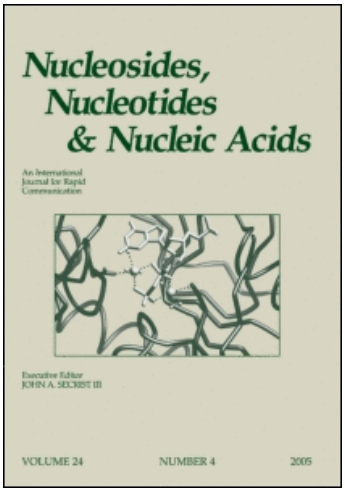

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Activation of c-*jun* and c-*fos* Genes in dNTP Imbalance Cell Death Induced With 5-Fluoro-2'-Deoxyuridine in Mouse Mammary Tumor FM3A Cell Line

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ACTIVATION OF c-*jun* AND c-*fos* GENES IN dNTP IMBALANCE CELL DEATH INDUCED WITH 5-FLUORO-2'-DEOXYURIDINE IN MOUSE MAMMARY TUMOR FM3A CELL LINE

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ABSTRACT: 5-Fluoro-2'-deoxyuridine (FdUrd)-induced death of mouse mammary FM3A cells was found to be associated with an increased expression of cellular c-*jun* and c-*fos* genes. The increase in these gene expressions was mediated through the protein kinase C-dependent pathway. Blockage of the expression with the use of antisense oligodeoxynucleotide for c-*jun* delayed the cell death. These findings suggest that the activation of c-*jun* and c-*fos* genes, which encode transcription factors participating in cell proliferation, plays a role in FdUrd-induced cell death.

INTRODUCTION

The process of DNA replication needs a balanced supply of four deoxyribonucleoside triphosphates (dNTPs).[1,2] Among the dNTPs, dTTP has to be synthesized in a more complex pathway than those for other dNTPs, and because of this complexity the thymidylate synthesis pathway is often a target of attack by anticancer and antiviral agents. The resulting disturbance of dNTP pool is common in the actions of these agents. It is known that a severe imbalance in dNTPs induces cell death and a variety of genetic and cytological effects in prokaryotic and eucaryotic cells.[1-4] However, molecular mechanisms underlying these events is not well understood.

In our previous studies, we found that when mouse mammary tumor FM3A cells in culture were treated with 5-fluoro-2'-deoxyuridine (FdUrd), an imbalance in the cellular dNTP pool was induced. In this experiment, a depletion of the dTTP and dGTP pool, and an increase in the dATP pool were observed, followed by double strand breaks in

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mature DNA before the cell death took place.[5] FdUrd and 5-fluorouracil are agents effective in the treatment of metastatic cancers. These agents form a common cytotoxic metabolite, 5-fluorodeoxyuridine 5'-phosphate, which inhibits thymidylate synthase (TS).[6] Furthermore, we found that the DNA double strand breaks and cell death can be prevented by addition of cycloheximide, an inhibitor of protein biosynthesis.[5] These observations lead us to propose that a newly synthesized endonuclease may play an integral role in this FdUrd-induced cell death; and we termed this mechanism 'dNTP pool imbalance death'.[5] Indeed, we have recently succeeded in isolating a novel endonuclease from FdUrd-treated FM3A cells that causes double strand breaks in the cellular DNA.[7] To investigate the mechanism of this nuclease induction, it seemed of interest to explore the possibility that transcription factors c-*jun* and c-*fos* oncogenes might be activated during an early stage of the FdUrd treatment. It is known that these genes are expressed following treatment of cells with protein kinase C (PKC)-activators such as phorbol esters and certain growth factors.[8-10] Moreover, it is reported that other cytotoxic agents and factors activate c-*jun* and c-*fos* oncogenes in cell death.[11-16]

We wish to report here that the treatment of FM3A cells with FdUrd is associated with expression of c-*jun* and c-*fos* genes. Both the FdUrd-induced activation of the transcription of these genes and the cell death were inhibited by PKC inhibitors, H-7 and calphostin C. Treatment with antisense oligonucleotide against c-*jun* mRNA delayed FdUrd-induced cell death. These findings suggest that the activation of c-*jun* and c-*fos* genes is a signal in the FdUrd-induced cell death.

