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Reversal of Tumor Necrosis Factor Resistance in Tumor Cells by Adriamycin via Suppression of Intracellular Resistance Factors

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Tumor necrosis factor (TNF) and various chemotherapeutic drugs show synergistic antitumor effects *in vitro* and *in vivo*, though the mechanism is not clear. Based on our previous finding that endogenous TNF (enTNF) acts as an intracellular resistance factor against exogenous TNF by scavenging oxygen free radicals (OFR) with induced manganous superoxide dismutase (MnSOD), we examined the suppression of these resistance factors by chemotherapeutic drugs and the resulting increase in TNF cytotoxicity. Pretreatment of HeLa cells, which produce an appreciable amount of enTNF and show apparent TNF resistance, with TNF followed by adriamycin (ADM) resulted in an additive effect, whereas pretreatment with ADM followed by TNF resulted in a synergistic effect. After treatment of HeLa cells with ADM, the expression of enTNF was remarkably suppressed and MnSOD activity was decreased by one-half. These results indicate that suppression of the intracellular resistance factors, i.e., enTNF and MnSOD, by ADM plays an important role in the mechanism of the synergistic antitumor effect of TNF in combination with ADM.

Key words: Tumor necrosis factor — Adriamycin — Resistance factor

Tumor necrosis factor (TNF) is known to possess potent antitumor activity both *in vitro*[1-4] and *in vivo*.[5-10] However, its spectrum of cytotoxicity is somewhat limited.[3,5,11] A number of recent studies have attempted to overcome this resistance by combining TNF with other effective modalities such as chemotherapeutic drugs, i.e., adriamycin (ADM) and cisplatin. Recent experiments in our laboratory and others have shown that combinations of certain chemotherapeutic drugs and TNF can synergistically kill TNF-resistant tumor cells *in vitro*[12-15] and *in vivo*.[16,17] This ability to overcome TNF-resistance should be of prime importance in the treatment of resistant tumors. Unfortunately, little is known about the mechanisms involved in the reversal of resistance with combinations of TNF and chemotherapeutic drugs.

We have reported that intracellular oxygen free radical (OFR) production is involved in the cytotoxicity of exogenous TNF.[18-21] We also found that endogenous TNF (enTNF) exerts an intracellular protective effect against exogenous TNF-induced cytotoxicity by scavenging OFR with induced manganous superoxide dismutase (MnSOD).[22-27]

In the present study, we examined whether the combination of TNF and ADM, which has been widely used to treat hematological malignancies and solid tumors, overcomes TNF resistance and, as a possible mechanism, whether ADM down-regulates the intracellular resistance factors to TNF cytotoxicity, i.e., enTNF and MnSOD, in TNF-resistant HeLa (human uterine cervical cancer) cells.[27]

MATERIALS AND METHODS

Reagents TNF, produced in *Escherichia coli* and purified, and rabbit anti-rhTNF polyclonal antibody, which neutralized rhTNF activity, were generously provided by Asahi Chemical Industry Co., Ltd. (Tokyo). TNF had a specific activity of 2.3×10^6 U/mg protein and, as a trimer, a molecular mass of 51 kilodaltons.[28] ADM was generously supplied by Kyowa Hakko Kogyo Co., Ltd. (Tokyo).

Cell culture HeLa (human uterine cervical cancer) cells[26,27] were cultured in Eagle's MEM supplemented with 10% FBS (Flow Laboratories, Inc., North Ryde, Australia) at 37°C in a humidified 5% CO_2 incubator.

Cytotoxicity assay Cells (1×10^4 cells/100 μl) were added to the wells of a 96-well microculture plate (Falcon) and incubated at 37°C for 18 h in 5% CO_2. Then, TNF (1 to 1×10^4 U/100 μl) and ADM (1 to 1×10^3 μM/100 μl) were added, followed by incubation at 37°C for 24 h in 5% CO_2. Cytotoxic activity was then assayed by the dye-uptake method as described earlier.[25-27] Synergistic cytotoxicity was estimated by using isobologram analysis.[13]

Western blotting ADM (0.5 μM)-treated cells were solubilized with 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 20 mM Tris-HCl (pH 7.4) and centrifuged (10,000g for 10 min). Supernatants were

