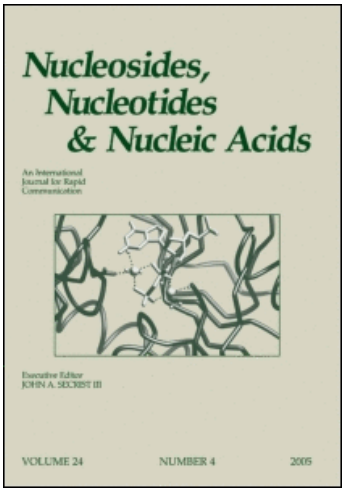

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Molecular Mechanism of 5-Fluoro-2'-deoxyuridine-induced dNTP Imbalance Cell Death: Purification of an Endonuclease Involved in DNA Double Strand Breaks During dNTP Imbalance Death

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MOLECULAR MECHANISM OF 5-FLUORO-2'-DEOXYURIDINE-INDUCED dNTP IMBALANCE CELL DEATH : PURIFICATION OF AN ENDONUCLEASE INVOLVED IN DNA DOUBLE STRAND BREAKS DURING dNTP IMBALANCE DEATH

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ABSTRACT: We have detected, isolated and purified an endonuclease from 5-fluoro-2'-deoxyuridine-treated FM3A cells. The molecular mass of the endonuclease was approximately 40 kDa as judged by sodium dodecyl sulfate-polyacrylamide DNA-containing gel electrophoresis. The endonuclease causes double strand breaks in DNA, with an optimum pH at 6.

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 are 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: a severe depletion of dTTP and dGTP and an increase in dATP.[5] This dNTP pool imbalance was followed by double strand breaks in the mature DNA and subsequent cell death.[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, and this metabolite inhibits thymidylate synthase.[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] From these observations, we presented an assumption that the intracellular dNTP pool imbalance induced by FdUrd would be a trigger for activating an endonuclease gene and that the resulting production of an endonuclease will cause the double strand breaks and subsequent cell death; and we termed this mechanism 'dNTP pool imbalance death'.[5]

In the present study, we have isolated the induced endonuclease and studied its properties.

MATERIALS AND METHODS

Materials

ES medium was purchased from Nissui Seiyaku (Tokyo, Japan), and fetal bovine serum from Gibco (Grant Island, NY). DEAE-Bio Gel Agarose, CM-Bio Gel Agarose, Affi-Gel Heparin (heparin-agarose) and Bio-Gel Hydroxylapatite were the products of Bio-Rad (Richmond, CA). FdUrd was obtained from Sigma (St. Louis, MO). Centriprep 10 and centricon 10 were from Amicon (Beverly, MA). Random Primer Labeling Kit was from Takara (Shiga, Japan). [5'-α-^{32}P] dCTP was from Amersham (Amersham, UK). All other reagents were of the highest grade available.

Buffers

Buffer A contained 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-Na (pH 8.0), 10% glycerol and 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF). Lysis buffer contained 0.5 M EDTA (pH 9.0), 1% lauroyl sarcosine, and 1 mg/ml proteinase K. Buffer B contained 10 mM HEPES-Na (pH 7.2), 10% glycerol and 0.1 mM *p*-APMSF. Buffer C contained 10 mM potassium-phosphate (pH 7.0), 10% glycerol and 0.1 mM *p*-APMSF. Buffer D contained 10 mM potassium-phosphate (pH 7.7), 10% glycerol and 0.1 mM *p*-APMSF.

Cell Culture and Drug Treatment

The mouse mammary tumor FM3A cell (wild type, F28-7)[7] was supplied by the Japanese Cancer Research Resources Bank (JCRB). FM3A cells were grown at 37°C in a 5% CO_2 atmosphere in ES medium containing 2% heat-inactivated fetal bovine serum.[5] For exposure experiments, FdUrd was added when the cell density reached $2x10^5$ cells/ml.

Detection of Endonuclease Activity by Orthogonal-Field Alternation Gel Electrophoresis

The apparatus used for the orthogonal-field-alternation gel electrophoresis (OFAGE) was as described previously.[8,9] The cell lysates were loaded onto a DEAE-agarose column

and eluted with buffer A containing 0, 5, 10 and 20 mM NaCl. Agarose blocks containing DNA from intact FM3A cells were incubated with individual fractions of the DEAE-agarose column chromatography. The incubation was at 37°C for 48 hr. The gel blocks were reincubated with lysis buffer for 24 hr at 50°C. The agarose blocks were loaded into the wells and the electrophoresis was performed at 13°C for 18 hr using linear gradient pulses of 50 to 100s (see ref. 10). The gel was stained with ethidium bromide and visualized on a UV trans-illuminator (with 300 nm light).

