of death receptor-activated caspase-8 in mediating the synergy of doxorubicin and MSA in apoptosis stimulation; (ii) identify the contribution of different death receptor pathways; and (iii) elucidate the mechanism of death receptor signaling modulation by doxorubicin and MSA.

Materials and Methods

Cell culture. The MCF-7 breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4 mmol/L of glutamine, and 1% penicillin-streptomycin at 37˚C in a 5% CO_2 incubator. At 48 h after seeding, when the culture was 60-70% confluent, the medium was changed before starting the treatment with methylseleninic acid (MSA), doxorubicin or the combination. The concentrations of MSA and doxorubicin in the culture were 5 μM and 400 nM, respectively. These concentrations were chosen based on our previous studies with the same cell model (2).

Chemicals and reagents. Doxorubicin was purchased from Sigma (St. Louis, MO, USA). MSA, now available commercially from PharmSe (Lubbock, TX, USA), was developed by our laboratory specifically for *in vitro* studies (24). Antibodies specific to cleaved PARP, cleaved caspase-8 and FasL were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific to DR4 (TRAIL-R1), DR5 (TRAIL-R2), FADD and Fas were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-8 specific inhibitor, z-IETD-FMK, was purchased from Calbiochem (San Diego, CA, USA). TRAIL neutralizing antibody (2E5) was purchased from AXXORA (San Diego, CA, USA). Anti-Fas blocking antibody (ZB4) was purchased from Beckman Coulter (Fullerton, CA, USA). Human cFas ligand was purchased from Biovision (Mountain View, CA, USA).

Western Blot analysis. Cells were harvested and lysed by 1X lysis buffer (Cell Signaling Technology, Beverly, MA, USA) containing 1 mmol/L phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA), 50 mmol/L NaF, and 1 tablet/7 mL of Mini Complete Protease Inhibitor (Roche, Indianapolis, IN, USA). Protein concentration of the lysate was determined by using the Bicinchoninic Acid (BCA) Protein Assay kit (Pierce). In preparing for SDS-PAGE, the cell lysate was mixed with 1/3 volume of SDS sample buffer (200 mmol/L Tris-HCl, pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol, 60 μL/mL of β-mercaptoethanol) and heated at 100˚C for 10 min. Protein bands were visualized by the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) or the ECL Plus Western Blotting Detection System (Amersham, Piscataway, NJ, USA).

Chemical cross-linking and immunoprecipitation. Cells were collected by gentle scraping from the culture dish and washed twice in PBS. The pellet was resuspended in 1 ml of PBS and treated with 2 mmol/L of the cleavable cross-linker, 3,3'-dithio*bis* (sulfosuccinimidyl proprionate) for 15 min on ice, and the reaction was quenched with 50 mmol/L of glycine for 5 min (25). The cells were pelleted and washed twice in PBS, and lysed with 500 ml of lysis buffer (1% NP-40, 20 mmol/L Tris, pH 7.4, 140 mmol/L NaCl, 10% glycerol, and 2 mmol/L EDTA) containing a protease inhibitor mixture (Roche). The suspension was left on ice for 30 min, followed by vortexing every 10 min. The lysate was then used for immunoprecipitation with a Fas antibody or FADD antibody. The immune complex was precipitated using Protein G Agarose beads (Pierce) and washed three times in lysis buffer. The precipitate was resuspended in Laemmli buffer, boiled for 10 min, and resolved on SDS-polyacrylamide gel electrophoresis. The sample was transferred onto a polyvinylidene difluoride membrane for Western blot analysis. The presence of Fas, FasL and caspase-8 was detected by using the appropriate primary and secondary antibodies.

Results

Involvement of caspase-8 in apoptosis induction by doxorubicin and MSA. Cleaved caspase-8 is a benchmark indicator of death receptor pathway activation. Cells were treated with doxorubicin, MSA, or the combination for 24 h. Western blot analysis of cleaved caspase-8 in the cell lysate is shown in Figure 1A. Doxorubicin or MSA by itself produced very little caspase-8 cleavage compared to the control. In contrast, a combination of the two drugs induced a noticeable increase of cleaved caspase-8. PARP cleavage is a well accepted biochemical assay for apoptosis. The ability of doxorubicin/MSA to induce PARP cleavage was also evaluated. The results were similar to the cleaved caspase-8 data in that neither drug alone was effective, whereas the combination caused a sizeable increase of PARP cleavage.

