

Profiles of Caspase Activation and Gene Expression in Human Breast Cancer Cell Line MCF-7, after Cyclophosphamide, Doxorubicin, 5-Fluorouracil (CDF) Multi-Drug Administration

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The multidrug treatment, CDF [cyclophosphamide (CPA), doxorubicin (DXR), and 5-fluorouracil (5-FU)], is a common chemotherapy protocol for breast cancer. However, the molecular mechanisms underlying its toxicity for breast cancer cells remain unclear. As a laboratory model of breast cancer chemotherapy, the human breast cancer cell line MCF-7 was treated with CDF or the individual CDF reagents. Western blotting analysis revealed that two effector caspases (-6 and -7) were activated following the administration of DXR, 5-FU, or the CDF multi-drug. However, after treatment with CPA alone, caspase-7 was activated, but caspase-6 was not. We next used the RNA arbitrarily primed-PCR differential display (RAP-PCR DD) method, a derivative of the differential display method, to reveal changes in gene expression with the individual or multi-anticancer drug treatment. RAP-PCR DD was performed using arbitrary PCR primers. Independent cDNA bands representing at least nine mRNA species were amplified after drug treatment. Our results strongly suggested that the administration of different single and multi-anticancer drug treatments induced the expression of several different genes, whose products may be involved in the induction of cancer cell death.

Key words—caspase activation, RNA arbitrarily primed-PCR differential display, MCF-7, doxorubicin, 5-fluorouracil, cyclophosphamide

INTRODUCTION

Multi-drug administration is well known to be advantageous over single-drug treatment in chemotherapies, especially in cancer chemotherapy. The administration of CDF [cyclophosphamide (CPA), doxorubicin (DXR), 5-fluorouracil (5-FU)] to treat human breast cancer is a representative multi-drug chemotherapeutic protocol. In cancer chemotherapy, it has been shown empirically that the administration of multiple drugs kills cancer cells more effectively than administration of a single drug, especially when the pharmacological efficacy of each drug is different. However, the efficacy of these chemotherapies has not always been confirmed by rigorous cell-biological studies, and some multi-drug protocols for cancer chemotherapy remain controversial with regard to their effectiveness for killing cancer cells.

Some anticancer drugs allegedly induce apoptosis; CPA,¹⁾ DXR,²⁾ and 5-FU³⁾ are well-known examples. Since apoptotic cell death does not induce an inflammatory reaction, therapies that kill cancer cells by apoptosis can promote better healing in cancer patients. Although CDF multi-drug administration is a common chemotherapy protocol for breast cancer, the detailed mechanisms of its cytotoxicity for breast cancer cells remain unclear.

Previously, we examined the physiological aspects of the cancer cell death caused by CDF treatment, using the human breast cancer cell line MCF-7 as a model.⁴⁾ Our results indicated that the MCF-7 cell death induced by each of these anticancer drugs administered individually is a mixture of apoptotic and non-apoptotic cell death. MCF-7 cells seem to die predominantly by apoptosis after the administration of either CPA or 5-FU, but the dying cells are morphologically non-apoptotic after DXR or CDF multi-drug treatment.

To gain insight into the mechanisms underlying these complicated but interesting results, here we investigated the activation of effector caspases (caspases-6 and -7) after individual treatment with CPA, DXR, or 5-FU, and after CDF multi-drug treatment. In addition, we examined the effects of the anticancer drugs on gene expression in the

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MCF-7 cells. Our previous results indicated that the viable cell population was reduced by 50% two to three days after the administration of anticancer drugs.⁴⁾ Thus, there seems to be sufficient time between the addition of the drugs and the beginning of cell death to permit changes in the expression of genes associated with apoptotic or non-apoptotic cell death. To investigate this possibility, we used the RNA arbitrarily primed-PCR differential display method (RAP-PCR DD), a derivative of the differential display method. We found that the gene-expression pattern in the MCF-7 cells was different after treatment with each individual drug and with CDF.