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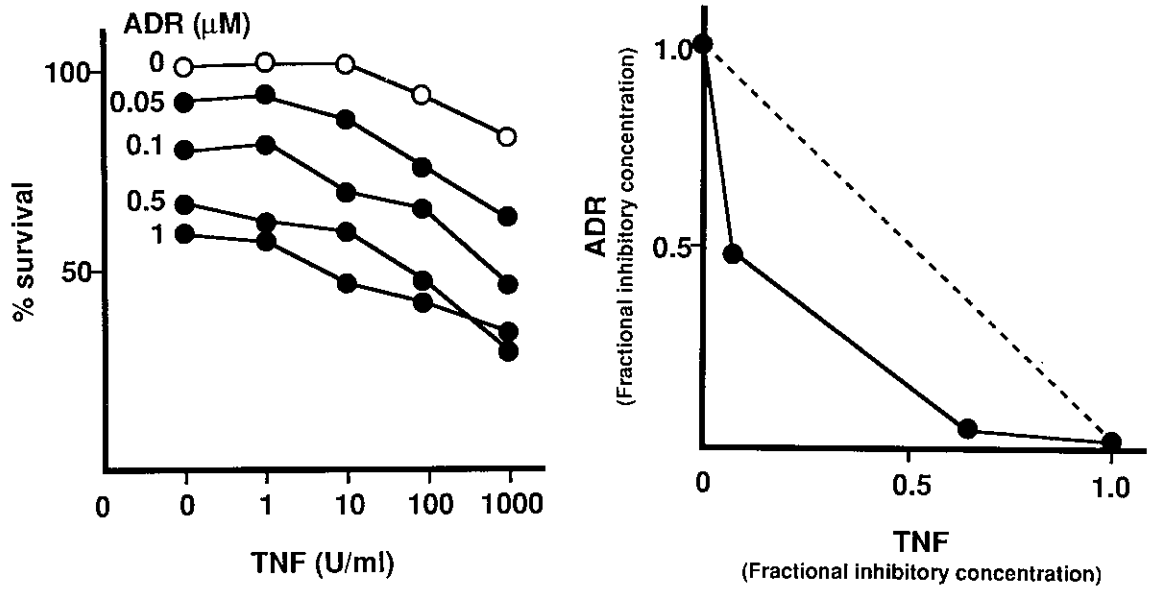

Fig. 1. Cytotoxic effect on HeLa cells by a combination of TNF and ADM. HeLa cells (1×10^5/ml) were cultured with TNF (10 to 1×10^5 U/ml) and ADM (10 to 1×10^4 μM/ml) for 24 h and then the cytotoxic activity was determined by dye-uptake assay. The cytotoxicity is expressed as percent survival (left column) and an isobologram analysis (right column) was made.

then subjected to 15% polyacrylamide-sodium dodecyl sulfate (SDS) gel electrophoresis.[29] After electrophoresis, the fractionated protein was transferred to a polyvinylidine difluoride membrane (Millipore Products Division, Bedford, MA, USA) in 20% methanol:48 mM Tris:39 mM glycine:1.3 mM SDS (pH 9.2) by using a Trans-Blot SD Cell (BioRad Laboratories, Richmond, CA, USA). Membranes were incubated for 3 h at room temperature with 3 μg/ml of ^{125}I-anti-TNF polyclonal antibody,[25,26] which was radiolabeled using Enzymobead reagent (BioRad). Membranes were then washed with phosphate-buffered saline (pH 7.4) 10 times and exposed to Kodak X-Omat film.

Determination of intracellular MnSOD MnSOD activity was assayed by the nitroblue tetrazolium method as described by Oberley and Spitz.[30] Protein concentrations were determined by means of the BioRad protein assay. MnSOD activity was expressed in units per mg of protein.

RESULTS

Augmented cytotoxicity of the combination of TNF and ADM against HeLa cells The results with TNF and ADM alone and in combination for 24 h are shown in Fig. 1. The synergistic effect with the combination of TNF and ADM is clearly shown by the isobologram. Furthermore, we examined the effect of sequential treat-

