The Effect of Cyclophosphamide on Fas-mediated Apoptosis

Fas is a cell surface protein that can mediate apoptosis and belongs to the tumor necrosis factor (TNF) receptor family. Anti-Fas antibody induces apoptotic cell death in sensitive cells. Because many chemotherapeutic drugs are capable of initiating pathways leading to apoptosis, we determined the effect of cyclophosphamide, one of the most widely used anticancer drugs, on Fas mediated apoptosis in human lymphoma cell lines; SKW6.4 and Jurkat. Cell lines were cultured for 3 days alone in a medium or with cyclophosphamide (2μg/ml). Anti-Fas IgM of various concentrations was added after treatment. Apoptosis was measured by electrophoresis of DNA fragmentation and surface expression of Fas was measured by flow cytometry. These cell lines were found to express Fas and were very sensitive to anti-Fas mediated apoptosis in a dose dependent manner. Up regulation of Fas was induced by cyclophosphamide in Jurkat except SKW6.4. Cyclophosphamide augumented apoptosis mediated by anti-Fas, synergistically. These results suggested that the anti-cancer drug might be mediated via the pathway of Fas mediated apoptosis in the lymphoma cell lines. *(JKMS 1997; 12: 185~9)*

Key Words : *Apoptosis, Anti-Fas, Anti-cancer drug, Lymphoma cell line*

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Received : December 11, 1996
Accepted : March 3, 1997

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INTRODUCTION

Apoptosis is a distinct mode of cell death that is responsible for the deletion of cells in normal tissues as well as in specific pathologic condition. Morphologically it is defined by surface membrane blebbing, contraction in cell size, chromatin condensation and formation of membrane enclosed apoptotic bodies which are phagocytosed and are not associated with inflammation. A characteristic biochemical feature is a double strand cleavage of nuclear DNA leading to production of oligonucleosomal DNA fragmentation (1).

Apoptosis occurs spontaneously in malignant tumors and is increased in tumors responding to irradiation, cytotoxic chemotherapeutic drugs, heating and hormone ablation (2). Another factor which modulates apoptosis induction, is antibody engagement of the cell surface protein designated Fas/Apo-1 (CD95) (3, 4). Fas/Apo-1 was defined during studies of monoclonal antibodies raised against a human B lymphoblastoid cell line. It was found to induce apoptosis of activated human B- and T- lymphocytes and of a variety of human lymphoid tumor derived cell lines. It belongs to the nerve growth factor receptor/ TNF receptor superfamily (5). The induction of apoptosis as an antitumor strategy has already been reported (4). Injection of anti-CD95 causes rapid regression of murine xenografts of CD95 expressing human lymphoid cell lines with the regression being accompanied by greatly enhanced apoptosis of the grafted cells (6).

A variety of anticancer drugs have been shown to induce extensive apoptosis in rapidly proliferating normal cells, lymphoid tissues and tumors (7, 8). Thus enhanced apoptosis is responsible for many of the adverse effects of chemotherapy and for tumor regression. The way in which anti-cancer drugs induce apoptosis is unknown (9). Clearly better understanding of the processes involved might be expected to lead to improved treatment regimens. Anticancer drugs mediate their therapeutic effect by triggering apoptosis. We hypothesized that apoptosis induced by cytotoxic drugs in lymphoma cell line may involved Fas/Apo-1 system. We used the anti-cancer drugs including adriamycin (0.2 μg/ml), vincristine (0.1 μg/ml), etoposide (50 μmol) and hypericine (1 μmol) and found cell death within 3 to 24 hours of treatments (data not shown). Cell death was too rapid to evaluate expression of Fas by flowcytometry.

Therefore, we determined the effect of cyclophosphamide at sublethal dose, one of the most widely used broad spectrum alkylating drugs, on Fas mediated apoptosis in human lymphoma cell lines.

MATERIALS AND METHODS

Cell lines and cell culture

All experiments were performed on SKW6.4 and Jurkat lymphoma cell lines, obtained from the American Type Culture Collection and maintained as described previously. The tumor cell lines were cultured in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum at 37℃ in a water saturated with 5% CO_2 atmosphere.

Antibodies, anticancer drugs and reagents

Mouse anti-human anti-Fas (CD95) IgM for induction of apoptosis was obtained from Pharmingen (San Diego, U.S.A). Cyclophosphamide was obtained from Sigma Chemical C. (St. Louis, MO). Cell lines were cultured in medium alone or with cyclophosphamide (2 μg/ml). Anti-Fas mouse IgM Ab (100 ng/ml~1 μg/ml) was added after treatment.