In order to delineate the significance of caspase-8 in apoptosis induction, we used a caspase-8 specific inhibitor, z-IETD-FMK, to block its activity. Cells were treated with doxorubicin/MSA for 24 h with or without 50 μM of the inhibitor. As shown in Figure 1B, the PARP cleavage data suggest that apoptosis induction by the combination was decidedly reduced when caspase-8 was inhibited. The z-IETD-FMK inhibitor did not prevent the cleavage of procaspase-8 (as shown by the Western blot data), it only interfered with the activity of caspase-8. The above finding is interpreted to mean that activation of the death receptor pathway is involved in the action of the drugs. The fact that the caspase-8 inhibitor was unable to completely reverse apoptosis was reasonable because we have previously shown that mitochondrial activation of caspase-9 also plays a role in apoptosis induction by doxorubicin/MSA (2).

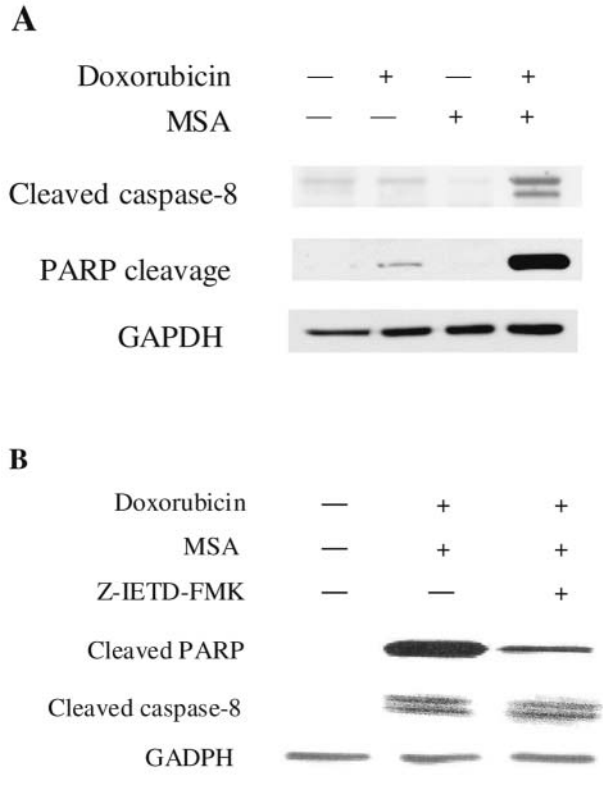

Figure 1. *Activation of death receptor pathway by doxorubicin/MSA, either individually or in combination. A) Western blot analysis of cleaved caspase-8 and PARP. B) Effect of inhibiting caspase-8 activity by z-IETD-FMK on cleaved caspase-8 and PARP.*