MATERIALS AND METHODS

Cell Line and Cell Culture — The human breast cancer cell line MCF-7 was used in this study. MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum and appropriate antibiotics. The MCF-7 cells were grown under 100% humidity in a 5% CO₂ atmosphere at 37°C. The details of the cell culture were as described pre-

viously.⁴⁾

Administration of Anticancer Drugs — The anticancer drugs CPA, DXR, and 5-FU were added alone or in combination to logarithmically growing MCF-7 cells at a final concentration of 500 µg/ml CPA, 5 µg/ml DXR, and 25 µg/ml 5-FU. The cells were kept in a CO₂ incubator until they were harvested. CPA and DXR were dissolved in H₂O, and 5-FU was dissolved in dimethyl sulfoxide (DMSO). The concentrations of anticancer drugs were the same as in our previous report.⁴⁾

Western Blotting — To detect caspases-6 and -7, MCF-7 cells that were untreated or treated with anti-cancer drugs were harvested by trypsinization at the sampling times indicated in Fig. 1. The cells were then washed with ice-cold phosphate-buffered saline without CaCl₂ and MgCl₂ (PBS(-)) and pelleted by centrifugation at 700 × *g*, 4°C, 5 min. The pellet was suspended in 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, Complete™ EDTA-free (protease inhibitor cocktail, Roche Diagnostics, Mannheim, Germany), and then disrupted using a Dounce homogenizer with 100 strokes on ice. The cytosolic fraction was prepared by standard cell-fractionation methods, and then 10–40 µg protein of the cytosolic fraction

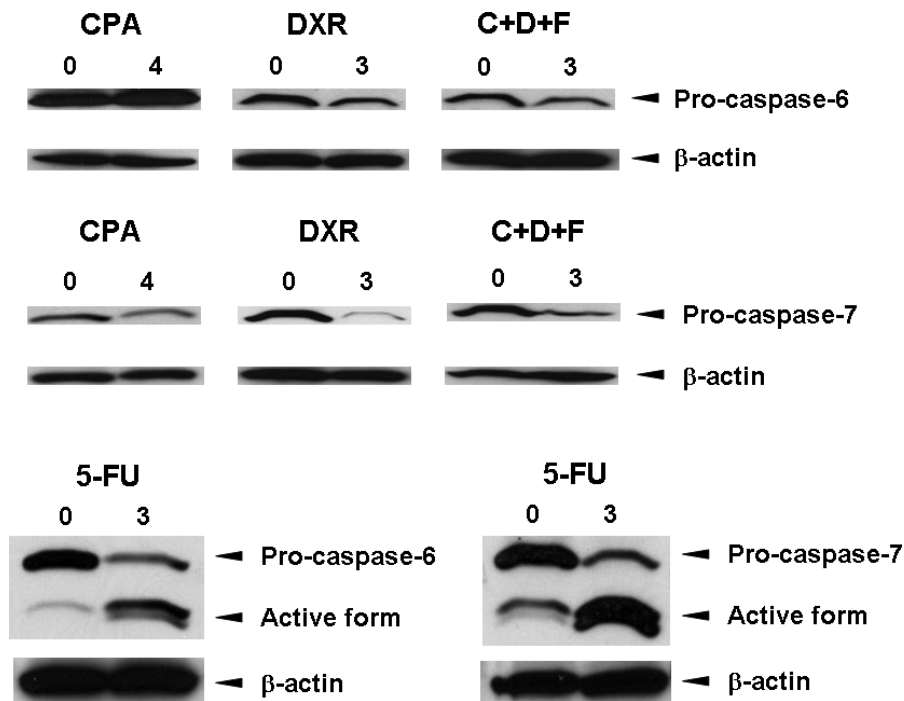


Fig. 1. Activation of Caspase-6 and -7 in the MCF-7 Cell Death Caused by Anti-Cancer Drugs

Number above each lane indicates days after drug administration. C+D+F means simultaneous administration of each drug. Beta-actin is indicated as a loading control. The active forms of caspase-6 and -7 after 5-FU administration are indicated in the lower two panels. (The lower band seen with the day-3 active form is an unidentified cross-reacting band.)