Analysis of Fas/Apo-1 antigen on lymphoma cell lines

The cells were fixed in 1% formaldehyde in PBS (pH 7.4) for 15 min on ice. Then after washing in PBS, cells were resuspended in 70% ice cold ethanol for flow-cytometry. Direct immunofluorescence was performed. Freshly isolated cells were analyzed after staining with the anti-Fas mouse IgG Ab conjugated with fluorescein isothiocyanate (FITC), obtained from Pharmingen or FITC conjugated goat anti-mouse IgG. 10^6 cells were washed twice, resuspended in 10 μg/ml anti-Fas IgG, incubated for 30 minutes on ice, in the dark. Then, after washing twice were anlyzed on a FACScan (Becton Dickinson).

MTT assay

This was performed by the previously reported standard method (10). Briefly, the cell suspension (1×10^6) was inoculated 96 well titer plate (Falcon 3075, Becton Dickson Co, Oxnard USA). Cyclophosphamide and/or anti-Fas IgM Ab were added at various concentrations. This plate was incubated at 37℃ in a humidified atmosphere of 5% CO_2 in air for 48 hr. Then, MTT (3-(4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide) solution (5 mg/ml) in PBS (50 μl) was added to each well. After 4 hr of exposure, the medium was removed and washed with PBS and then 50 μl of DMSO (dimethyl sulfoxide) was added to each plate to solubilize the precipitates. The plate was transferred to an ELISA reader to measure the absorbance at 630 nm. All experiments were performed at least 3 times.

Electrophoresis of DNA fragmentation assay

For DNA fragmentation analysis cell lysates were obtained by incubating a cell pellet containing 10^6 cells in 20 μl lysis buffer (10mM EDTA; 50 mM Tris, pH 8 ; 0.5% sarcosyl ; 0.5 mg/ml proteinase K) for one hr at 50℃. After the addition of 5 μl of RNase (1 mg/ml) and another incubation of one hr at 50℃, lysates were electrophoresed in a 2% agarose gel containing ethidium bromide. The gel was runned in a solution containing 26 mM Na_2HPO_4, 33 mM NaH_2PO_4, 10 mM EDTA and photographed under UV illumination.

RESULTS

Cyclophosphamide upregulates Fas antigen (CD95) expression

We examined Fas Ag in human lymphoma cell lines, SKW6.4 and Jurkat. As shown in Fig. 1, cyclophosphamide (2 μg/ml) results in increased Fas Ag expression of Jurkat cell line, except SKW6.4.

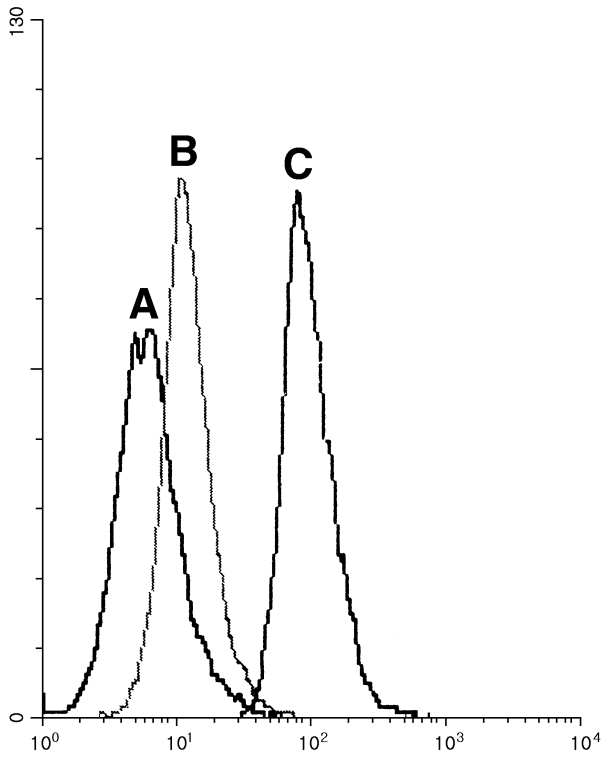

Fig. 1. Flowcytometric analysis of Fas Ag (CD95) on Jurkat cell line : The histogram analysis of fluorescence is plotted as log function. The (B) line represents the control cells without cyclophosphamide (2 μg/ml), while the overlaid line (C) plot demonstrates the treated cells. The (A) line shows the cells reacted with FITC goat anti-mouse IgG.