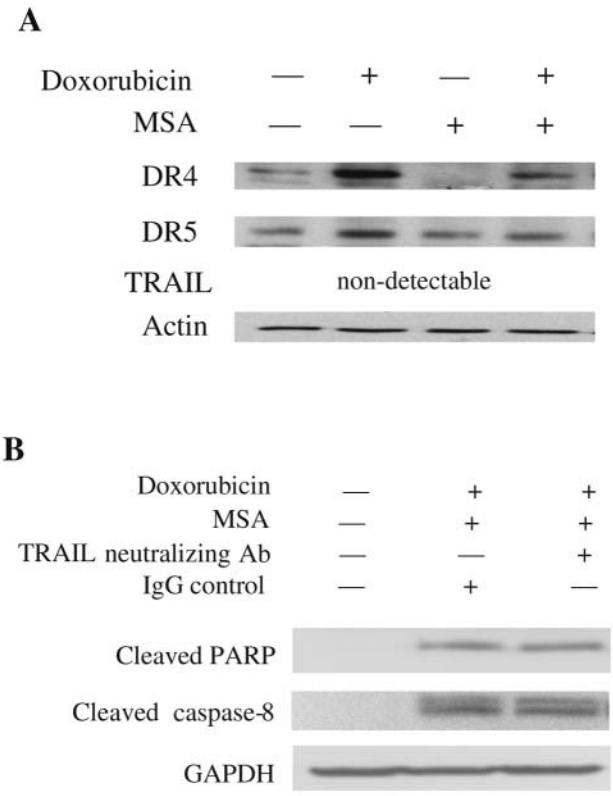

Figure 2. *Effect of doxorubicin/MSA on TRAIL signaling pathway. A) Western blot analysis of TRAIL signaling regulatory molecules. B) Evaluation of TRAIL neutralizing antibody (3 μg/ml) on increases of cleaved PARP and caspase-8 by doxorubicin/MSA combination. Mouse IgG (3 μg/ml) was used as the control.*

Effect of doxorubicin/MSA on TRAIL signaling. We then studied the modulation of TRAIL receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), by doxorubicin and MSA. The Western blot data from the 24-h cultures are shown in Figure 2A. Doxorubicin increased the expression of both DR4 and DR5, while MSA down-regulated DR4, but had no effect on DR5. Treatment with the combination increased slightly the expression of both receptors; however, the increase was much less than that caused by doxorubicin alone. It appears that MSA might have negated the effect of doxorubicin. TRAIL protein expression was not detectable by Western blot analysis. Our observation is consistent with the lack of TRAIL transcription in MCF-7 cells as reported previously (26).

In order to confirm that TRAIL signaling was indeed non-operative, we used a TRAIL neutralizing antibody to see whether it would suppress the increased cleavage of PARP and caspase-8 by doxorubicin/MSA. A non-specific mouse IgG was used as the control. The data in Figure 2B show that the TRAIL neutralizing antibody failed to reverse the effect of doxorubicin/MSA. Based on the above observations, we are ruling out TRAIL signaling in mediating the action of doxorubicin/MSA in the MCF-7 cell model. The main reasons are: (i) the ligand is either missing or non-functional, and (ii) the change of TRAIL receptor expression is inconsistent with the activation of caspase-8.

Effect of doxorubicin/MSA on Fas signaling. Next we studied a number of key molecules involved in Fas signaling, including FasL, Fas, FADD and FLIP. The Western blot data in Figure 3 show that neither FasL nor Fas was affected by doxorubicin, MSA, or the combination. As noted earlier, FADD is an important adaptor protein which facilitates the recruitment of caspase-8 to the activated death receptors. We found that FADD expression was almost undetectable in the control MCF-7 cells. Doxorubicin slightly increased FADD expression. MSA, on the other hand, resulted in a more robust increase, and so did the combination. FLIP is known to prevent the recruitment of procaspase-8 to DISC. Our data show that FLIP expression was not changed by any drug treatment.

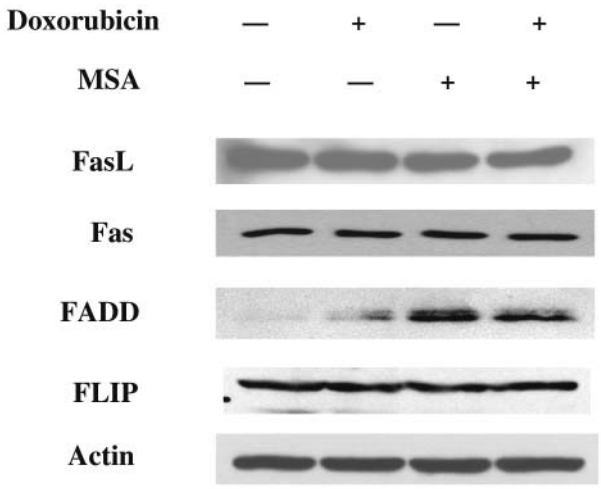

Figure 3. *Effect of doxorubicin/MSA on Fas signaling molecules as determined by Western blot analysis.*