was subjected to 10–20% sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis (SDS-PAGE) followed by western blotting, as described previously.⁵⁾ The primary antibodies used in this experiment were mouse anti-human-caspase-6 antibody (diluted 1 : 100; MBL, Nagoya, Japan) and mouse anti-human-caspase-7 monoclonal antibody (1 : 100; MBL, Nagoya, Japan). The second antibody was horseradish peroxidase-conjugated anti-mouse IgG (1 : 5000; GE Healthcare Biosciences, Pittsburgh, PA, U.S.A.). Beta-actin was used as a loading control in the western blot analyses. The signals in western blots were detected by using the SuperSignal™ West Pico Chemiluminescent Substrate (Pierce, Rockford, CA, U.S.A.).

mRNA Purification from MCF-7 Cells and First-Strand cDNA Synthesis for RNA Arbitrarily RAP-PCR DD— Poly-A-tailed mRNA was purified from untreated and anticancer drug-treated MCF-7 cells as described previously.⁶⁾ In brief, the total RNA was isolated, and then the poly-A-tailed mRNA was purified from it. To isolate total RNA, MCF-7 cells were collected after 1 day of drug treatment, rinsed twice with ice-cold PBS(–), and processed with the RNeasy™ Mini kit (Qiagen, Valencia, CA, U.S.A.) followed by using the QuickPrep™ mRNA purification Kit (GE Healthcare Bio-Sciences). The purified mRNA was then used to synthesize cDNA using the SuperScript™ First-Strand Synthesis System for reverse transcription (RT)-PCR kit (Invitrogen, Carlsbad, CA, U.S.A.). For the RT procedure, the reaction mixture (100 μ l) contained 520 ng of mRNA, 100 ng of a fully degenerate 6-oligomer, NNN NNN, 2.5 mM MgCl₂, 100 μ g/ml bovine serum albumin (BSA), 30 units of ribonuclease (RNase) inhibitor, and 400 units of SuperScript II™ reverse transcriptase (Invitrogen). RT was carried out at 20°C for 15 min, 42°C for 50 min, 99°C for 6 min, and 95°C for 5 min. After the reaction, RNase H was added to the reaction mixture to remove the mRNA.

RAP-PCR DD— For the second RAP-PCR amplification step, 3 μ l of the first-strand synthesized DNA solution was added to a 100 μ l (final volume) reaction mixture containing 5 units of TaKaRa Taq polymerase™, 10 mM dNTP mixture, 25 mM MgCl₂ (TaKaRa Bio, Kyoto, Japan), and a combination of three “arbitrary but defined” primer mixtures (Table 1), each at 2 μ M. The thermocycling was conducted at 94°C for 25 sec, 32°C for 1 min, 72°C for 30 sec, for 45 cycles, followed by 10 min at 72°C. The RAP-PCR products were ana-

Table 1. PCR Primers with Arbitrary but Fixed Sequences Used for the Second Step of Differential Display

Arbitrary Primers	Length (bp)	GC content (%)
BS-52: CAAGCGAGGT	10	60
BS-54: AACGCGCAAC	10	60
BS-55: GTGGAAGCGT	10	60
BS-57: GGAAGCAGCT	10	60
BS-58: CAGTGAGCGT	10	60

lyzed by agarose-gel electrophoresis with 2% low-melting agarose. The DNA bands were stained with ethidium bromide and visualized under 364-nm UV light.

RESULTS AND DISCUSSION

The three well-known anticancer drugs used in this study belong to different chemical groups: CPA is an alkylating agent, DXR is an anthracycline, and 5-FU is a pyrimidine analog. To obtain effective anti-proliferation activity against human cancers, cancer patients are commonly treated with a combination of anticancer drugs derived from different chemical compounds.⁷⁾ In the breast cancer chemotherapeutic protocol, each anticancer drug (CPA, DXR, and 5-FU) allegedly works by inducing apoptosis. Therefore, in our previous study, we examined the cancer cell death induced by these drugs using the MCF-7 human breast cancer cell line.⁴⁾ We focused on the morphological and cell biological events (by phase-contrast microscopy, examination of nuclear blebbing/chromatin condensation, FACS analysis, and examination of DNA ladder formation) associated with the cell death caused by CPA, DXR, and 5-FU administration alone or in combination. Interestingly, apoptosis-specific morphological features of the MCF-7 cells were observed when CPA and 5-FU were administered alone, but these features were absent when DXR alone or the three-drug combination was administered.⁴⁾