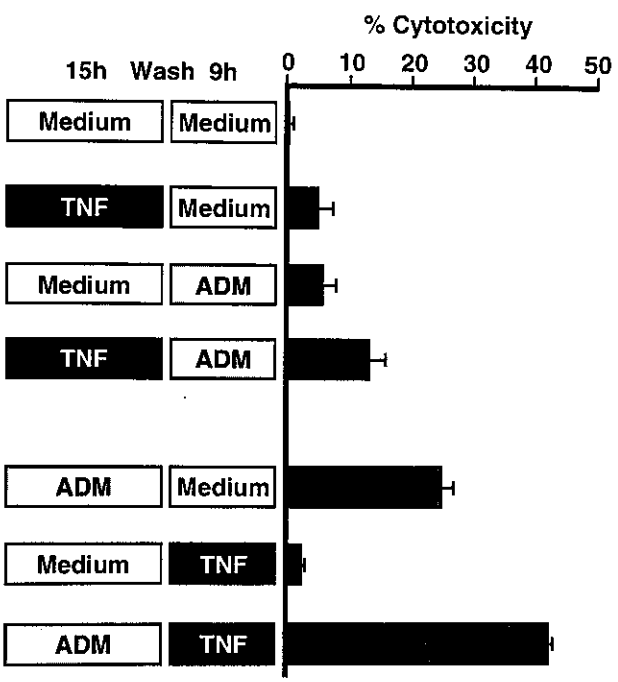

Fig. 2. Effect of sequential treatment with TNF and ADM on cytotoxicity against HeLa cells. TNF (10 U/ml) or ADM (0.5 μM) at 15 h was used for pretreatment followed by washing and incubation for 9 h with the other agent, and cytotoxicity was assessed by dye-uptake assay. Values are mean±SD (bars) of three separate experiments.

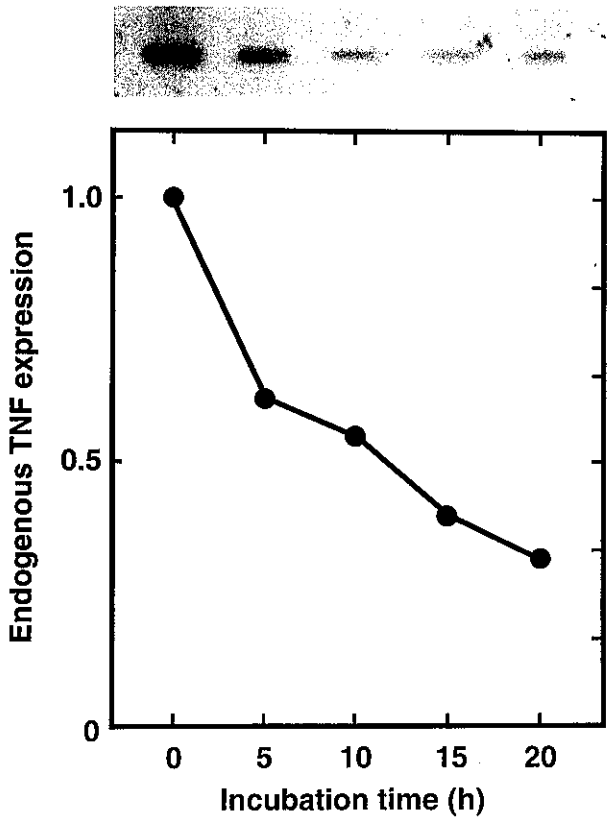

Fig. 3. Effect of ADM treatment on enTNF expression of HeLa cells. Cell extracts from HeLa cells treated with ADM (0.5 μM) for 0, 5, 10, 15 and 20 h were subjected to 15% polyacrylamide-SDS gel electrophoresis and transferred to a polyvinylidine difluoride membrane. An immunoblot was developed using ^{125}I-labeled anti-rhTNF polyclonal antibody (rabbit). The membrane was exposed to Kodak X-Omat film for 48 h at −70°C (upper panel). Lanes represent HeLa cells treated with ADM for 0, 5, 10, 15 and 20 h, respectively. Using densitometry, the relative density of each band was estimated by arbitrarily normalizing the results with respect to ADM-untreated HeLa cells (lower panel).