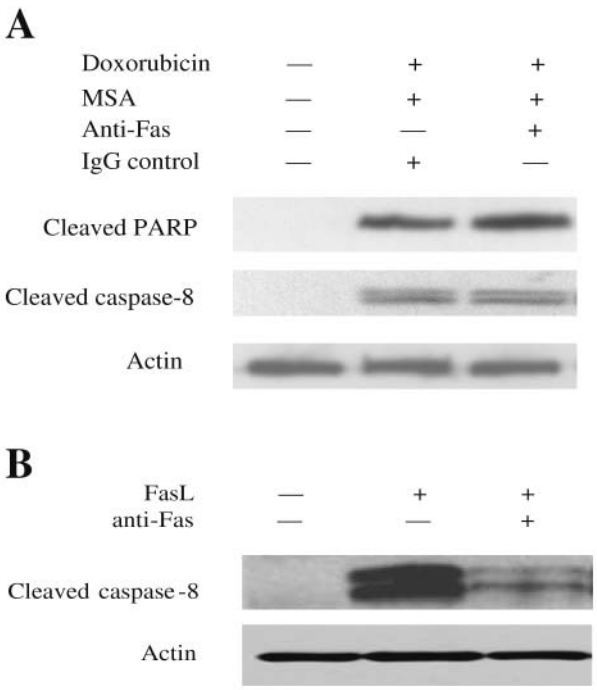

Figure 4. *A) Evaluation of anti-Fas blocking antibody (1 μg/ml) on increases of cleaved PARP and caspase-8 by doxorubicin/MSA combination. Rabbit IgG (1 μg/ml) was used as the control. B) FasL (30 ng/ml) was used as a positive control to verify the efficacy of the anti-Fas blocking antibody (1 μg/ml). Cleaved caspase-8 was the end-point of analysis.*

Lack of response to disruption of FasL-Fas interaction. In order to determine whether FasL-Fas signaling was integral to the activation of caspase-8 and PARP cleavage by doxorubicin/MSA, we used an anti-Fas blocking antibody to disrupt its interaction with FasL. The results are shown in Figure 4A. A non-specific rabbit IgG served as the negative control. Much to our surprise, the blocking antibody failed to suppress caspase-8 activation or PARP cleavage. Figure 4B shows that the anti-Fas blocking antibody was effective, since the same concentration of the blocking antibody was able to greatly compromise the efficacy of exogenous FasL in inducing caspase-8 cleavage. At first glance, our finding tends to suggest that FasL-Fas signaling is not relevant to the action of doxorubicin/MSA. We searched the literature and discovered that similar observations have been reported in which a Fas blocking antibody was ineffective in reversing Fas signaling. Many have argued that this kind of Fas activation is FasL-independent (9-15). We decided to pursue the above possibility more vigorously before ruling out the involvement of Fas.

Fas oligomerization is independent of FasL-Fas interaction. When Fas is activated, Fas oligomer is formed. Our next step was to follow a previously published procedure in which a limiting amount of antibody was used to preferentially immunoprecipitate the Fas oligomers (9). The principle of the assay is shown in Figure 5A. In a condition of limited antibody (left panel), a greater amount of Fas protein would be precipitated if oligomerization occurs. As a result, there will be a stronger Fas band in the Western blot. In contrast, when the antibody is present in excess (right panel), an equal amount of Fas would be immunoprecipitated, with or without oligomerization, provided that Fas expression is not affected. As shown in Figure 5B, all treated cells (doxorubicin, MSA, or the combination) had the same amount of Fas as the control when we immunoprecipitated with an excess of Fas antibody (10 μg/ml). This result was consistent with that from whole cell lysate (Figure 3). However, cells treated with doxorubicin or the combination (but not MSA) had more Fas when we immunoprecipitated with a limited amount of Fas antibody (0.5 μg/ml), suggesting that doxorubicin caused Fas oligomerization. In order to confirm that Fas oligomerization was independent of FasL-Fas interaction, we first immunoprecipitated Fas and then probed with the FasL antibody by Western blot. The analysis did not reveal the presence of FasL in the Fas immunoprecipitate (Figure 5C), suggesting that FasL-Fas interaction might not be necessary for Fas oligomerization to take place. It should be noted that the absence of FasL in the Fas immunoprecipitate was not an artifact. As noted in Materials and Methods, the cells were treated with a cross-linker to stabilize the interaction between FasL and Fas (if any) before lysis. Furthermore, sFasL was detected in the Fas immunoprecipitate by the same antibody which is able to detect both FasL and sFasL, suggesting that the presence of sFasL in the cell lysate was from the sFasL-Fas complex.