In apoptosis, caspases are indispensable enzymes that control the apoptotic pathway. There are at least 15 known caspases,⁸⁾ which are classified into two groups: initiator and effector caspases.^{9,10)} Initiator caspases are located upstream of effector caspases, and they transmit the apoptotic signal by cleaving the effector pro-caspases located downstream.¹¹⁾ Effector caspases usually in-

clude caspases-3, -6, and -7. To gain insight into the mechanisms underlying the results of our previous study, here we first examined the activation of these effector caspases in MCF-7 cells after the administration of each anticancer drug alone or in combination.

The MCF-7 breast cancer cell line was established from the breast cancer tumor of a Caucasian woman, and it does not express caspase-3, one of the “hub” genes that promote apoptotic events.^{12,13)} Since caspases-6 and -7 usually act downstream of caspase-3, we evaluated the expression of these two caspases by western blotting. Ten to forty micrograms of the cytosolic protein fraction extracted from anticancer drug-treated and non-treated MCF-7 cells was fractionated by electrophoresis in a 10–20% SDS-polyacrylamide gradient gel, followed by western blotting using anti-procaspase-6 and -7 monoclonal antibodies.

Figure 1 indicates the results of caspase-6 and -7 activation after the individual or multiple administration of anticancer drugs. (For the 5-FU-treated cells, both the pro- and active forms of the caspases are shown.) Caspase-6 was activated after the individual administration of DXR or 5-FU and the multiple administration of CDF, but not after CPA. On the other hand, caspase-7 was activated after the administration of every anticancer drug, whether individually or in combination.

As mentioned above, MCF-7 cells treated with CPA or 5-FU caused predominantly apoptotic features, while those treated with DXR or the CDF multi-drug had morphologically non-apoptotic features in our previous study.⁴⁾ However, interestingly, the effector caspases (caspases-6 and -7) were both activated after 5-FU, DXR, or CDF administration, but only caspase-7 was activated after CPA administration. We admit that these findings do not definitively show the type of cell death induced in MCF-7 cells by CPA, DXR, and 5-FU whether added independently or in combination. However, the present findings on effector caspase activation along with our previous morphological studies⁴⁾ strongly suggest that the MCF-7 cell death after the independent administration of CPA and 5-FU is apoptotic.

On the other hand, the nature of the MCF-7 cell death caused by DXR and by CDF multi-drug administration is more complicated. Although two effector caspases were activated by these treatments, the typical morphologic features of apoptosis (nuclear blebbing/chromatin condensation, DNA

fragmentation) were not observed.⁴⁾ It is currently widely acknowledged that cell death is not simply divided into two categories, apoptotic or necrotic. There have been several reports of cell death that is morphologically “non-apoptotic,” but accompanied by caspase activation.^{14–16)} Although we performed western blotting of only caspase-6 and -7 in this report, the MCF-7 cell death caused by DXR or CDF appears to be necrotic with effector caspase activation rather than apoptotic, given the lack of nuclear blebbing (or chromatin condensation) of MCF-7 cells after DXR and CDF administration,⁴⁾ which is the fundamental morphological feature of apoptosis. It will be important to measure inflammatory cytokines released from the MCF-7 cells to confirm this idea.¹⁶⁾

In our previous study, CPA administration did not cause DNA fragmentation but did cause nuclear blebbing/chromatin condensation.⁴⁾ Although the roles of caspases-6 and -7 are still controversial, some interesting evidence regarding their contributions to the classic features of apoptosis was reported by Eguchi *et al.*¹⁷⁾ Using tube-forming human umbilical vein endothelial cells (HUVECs), Eguchi *et al.* investigated the role of effector caspases in the apoptotic physiological events occurring during hypoxia. They concluded that caspase-6 is responsible for formation of the DNA ladder and caspase-7 for chromatin condensation.¹⁷⁾ A similar result was reported by Johnson *et al.* using Jurkat T cells treated with staurosporine.¹⁸⁾ These reports are consistent with our findings in MCF-7 cells after CPA administration. Namely, caspase-6 may also be involved in the DNA ladder formation in these MCF-7 cells, although the detailed mechanism remains unsolved.