MATERIALS AND METHODS

Materials

FdUrd was obtained from Sigma (St. Louis, MO). ES medium was purchased from Nissui Pharmaceuticals (Tokyo, Japan) and fetal bovine serum from Gibco (Grand Island, NY). Calphostin C was a product of Kyowa Medex (Tokyo, Japan), and H-7 and HA1004 were those of Seikagaku Kogyo (Tokyo, Japan). [α-^{32}P] dCTP, [γ-^{32}P] ATP, nylon membrane (Hybond-N+) and Rapid-hyb buffer were from Amersham (Amersham, UK). All probes were products of Oncogene Science (Uniondale, NY). Phosphorothioate oligonucleotides were synthesized by Sawady Technology (Tokyo, Japan). All other reagents were of the highest grade available.

Cell Culture and Drug Treatment

Mouse mammary tumor FM3A cells (wild type, F28-7) were obtained from the Japanese Cancer Research Resources Bank. The cells were grown at 37℃ in 5% CO_2 in ES medium containing 2% heat-inactivated fetal bovine serum.[17] Cell viability during treatment with agents was estimated with a hemocytometer by the trypan blue dye exclusion. FdUrd was added when the cell density reached 2 x 10^5 cells/ml. Calphostin

C was dissolved in dimethylsulfoxide (DMSO). For the activation of calphostin C, cell suspension containing calphostin C, was incubated under irradiation of visual light.[18]

RNA Isolation and Northern Blot Hybridization

For rapid and reproducible preparation of total RNA, the QIAshredder and RNeasy kits were used following the manufacture's instructions (Qiagen, Hilden, Germany). Samples of total RNA (7-10 μg) were denatured in formaldehyde and electrophoresed through 1% agarose-formaldehyde gels. The RNA was visualized by UV illumination after the gel was treated with ethidium bromide. The RNA was then transferred to Hybond-N+ nylon membrane and hybridized in Rapid-hyb buffer to ^{32}P-labeled DNA probes.[19] The membrane was usable for reprobing. Autoradiography and densitometry were done with Bio-imaging analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan).

Treatment with Oligonucleotides

For investigating the relationship between FdUrd-induced cell death and c-*jun* and c-*fos* expressions, we designed 18-mers (phosphorothioate) corresponding to the antisense sequences flanking the translation initiation region of murine c-*jun* and c-*fos* mRNAs[12], the sense sequences of the same region, and the reversal sequences (rev-antisense) against these antisense sequences for control experiments.

The sequences of six oligonucleotides are shown below.

c-*jun* antisense : 5'-**CGTTTCCATCTTTGCAGT**-3'
c-*jun* sense : 5'-**ACTGCAAAGATGGAAACG**-3'
c-*jun* rev-antisense : 5'-**TGACGTTTCTACCTTTGC**-3'
c-*fos* antisense : 5'-**GGCGTTGAAACCCGAGAA**-3'
c-*fos* sense : 5'-**TTCTCGGGTTTCAACGCC**-3'
c-*fos* rev-antisense : 5'-**AAGAGCCCAAAGTTGCGG**-3'

Though it was suspected that thymidine, which was released from oligonucleotide by degradation, should induce competitive inhibition to FdUrd-induced cell death, the degradation of oligonucleotides was prevented by modification of oligonucleotides to phosphorothioate. Besides we evaluated antisense-effects comparing the results of antisense oligonucleotide with those of rev-antisense which had the sequence with the same base composition as antisense.

FM3A cells were treated with 1 μM oligonucleotide for 6 h,[20] and then the FdUrd treatment was started with 1 μM FdUrd and additional 1 μM oligonucleotide.

RESULTS

Cell Viability

The cytotoxicity of FdUrd to FM3A cells was examined. In cells treated with 1 μM FdUrd, the viability did not change within 8 h, began to decrease from 12 h (viability; 94%), and then became about 20% at 24 h.

Induction of c-*jun*, c-*fos*, c-*myc*, and *p53* mRNAs

We examined the levels of mRNAs for transcription factor genes c-*jun*, c-*fos*, c-*myc*,[21] and *p53*[22] in FdUrd-treated cells. FIG. 1 shows these mRNA levels during the 1 μM FdUrd treatment. The c-*jun* mRNA was detectable at a low level in the beginning of the treatment, and the c-*fos* mRNA was not detectable within 4 h. The c-*jun* and c-*fos* mRNA increased from 8 h to 12 h simultaneously, and then their amounts decreased after 16 h. The mRNA for c-*myc* did not change significantly within 8 h, but increased until 20 h. The band of *p53* mRNA did not change significantly within 24 h. The level of β-actin mRNA was used for normalization of these mRNA levels. Thus, maximum induction of c-*jun* and c-*fos* genes occurred at 12 h (c-*jun*, 29-fold increase; c-*fos*, 18-fold). In contrast, that of c-*myc* and *p53* occurred at 20 h (c-*myc*, 7-fold; *p53*, 2-fold). From these results, we assumed that the induction of cell death with FdUrd is related to the expression of c-*jun* and c-*fos* mRNAs. Moreover, we observed that the induction of c-*jun* and c-*fos* transcriptions at 12 h by FdUrd (0.01-1 μM) was a dose-dependent manner (data not shown).