Purification of the Endonuclease

The work-up was done at 0-4°C. The FdUrd-treated cells in 80 liters of culture medium were harvested and washed twice with phosphate-buffered saline (PBS), and then were suspended in buffer A (166 ml). The cells were lysed with a Branson Sonifier (Model 250). The mixture was centrifuged at 100,000 x g for 2 hr to obtain the cell lysate as supernatant.

The endonuclease was purified from the cell lysate by a serial chromatography on DEAE-agarose, CM-agarose, heparin-agarose, and hydroxylapatite columns. Cell lysate (1.16 g) was loaded onto a DEAE-agarose column (5×10.2 cm) and eluted with buffer A containing 5 mM NaCl (total 1 liter). The enzyme fraction was dialyzed against buffer B, concentrated and applied to a CM-agarose column (2.5×10 cm). The column was washed with a linear gradient of 0-200 mM NaCl in buffer B (total 200 ml). Endonuclease was eluted with about 50 mM NaCl. The fraction was dialyzed against buffer C, concentrated and applied to a heparin-agarose column (1.0×10 cm). The column was washed with a linear gradient of 0-200 mM NaCl in buffer C (total 100 ml). The column chromatography was repeated again. The endonuclease was eluted with about 40 mM NaCl in buffer C. The fraction was dialyzed against buffer D, concentrated and applied to a hydroxylapatite column (0.7×5 cm). The column was washed with a linear gradient of 0-200 mM potassium-phosphate buffer (pH 7.7, total 20 ml). The endonuclease was eluted with about 35 mM potassium-phosphate (pH 7.7), concentrated and dialyzed against buffer D. Concentration and dialysis in the purification steps were done with the use of centriprep 10 or centricon 10. The fraction thus prepared was used for characterizing the enzyme.

Nuclease Detection in SDS-Polyacrylamide Gel containing DNA

To identify the endonuclease, we used sodium dodecyl sulfate (SDS)-polyacrylamide gel containing DNA.[11,12] The 10% separating gel matrix contained 1 mg/ml heat-denatured calf thymus DNA, along with 75,000 cpm/ml of [^{32}P]double-stranded DNA prepared from a Random Primer Labeling Kit. Micrococcal nuclease was run as a positive control on gels. After electrophoresis, the gel was washed with 10 mM sodium-phosphate buffer (pH 6.0) containing 25% isopropyl alcohol to remove SDS,[13] thereby allowing protein renaturation. Incubation of the gel in 10 mM sodium-phosphate buffer (pH 6.0)

was then performed for 14 hr at 37°C in order to digest the DNA in the gel. The gels were stained with ethidium bromide and photographed under a UV trans-illuminator. The gels were next subjected to autoradiography using a BAS 2000 imaging analyzer (Fuji, Tokyo, Japan). With this process a nuclease can be distinguished from a DNA-binding protein.

Endonuclease Activity Assay

We used the replicative form I (RFI) DNA of M13mp18 to measure the endonuclease activity. The assay mixture (10 μl) contained 10 mM sodium-phosphate buffer (pH 6.0), RFI DNA (0.15 μg), and enzyme (0.5 unit). The reaction mixture was incubated at 37°C for 5 min. The resulting mixture was then loaded onto a 1% low melting agarose gel (Bio-Rad Lab.), and electrophoresis was performed with 1 x TBE buffer (88 mM Tris, 88 mM boric acid, and 2 mM EDTA, pH 8.3) at 17 volts/cm for 1.5 hr. The gel was stained with ethidium bromide and photographed under the trans-illuminator. The measurement of endonuclease activity was determined by ethidium fluorescence assay of Morgan et al.[14] One unit of endonuclease activity is defined as the amount of enzyme required to produce a single cleavage in 1 *p*mol of RFI DNA circles under standard conditions.

Other Methods

Protein concentrations were determined by the method of Bradford.[15] The basic protocol for SDS-PAGE preparation and running was that of Laemmli.[16] For SDS-PAGE, a precast gradient gel 4/20 (Daiichi, Tokyo, Japan) was used.

For amino acid sequencing, the protein was eluted from SDS-polyacrylamide gel containing DNA by electrophoretic transfer to PVDF membranes, and subjected to sequencing on an Applied Biosystems 473 Sequencer.[17] For proteins having blocked NH_2 termini, they were treated with endoproteinase Lys-C, and the resulting peptides were isolated by reverse phase HPLC. These peptides were then subjected to sequencing on an Applied Biosystems 473 Sequencer.[18,19]

RESULTS

Detection of an Endonuclease Activity in FdUrd-Treated Cell Lysate

Figure 1*a* shows an OFAGE plate in which nuclease activity in the FdUrd-treated cell lysate fractions is seen. With the 5 mM NaCl fraction, FM3A DNA as substrate was cleaved to give fragments of sizes 100-200 kbp. No endonuclease activity was detected in untreated FM3A cells (Fig. 1*b*).