The doses of CPA, DXR, and 5-FU used were based on the clinical dosage, as explained in our previous paper.⁴⁾ Our results indicated that, at these doses, the cell death of MCF-7 progresses relatively slowly. The viability of MCF-7 cells was reduced to 40% (DXR, 5-FU, and CDF) and 80% (CPA) on day 4. This “slow mortality” of MCF-7 cells after anticancer drug administration was notable, particularly because it was consistent with the idea that changes in gene expression could be triggered by the administration of the individual anti-cancer drugs or the CDF multi-drug treatment. To investigate this possibility, we chose the RAP-PCR DD method,^{19,20)} a rapid, simple, and cost-effective way to visually identify genes of interest.

The differential display technique was first in-

troduced in 1992 by Ling and Pardee to identify differentially expressed genes.¹⁹⁾ Although convenient for gene hunting, a number of problems hampered its use. One serious problem was the generation of first-strand cDNAs that predominantly encoded the 3'-non-coding region. Following the second round of amplification, 3' sequences may be over-represented among the PCR products. This unwelcome outcome was caused by the usage of oligo-dT primers for the first-strand cDNA synthesis in the original paper. To avoid this problem, Sokolov and Prockop introduced "RNA arbitrarily primed-PCR" into the differential display method (RAP-PCR DD).²¹⁾ They used fully degenerate 6-mer primers for the first-strand cDNA synthesis, and then introduced sets of arbitrary but defined 10-bp nucleotide primers for the PCR amplification step, to detect differentially expressed cDNAs.

Here we found RAP-PCR DD to be a convenient and effective tool for amplifying and visualizing genes expressed specifically in MCF-7 cells after the administration of anticancer drugs. Table 1 lists the five arbitrary primers used in this study. They were 10-bp oligonucleotides with a 60% GC content that were chosen randomly from among the 27 arbitrary primers in Sokolov and Prockop's report.²¹⁾ We tested them in combinations of three primer pairs for the second-strand synthesis in this study. The total RNA samples for the RAP-PCR DD were extracted from untreated and anticancer drug-treated MCF-7 cells, harvested one day after drug administration. We chose this time point to detect the activation of gene expression because the decrease in MCF-7 cell viability one day after drug administration was less than 15%. After cDNAs were obtained by reverse transcription, we tested several primer combinations and annealing conditions to optimize the RAP-PCR DD conditions to distinguish clear PCR bands. These conditions are described in the Materials and Methods section. Under these conditions, we visually confirmed at least nine distinct differentially expressed DNA fragments (Fig. 2, arrows). Judging from the molecular weights in Fig. 2, the three DNA bands of around 1.0 kb indicated by arrows in #2-D, -F, -3; three DNA bands of around 1.0 kb indicated by arrows in #4-D, -F, -3; four DNA bands of around 770 bp indicated by arrows in #5-C, -D, -F, -3; and three DNA bands of around 1.0 kb indicated by arrows in #6-D, -F, -3, are probably the same DNAs.

Since the differentially expressed DNA fragments were revealed on a 2% agarose gel, we fo-