Effect of H-7 on c-*jun* and c-*fos* mRNA Levels in Cells Treated with FdUrd

The effect of a PKC inhibitor, H-7[23], on c-*jun* and c-*fos* transcriptions in cells treated with 1 μM FdUrd for 12 or 24 h is shown in FIG. 2. For a control, HA1004, an analog of H-7 without potent PKC inhibition activity, was used.[24] When cells were treated with FdUrd and 50 μM H-7 simultaneously, the levels of these mRNAs at 12 h (lane 4) were as low as that of control (lane 1), and increased at 24 h (lane 5). H-7 or HA1004 themselves did not cause these gene transcriptions. Thus, the FdUrd-induced increase in these mRNA levels was delayed with the addition of H-7. The effect of another PKC inhibitor, calphostin C[18], on c-*jun* and c-*fos* transcriptions in cells treated with 1 μM FdUrd for 12 h was investigated. The FdUrd-induced increase in these mRNA levels at 12 h was inhibited with the addition of 5 nM calphostin C (data not shown). These results indicate that the activation of c-*jun* and c-*fos* transcriptions in cells treated with FdUrd is mediated through the PKC-dependent pathway.

Effect of PKC Inhibitors on FdUrd-Induced Cell Death

The effect of PKC inhibitors on viability of cells treated with FdUrd is shown in FIG. 3. When FM3A cells were treated with 1 μM FdUrd plus 5 nM calphostin C, cell viability decrease was slowed down significantly. When FM3A cells were treated with 1 μM FdUrd and 50 μM H-7, again the cell viability decreased more slowly than in the treatment with FdUrd alone. Thus, calphostin C and H-7 delayed the decrease in cell viability. From these experiments, it appears that FdUrd-induced cell death is associated with PKC activation.

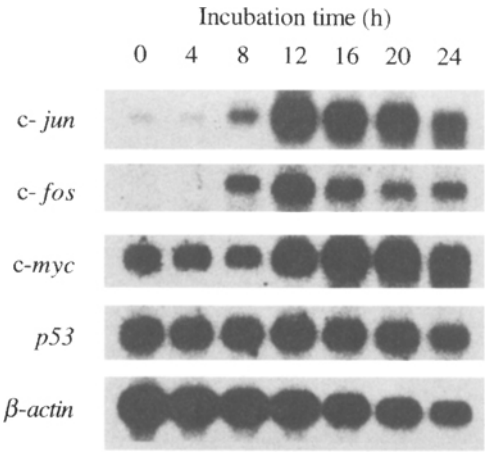
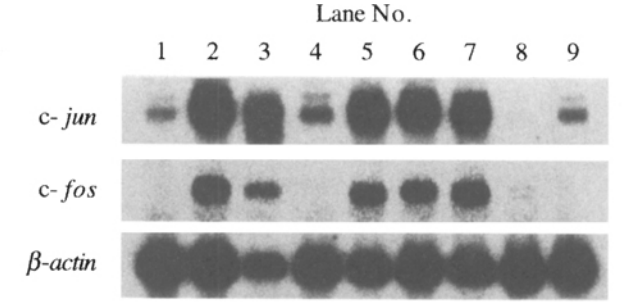


FIG. 1. Induction of c-*jun*, c-*fos*, c-*myc*, and *p53* mRNAs in FM3A cells treated with 1 μM FdUrd. Total RNA (7 μg) extracted from the cells was transferred to nylon membrane and hybridized with the ^{32}P-labeled probes. The membrane was usable for reprobing. The migration of these mRNAs relative to 18S rRNA was, c-*jun* 0.84, c-*fos* 0.88, c-*myc* 0.90, and *p53* 0.96. The level of β-actin mRNA was used for normalization of these mRNA levels.



Lane No.
1 : Control
2 : FdUrd for 12 h
3 : FdUrd for 24 h
4 : FdUrd + H-7 for 12 h
5 : FdUrd + H-7 for 24 h
6 : FdUrd + HA1004 for 12 h
7 : FdUrd + HA1004 for 24 h
8 : H-7 for 12 h
9 : HA1004 for 12 h

FIG. 2. Effect of H-7 on the FdUrd-induced transcription of c-*jun* and c-*fos* genes in FM3A cells. Total RNA (10 μg) was transferred to nylon membrane and hybridized with the ^{32}P-labeled probes.