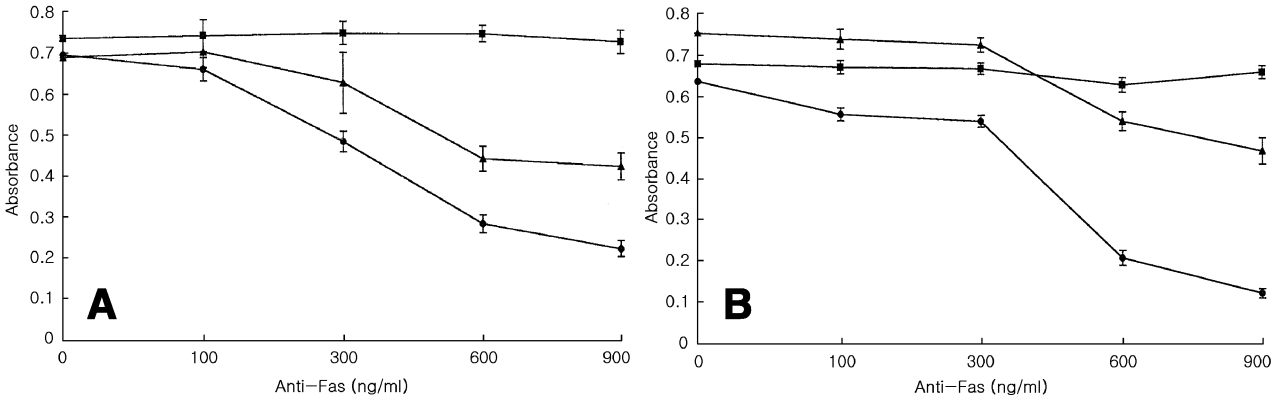

Fig. 2. Effect of cyclophosphamide on Fas mediated apoptosis in SKW 6.4 (A) and Jurkat (B). Cells (10^6/ml) were incubated for 3 days at 37℃ with or without 2 μg/ml of cyclophosphamide and then anti-Fas IgM of various dose was added. Cell viability was determined by the MTT assay. ●, cyclophosphamide 2 μg/ml and anti-Fas, ■, cyclophosphamide 2 μg/ml, ▲, anti-Fas of various dose. Data shown are the mean±SD for 3 repeated experiments.

The effect of cyclophosphamide on the anti-Fas mediated cell death

SKW6.4 and Jurkat cell lines were killed by anti-Fas IgM in a concentration- dependent manner. The cytolytic activity is enhanced when the cells are treated with cyclophosphamide and anti-Fas IgM in both cell lines. The synergistic effect of cyclophosphamide on the Fas mediated cell death is significantly found both in SKW6.4 and Jurkat cell lines (Fig. 2).

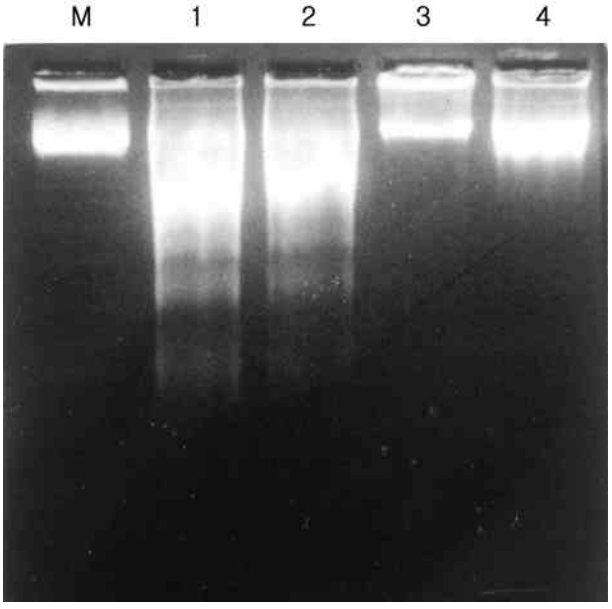

Fig. 3. Agarose gel electrophoresis of DNA extracted from cultured SKW6.4. treated with cyclophosphamide 2 μg/ml for 3 days. M: DNA marker, Lane 1: cells treated with anti-Fas after cyclophosphamide addition, Lane 2: cells treated with anti-Fas, Lane 3: cells treated with cyclophosphamide 2 μg/ml for 3 days, Lane 4: genomic DNA

Anti-Fas IgM mediates cell death via apoptosis

As shown in Fig. 3, DNA from the cells treated with anti-Fas IgM alone and with combined cyclophosphamide and anti-Fas IgM showed DNA framentation in a ladder fashion. On the other hand, no DNA fragmentation was observed in the cells incubated with cyclophosphamide at low toxic concentration (2 μg/ml) alone.