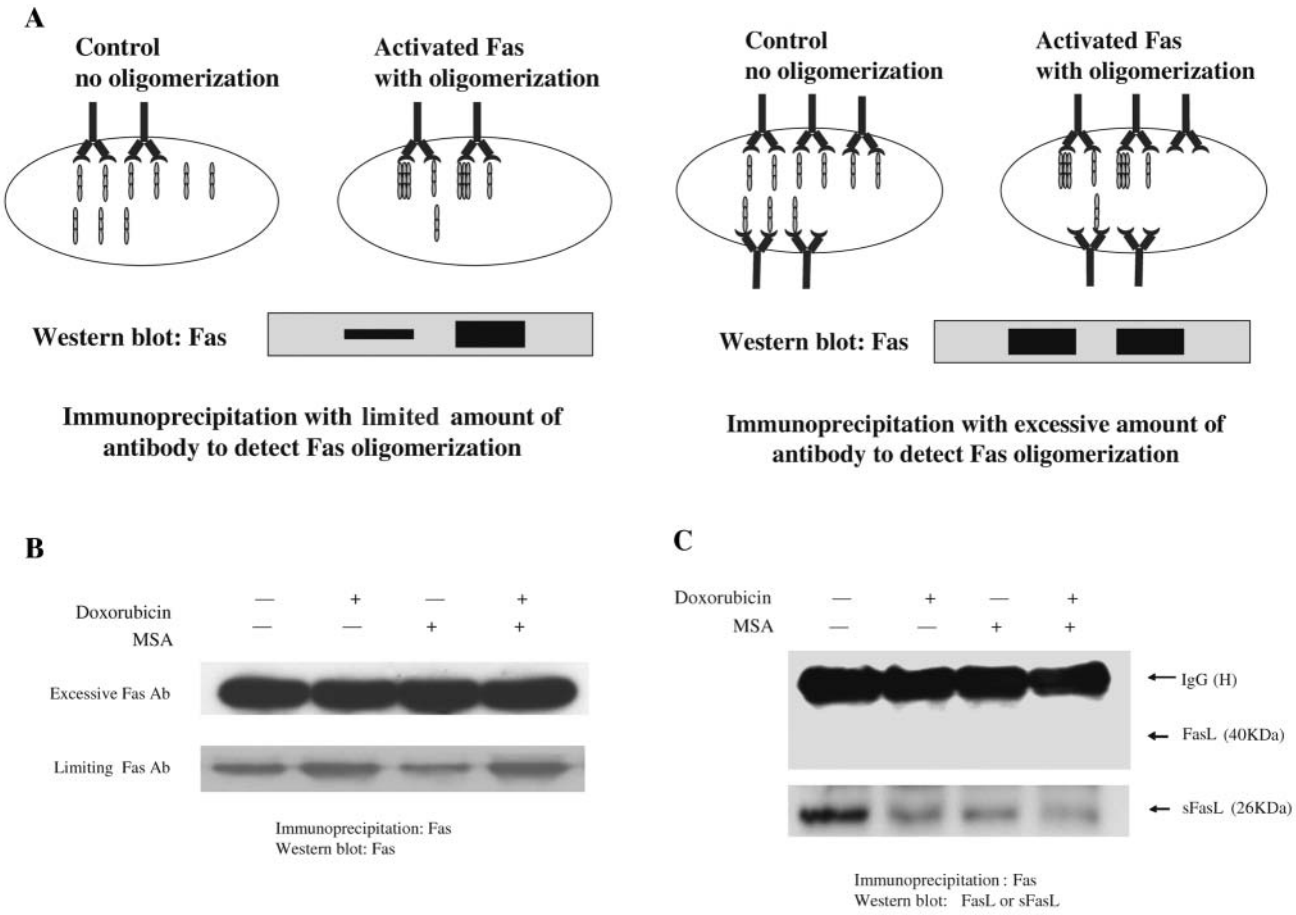

Figure 5. *Independence of Fas oligomerization on FasL-Fas interaction. A) Schematic representation of experimental design of using a limited or an excessive amount of Fas antibody to immunoprecipitate Fas oligomers. B) Western blot analysis of Fas under excessive or limiting Fas immunoprecipitation condition in cells treated with doxorubicin/MSA. C) Western blot analysis of FasL and sFasL in Fas immunoprecipate obtained from cells treated with doxorubicin/MSA.*

sFasL is produced *via* cleavage of membrane-bound FasL by metalloproteinases (27). However, it is about 1000-fold less potent than membrane-bound FasL (28).

Cleaved caspase-8 is present in Fas and FADD immunoprecipitate in doxorubicin/MSA-treated cells. DISC is formed by the association of activated Fas, FADD and caspase-8. In view of our observations that doxorubicin promoted Fas oligomerization (*i.e.* activation) and MSA increased FADD expression, we would expect to see a greater recruitment of caspase-8 to the complex in cells treated with doxorubicin and MSA than in cells treated with the single agent. We first immunoprecipitated Fas and probed with caspase-8 antibody by Western blot. The results are shown in Figure 6A. The cleaved caspase-8 band was much stronger in the doxorubicin/MSA sample than in the doxorubicin or MSA only sample. This is sound evidence that when cells were treated with doxorubicin and MSA, Fas was activated, and the signal