Purification of Endonuclease

The endonuclease associated with the DNA cleavage in the dNTP imbalance death was purified by a series of column chromatography. Figure 2 shows protein profiles on SDS-PAGE found for the endonuclease fractions in each chromatographic step. The hydroxylapatite fraction (lane *6*) gave a major band at approximately 40-45 kDa. In

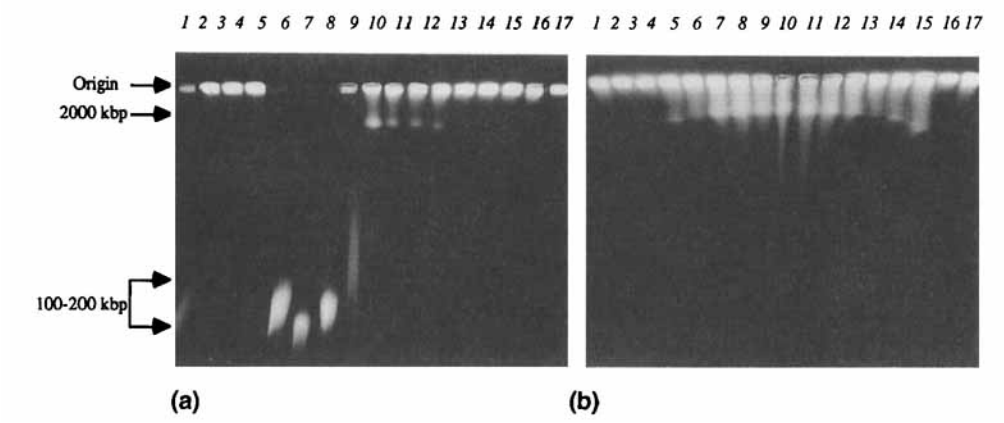

Fig. 1. Endonuclease activity in FdUrd-treated cell lysate.

a. FdUrd-treated cell lysate in 10 liters of culture medium was loaded onto DEAE-agarose column and eluted with 0, 5, 10 and 20 mM NaCl in buffer A. The individual fractions were incubated with gel blocks containing FM3A DNA, and the blocks were electrophoresed. *b*. Lysate from untreated FM3A cell was processed as in *a*. The lane numbers correspond to the fraction numbers of DEAE-agarose column chromatography. Fractions *1-4* were with 0 mM, *5-9* with 5 mM, *10-14* with 10 mM and *15-17* with 20 mM NaCl elution. The DNA sizes were determined by reference to yeast DNA markers.

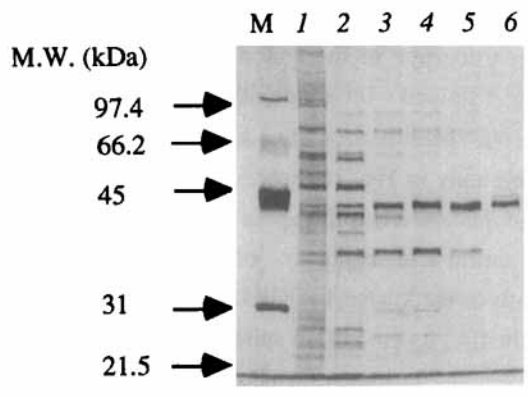

Fig. 2. SDS-PAGE analysis of endonuclease.

Aliquots of fractions after each purification step were electrophoresed in SDS-polyacrylamide gel (with a 4-20% acrylamide concentration gradient) and subsequently silver-stained. Lane *1*, cell lysate from FdUrd-treated cells; lane 2, DEAE-agarose column fraction; lane *3*, CM-agarose column fraction; lane *4*, 1st heparin-agarose column fraction; lane *5*, 2nd heparin-agarose column fraction, and lane *6*, hydroxylapatite column fraction. The arrows indicate the molecular mass markers (Bio-Rad) (lane M).