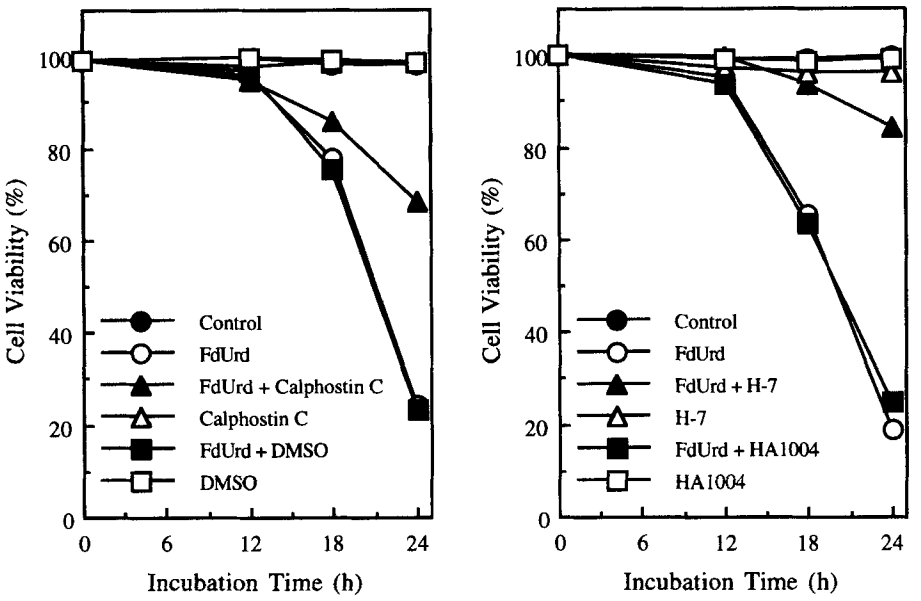


FIG. 3. Effect of PKC inhibitors on the viability of FM3A cells treated with 1 μM FdUrd.

Effect of Antisense Oligonucleotides against c-*jun* and c-*fos* mRNAs on FdUrd-Induced Cell Death

FIG. 4A shows the effect of phosphorothioate antisense oligonucleotides against c-*jun* and c-*fos* mRNAs on FdUrd-induced cell death. When FM3A cells were treated with 1 μM FdUrd in the presence of c-*jun* antisense oligonucleotide, the cell viability recovered from 18% (FdUrd alone) to 48% (FdUrd + c-*jun* antisense) at 24 h by the treatment. With sense or rev-antisense oligonucleotides for c-*jun*, this significant recovery was absent. Thus, the treatment with c-*jun* antisense oligonucleotide delayed the cell death. In contrast, however, the effect of c-*fos* antisense oligonucleotide on FdUrd-induced cell death was not significant as well as that of sense or rev-antisense oligonucleotides for c-*fos*. FIG. 4B shows the effect of these oligonucleotides on FdUrd-induced transcription of c-*jun* and c-*fos* genes at 12 h. The treatment with c-*jun* antisense reduced not only the level of c-*jun* mRNA but also c-*fos* (lane 3). These results suggest that FdUrd-induced cell death is dependent on the induction of c-*jun* and c-*fos* oncogene expressions. The c-*fos* antisense, however, did not affect significantly c-*fos* transcription.

Protein products of c-*jun* and c-*fos* were also investigated by Western blotting. An increase of these products, which should reflect the activation of these gene expressions in FdUrd-treated cells, was confirmed (data not shown).