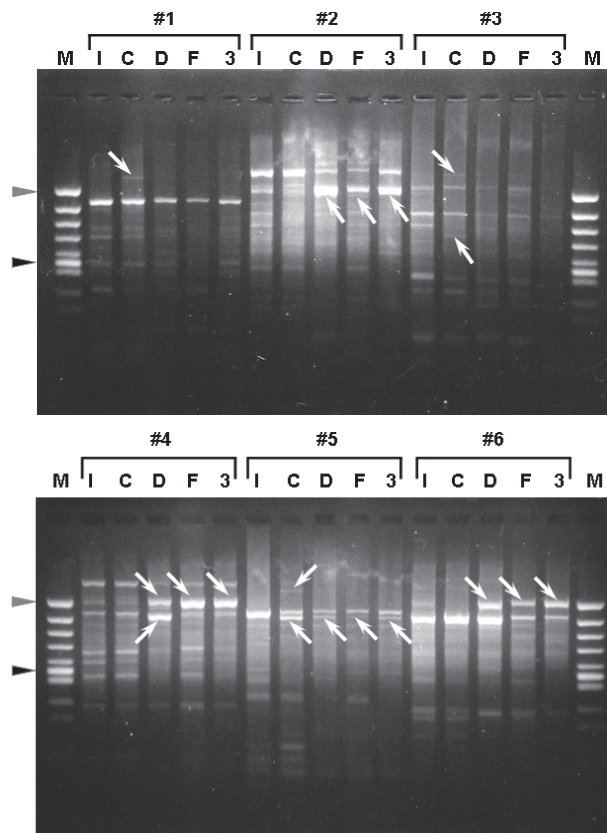


Fig. 2. RAP-PCR DD

Results from the RAP-PCR DD. The leftmost and rightmost lanes in both panels are the *phi*X-*Hinc* II-digest DNA molecular markers; the gray and black arrowheads indicate a 1057- and 341-bp band, respectively. Above the lanes, I, C, D, F, and 3 indicate, respectively, samples from untreated (Intact) MCF-7 cells, CPA-treated, DXR-treated, 5-FU-treated MCF-7 cells, and MCF-7 cells treated with the 3 combined anticancer drugs. The numbers above each square bracket indicate the arbitrary PCR primer pairs (Table 1) that were used for PCR amplification. #1, #2, #3, #4, #5, #6 indicate the primer pairs of BS: 52+54+55, 52+54+57, 52+54+58, 54+55+57, 54+55+58, 55+57+58, respectively. For example, lane #1-C shows the RAP-PCR DD results in which the mRNA source was MCF-7 cells treated with CPA, and the arbitrary PCR primers for the secondary PCR amplification were 52+54+55. The arrows indicate the differentially expressed DNA fragments.

cused on bands smaller than 1.0-kb to search for genes of interest. Although we did not sequence or characterize these amplified DNAs, our results indicate that at least some genes were still being transcribed after one day of anticancer drug administration. As mentioned above, the amplified PCR bands obtained in RAP-PCR DD may include false-positive bands. Thus, we have performed DNA microarray analysis to identify these genes (Kugawa *et al.*, manuscript in preparation) and gain further insight into the MCF-7 cell death caused by clinically applied anticancer drugs.

Our previous apoptosis study revealed that

polyubiquitin gene expression is triggered after the administration of buprenorphine hydrochloride (anesthesia) to the rodent-derived nerve cell line NG108-15.⁶⁾ Thus, the expressed genes shown in Fig. 2 might belong to the apoptosis-related or ubiquitin-proteasome system, both of which are involved in cell death. In 2006, Hernández-Vargas *et al.* administered 5-FU at 10 or 500 μM to MCF-7 cells, and then investigated the gene expression by DNA microarray analysis.²²⁾ Although the 5-FU concentration used by Hernández-Vargas was 1/40 of the concentration used in this study, they detected an upregulation of mitochondrial apoptosis-related genes after the drug treatment. Therefore, the identification and characterization of the different DNA bands that we found to be expressed in MCF-7 cells after anticancer drug treatment should help elucidate the cell-death mechanism(s).

In this study, we added two suggestive findings to our previous report⁴⁾ on MCF-7 human breast cancer cell death by anticancer drug administration. Our western data supported the idea that CPA and 5-FU activate apoptotic cell death, while DXR and CDF multiple administration may activate caspase-dependent non-apoptotic cell death in MCF-7 cells. In addition, the induced genes revealed in our molecular biological experiment should provide possible targets for future basic and clinical studies addressing the molecular mechanisms of chemotherapy in breast cancer cytotoxicity.

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