DISCUSSION

For successful pharmacological modulation of chemotherapy sensitivity, we investigated the cytotoxic activity of anti-Fas antibody in combination with cyclophosphamide at a sublethal dose (2 μg/ml), in human lymphoma cell lines, SKW6.4 and Jurkat. The reason for the choice was that these cell lines were well known for expression of Apo-1/Fas antigen and susceptibility to apoptosis induced by anti-Fas antibody (4). Anti-Fas is an IgM monoclonal antibody that reacts with Fas antigen expressed on the surface of tumor cells and mediates cytotoxic activity against sensitive tumor cell lines (3). We also found the presence of Fas antigen on these cell lines by flowcytometry (data not shown) and increased susceptibility of the cells to anti-Fas in a dose dependent pattern (Fig. 2). The present findings provide evidence that anti-Fas antibody in combination with cyclophosphamide at less toxic concentration, results in augmented or synergistic cytotoxic activity against cell lines SKW 6.4. and Jurkat. These results are consistent with previous observation on cell death induced by anti-Fas

antibody (5, 11, 12). It has been reported that cells infected with human immunodeficiency virus are more sentitive to anti-Fas antibody than uninfected cells (13). Antibodies have frequently been used as heteroconjugates with toxins or drugs to destroy tumor cells. Anti-Fas antibody can synergize in cytotoxicity with toxins and chemotherapeutic drugs and combination treatment can reverse resistance to TNF, toxins and/or drug (14). The cytotoxicity was augumented by pretreatment with gamma-interferon which upregulates Fas antigen expression (15). Induction of Fas expression following treatment with cytotoxic drug was also observed in neuroblastoma cell line (16). Therefore we made tests to try to explain the synergistic effect of cyclophosphamide on Fas mediated apoptosis. In our study, cyclophosphamide upregulates surface expression of Fas antigen in Jurkat (Fig. 1) and thus renders the cells more sensitive to anti-Fas antibody interaction. However, up-regulation of Fas antigen was not found in SKW6.4, while synergy of anti-Fas antibody with cyclophosphamide is significant. This could be explained by the fact that anti-Fas susceptibility is not uniformly correlated with expression of this protein but is correlated with a signal pathway involved in Fas mediated apoptosis. For example, Fas antigen expression is observed on both resting and activated human lymphocytes, although sensitivity to anti-Fas mediated apoptosis is restricted only to cells stimulated for 4 days or longer (17). The dissociation of Fas function and expression suggests that the downstream signaling pathway may be modified for elicitation of the death signal. The downstream factors involved in Fas signaling, such as Mort1/FADD protein are essential for Fas induced apoptosis (18). Another candidate of this signaling for apoptosis is Fas-ligand, a novel member of TNF family. Fas ligand is an activation inducible molecule and Fas ligand mRNA has been shown to be abundantly induced in splenocytes upon stimulation with phorbol ester and calcium ionophore (19). Induction of Fas-ligand mRNA following treatment with cytotoxic drugs has been described in human leukemia T cell, neuroblastoma, and hepatocellular carcinoma cell lines (20). It would be helpful to study the Fas-ligand mRNA for explanation of the synergistic effect of anticancer drug on Fas mediated apoptosis in our study.

Our data indicates that anti-Fas antibody alone induced apoptosis of human lymphoma cell lines, SKW6.4 and Jurkat in a dose dependent pattern. Fas antigen was up-regulated by cyclophosphamide in Jurkat but not in SKW6.4. Cyclophosphamide at low toxic dose augumented Fas mediated cell death. These results indicate that the anti-cancer drug, cyclophosphamide may be involved in the pathway of Fas mediated apoptosis. Combination therapy with anti-Fas antibody and drugs appears to be effective in various experimental tumor systems and humans.

Acknowledgments

This paper was presented at the 85th United States and Canadian Academy of Pathology and supported in part by Kyung Hee University research funds.

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