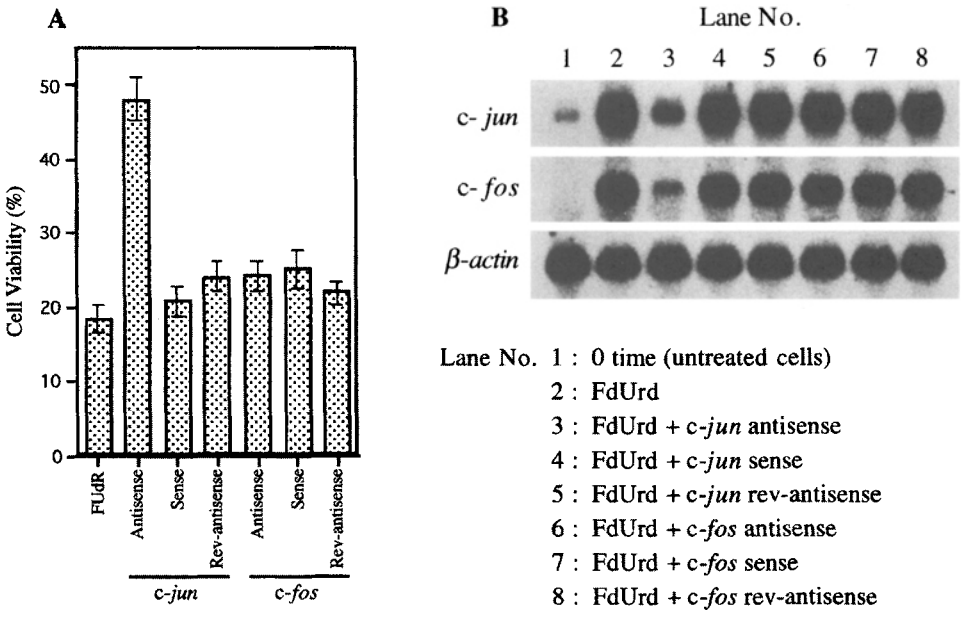


FIG. 4. Effect of antisense oligonucleotides against c-*jun* and c-*fos* mRNAs on FdUrd-induced cell death and these gene transcriptions. FM3A cells were treated with 1 μM phosphorothioate oligonucleotide for 6 h,[20] and then the FdUrd treatment was started with 1 μM FdUrd and additional 1 μM oligonucleotide. A: Cell viabilities at 24 h after the treatment with 1 μM FdUrd in presence or absence of oligonucleotides are expressed as percent (mean ± SD of three examinations). B: The levels of c-*jun* and c-*fos* mRNAs at 12 h are indicated.

DISCUSSION

We found a significant increase in expression of c-*jun* and c-*fos* genes in cells treated with FdUrd. Previously Kufe et al. have described that c-*jun* and c-*fos* oncogenes are expressed in cell death induced with other cytotoxic agents,[13, 16] but the role of the activation of these genes has not been clear. Our results of effect of PKC inhibitors and c-*jun* antisense oligonucleotide on FdUrd-treated cells provide the evidences that the induction of the cell death is mediated through the overexpression of c-*jun* gene and PKC-dependent pathway. Because c-*fos* antisense was not significantly effective in our experiments, the importance of the increase in c-*fos* gene expression in FdUrd-induced cell death has not been clear yet.

The profile of the c-*jun* and c-*fos* mRNA level changes was different from that of c-*myc* and *p53* (FIG. 1). These profiles suggest that the activation of c-*jun* and c-*fos* plays a signal in the early stage of FdUrd-induced cell death, and the other genes may be

associated with the later stage . Products of c-*jun* and c-*fos* genes form a hetero- and homodimer, transcriptional factor termed AP-1 (activator protein 1), participating cell proliferation, differentiation, transformation, and death.[10] However, the target genes of AP-1 in induction of cell death are still unidentified. Recently, Wetzel et al. described a gene *bad*, a member of the *bcl-2* gene family as a target gene of AP-1.[25]

It was interesting that c-*jun* antisense inhibited not only FdUrd-induced c-*jun* transcription but also c-*fos* transcription. The results suggest that overexpression of c-*jun* gene might induce c-*fos* transcription. Indeed AP-1 binding site-like sequence exists in c-*fos* promoter region.[26] We speculate that c-*jun* products could bind c-*fos* promoter region and induce transcription.

Cellular abnormalities (dNTP pool imbalance followed by double strand breaks in the mature DNA and subsequent cell death) similar to those in FdUrd-treated cells were previously observed when the cells were treated with 2'-deoxyadenosine,[27] 2-chloro-2'-deoxyadenosine,[28] and α,α-bis(2-hydroxy-6-isopropyltropon-3-yl)-4-methoxytoluene.[29] Also when TS-defective FM3A thy-21 cells were cultured in thymidine-less medium, these abnormalities[30] and activation of c-*jun*, c-*fos*, c-*myc*, and *p53* genes were observed (the details of this aspect will be reported elsewhere). We think that the activation of these genes may be a signal in the mechanism of dNTP imbalance cell death. From these findings, it appears that dNTP imbalance death may be comparable to thymine-less death in prokaryotic cells.[31] We hypothesize that certain cytotoxic agents induce dNTP pool imbalance, and the imbalance will perturb or stop the cell cycle, leading to the activation of c-*jun* and c-*fos* genes. These activations may be mediated by the PKC-dependent pathway, and lead to induction of an endonuclease[7], DNA fragmentation, and eventually cell death.

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