was functionally relayed downstream, thereby leading to the formation of DISC. The data also suggest that although doxorubicin was able to induce Fas activation (as evidenced by Fas oligomerization), the signal was not sufficiently amplified because of the low abundance of FADD. With MSA boosting the expression of FADD, the signal had now gained enough power to successfully recruit caspase-8 to the complex.

In order to strengthen our argument, we carried out a second immunoprecipitation experiment, this time of FADD. Again, we probed the immunoprecipitate with caspase-8 antibody. The results are shown in Figure 6B. With this setup, we detected a modest amount of caspase-8 in the complex in cells treated with doxorubicin or MSA alone. However, cells treated with the combination still accumulated much more caspase-8 in the complex. Why is there a slight discrepancy in caspase-8 abundance between the Fas and FADD immunoprecipitates, especially with doxorubicin treatment?

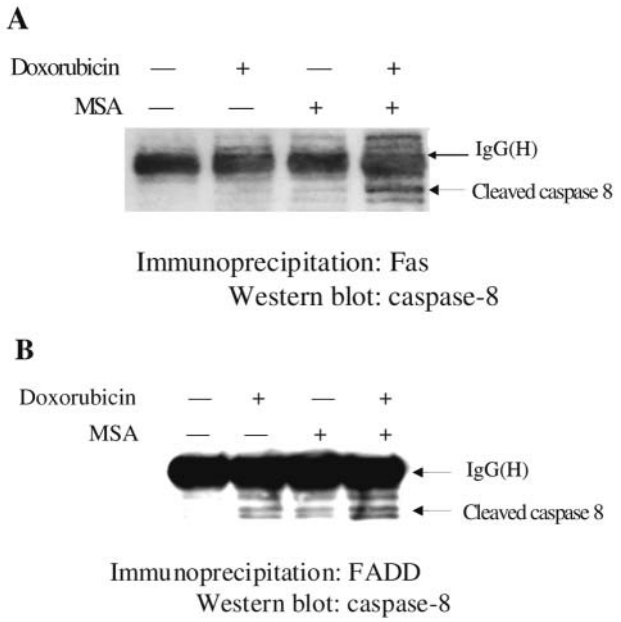

Figure 6. *A) Western blot analysis of cleaved caspase-8 in Fas immunoprecipitate of doxorubicin/MSA-treated cells. B) Western blot analysis of cleaved caspase-8 in FADD immunoprecipitate of doxorubicin/MSA-treated cells.*

One explanation could be due to the fact that FADD is the molecule that is directly associated with caspase-8. Therefore the detection of caspase-8 is sensitized when FADD antibody is used to immunoprecipitate the complex.