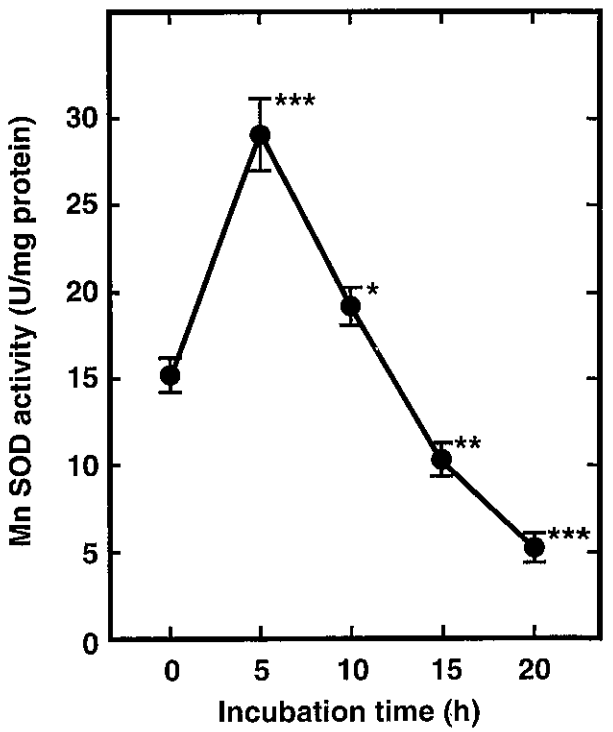

Fig. 4. Effect of ADM treatment on intracellular MnSOD activity. Cells were treated with ADM (0.5 μM) for 0, 5, 10, 15 and 20 h and MnSOD activity was assayed by nitroblue tetrazolium assay. MnSOD activity was expressed in U/mg protein. Values are mean±SD (bars) of three separate experiments. *, $P<0.02$; **, $P<0.01$; ***, $P<0.001$ by Student's t test.

ment on cytotoxic activity against HeLa cells. Cells were first treated for 15 h with one of the agents, then washed, and incubated for 9 h with the other agent, and cytotoxicity was assessed. The results, shown in Fig. 2, indicate that pretreatment with TNF followed by ADM results in an additive effect, whereas pretreatment with ADM followed by TNF results in a synergistic effect. These observations indicate that sequential treatment with ADM sensitizes the cells to the effect of TNF.

Effect of ADM treatment on the expression of enTNF by HeLa cells We have reported that enTNF acts as an intracellular resistance factor against exogenous TNF by inducing MnSOD.[23-27] Therefore, we used Western blotting to examine whether ADM treatment inhibits the synthesis of enTNF. As shown in Fig. 3, protein reactive to anti-TNF antibody was clearly identified in non-treated HeLa cells, while in ADM-treated HeLa cells, the intensity of the enTNF band was weak.

Changes in intracellular MnSOD levels with ADM treatment Next, we assessed the kinetics of intracellular MnSOD activity in HeLa cells after ADM treatment. Compared with non-treated HeLa cells, MnSOD activity was decreased by one-half at 15 h after treatment with ADM, although a transient increase was observed at 5 h (Fig. 4).

DISCUSSION

In the present study, we demonstrated that ADM can reverse TNF resistance in tumor cells by suppressing the expression of intracellular resistance factors, i.e., enTNF and MnSOD. Recently, Safrit and Bonavida reported that ADM down-regulates the expression of TNF

mRNA, resulting in the augmentation of TNF cytotoxicity.[31] These results are consistent with ours. However, they also found that the MnSOD mRNA level in TNF-resistant R4 human renal carcinoma cells was increased about two times by ADM treatment and concluded that MnSOD played no role in the reversal of TNF resistance by ADM. These findings differ from our observation that the intracellular MnSOD activity of HeLa cells was decreased by one-half at 15 h after treatment with ADM. The discrepancy between our results and these of Safrit and Bonavida may be due to the period of measuring the levels of MnSOD mRNA or MnSOD activity. They examined the expression of MnSOD mRNA only 1 h after ADM treatment. In this initial period after ADM treatment, we also observed a transient increase in MnSOD activity at 5 h.

Therefore, we consider that suppression of not only enTNF expression but also MnSOD synthesis plays an important role in the reversal of TNF resistance in tumor cells by ADM. This idea is consistent with our earlier reports that the TNF sensitivity of cells varied with the modulation of enTNF expression; i.e., L-M cells, originally non-producers of enTNF, transfected with secretory and nonsecretory types of human TNF-expressing vectors acquired TNF resistance[24, 25] and, conversely, introduction of an antisense TNF gene into HeLa cells enhanced TNF sensitivity.[24] MnSOD activity in these cells and transfectants showed a good correlation with TNF resistance.[23, 25, 26]

With regard to the question of whether TNF resistance induced by enTNF is due only to the induction of MnSOD synthesis, we have recently observed a remarkable increase in heat shock protein (HSP) 72, which is known to be an intracellular protective factor against various cellular stresses, including exogenous TNF,[32] in TNF gene-transfected cells after heat treatment.[33] These findings suggest that further study concerning the effect of ADM on the expression of other resistance factors, i.e. HSP families, is necessary to substantiate the mechanism of the synergy between TNF and ADM, and to develop effective combination therapies.

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