Discussion

We have made an interesting discovery that doxorubicin is capable of causing Fas oligomerization (*i.e.* activation) in a FasL-independent manner, without increasing the level of Fas. We have also found that MSA increases FADD expression. Enhanced Fas oligomerization and FADD expression are expected to promote the formation of DISC and caspase-8 activation. Inhibiting the activity of caspase-8 also reduces the synergy of doxorubicin and MSA in apoptosis induction. Collectively, the above observations are supportive of the role of the Fas signaling pathway in mediating the action of doxorubicin and MSA.

Our original plan to focus on TRAIL and FasL was based on our own data that MSA increases FOXO transactivation (2), and that TRAIL and FasL are known transcriptional targets of FOXO (22, 23). We were not able to find evidence of TRAIL or FasL up-regulation by MSA in the MCF-7 cells. This could be a cell type-specific issue. As a matter of fact, TRAIL was not even detectable in this cell line. The notion that doxorubicin and MSA are able to engage the Fas pathway without having to rely on the participation of FasL is a therapeutic advantage, since tumor cells often have a defect in death receptor ligand expression.

The idea that Fas can be activated independently of FasL has precedence. A number of DNA-damaging chemotherapeutic drugs are known to cause activation of Fas signaling free of FasL (9-15), although little information is available about the mechanism. Chen *et al.* found that persistent activation of JNK could trigger Fas signaling without involving FasL (12). Since most DNA damaging drugs can stimulate JNK, it is possible that JNK may mediate FasL-independent Fas signaling. Our hypothesis that doxorubicin is capable of activating Fas in this fashion is based on two lines of evidence: (i) exposure to anti-Fas blocking antibody did not protect against apoptosis, and (ii) the absence of FasL in DISC. Although we did not provide direct evidence of DISC formation, the detection of cleaved caspase-8 in either the Fas or FADD immunoprecipitate suggests that a functionally active DISC is formed. It has been suggested that the lack of efficacy of the anti-Fas blocking antibody, also seen in other studies, might be due to inaccessibility of the target to the reagent. Fas and FasL are known to be stored intracellularly and co-localized to the same compartment inside the cell, *e.g.* endoplasmic reticulum and/or Golgi (29). FasL may be able to trigger Fas signaling within the cell before the FasL-Fas complex is presented to the cell surface. However, it does not appear to be the case in our study, because there is no evidence of FasL interacting with Fas based on our co-immunoprecipitation data.

FADD is a key adaptor molecule charged with transmitting the signal initiated by the death receptors. By up-regulating FADD, MSA amplifies the Fas signal through more DISC formation and thus activation of caspase-8. MSA by itself did induce a small amount of activated caspase-8, which was only detected in the FADD immunoprecipitate (Figure 6B), but not in the whole cell lysate (Figure 1A). It is possible that this was due to signaling through other death receptors, since FADD is also the adaptor for DR3, DR4, DR5 and TNF-R1 (30-33). Furthermore, FADD may also activate caspase-8 in a death receptor-independent manner (34). Overexpression of FADD has been reported to induce apoptosis *via* the formation of death effector filaments (DEF), which in turn recruit and activate procaspase-8 (30). However, there is some controversy that DEF may be just an artifact resulting from protein overexpression. Its physiological relevance *in vivo* still remains questionable. Regardless, there is little debate that FADD is more than a transducer for death receptors. FADD plays an important role in controlling cell proliferation and cell cycle progression (35). The knowledge that MSA up-regulates FADD could have far reaching implication in how we use MSA as a chemotherapeutic modulator.

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