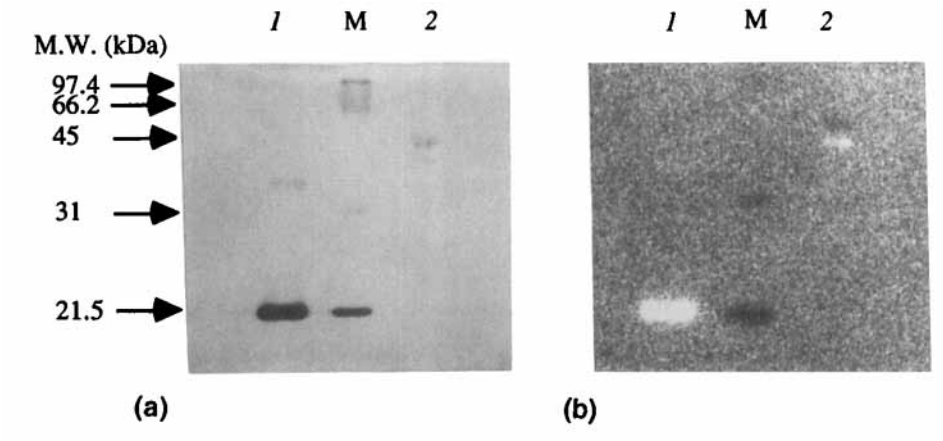

Fig. 3. Nuclease detection in electrophoresis on DNA-containing SDS-polyacrylamide gel (10% acrylamide concentration).

The enzymatic activities of hydroxylapatite fraction were detected after ethidium bromide staining (*a*) and subsequent autoradiography (*b*). Lane *1*, micrococcal nuclease (a positive control) ; lane M, protein standard markers; lane *2*, the hydroxylapatite fraction. The arrows indicate the mobility of molecular mass markers.

addition, gel filtration analysis on HiLoad 26/60 Sephacryl S-300 HR (Pharmacia, Uppsala, Sweden) gave a value of 40 kDa (data not shown).

Detection of Endonuclease by Electrophoresis on DNA-containing SDS-Polyacrylamide Gel

Figure 3*a* shows a photograph of the endonuclease fraction of the hydroxylapatite chromatography on the gel stained with ethidium bromide, in which two dark bands were detected. These bands could be either for an endonuclease or for a DNA binding protein. The results of autoradiography in Fig. 3*b*, however, show that the lower band (about 40 kDa) on lane 2 is an endonuclease and the upper band (about 45 kDa) is a DNA binding protein. We attempted amino acid sequencing of these proteins, and the upper band was revealed to be phosphoglycerate kinase-1 (PGK-1). The lower band corresponding to an endonuclease was too small in its amount in spite of its recognizable nucleolytic activity, and the attempt for sequencing has been unsuccessful at this stage. These two proteins were judged to be the components of the major protein bands observable in Fig. 2, lane *6*. The faint upper band in lane *6*, Fig. 2, was detected when the DNA gel of Fig. 3*b* was silver-stained, at a position above the DNA binding protein.

Characterization of Endonuclease

We determined the characteristics of the hydroxylapatite fraction, using M13mp18 RFI DNA as substrate. From this DNA, a single strand cleavage will give the *RFII form* (a

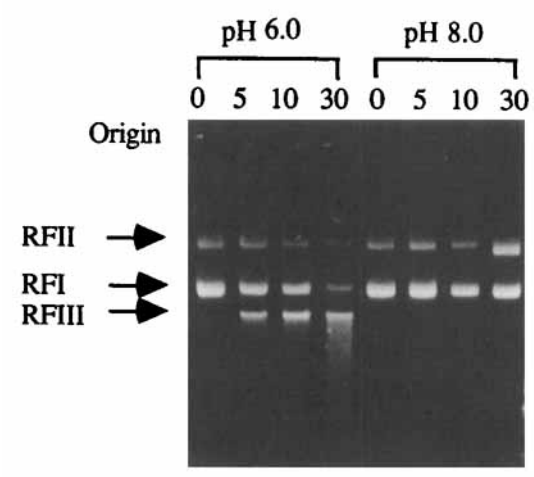

Fig. 4. DNA double-strand breaks caused by the endonuclease.
M13mp18 RFI DNA was incubated with the hydroxylapatite fraction at pH 6 and 8 (10 mM sodium-phosphate buffer) for the periods (min) indicated.

relaxed double-strand circular form), whereas a double strand cleavage will yield the RFIII form (a double-strand linear form). Figure 4*a* shows that at pH 6 the RFI DNA was directly converted to the RFIII form, indicating that double-strand cleavage is the mode of action of this nuclease. At pH 8, the DNA double-strand cleavage activity was not observed. The pH optimum for the double strand cleavage was 6.0-6.5, and the enzyme activity was lost on heating at 60°C for 30 min. The DNA cleavage activity of the enzyme did not require Ca^{2+}, Mg^{2+}, or Zn^{2+}: rather, these ions, particularly Zn^{2+}, inhibited the cleavage. In the presence of EDTA, on the other hand, the DNA cleavage activity was stimulated. The DNA cleaved by the enzyme had a phosphoryl residue at the 5'-termini end (data not shown). When the nuclei isolated from FM3A cells were treated with the hydroxylapatite fraction at pH 6.0-6.5, formation of the oligonucleosomal fragments was observed. During the FdUrd treatment, the intracellular pH gradually decreased, i.e., a 0.5 downward shift, as detected by the flowcytometer analysis. This acid-side shift would clearly favor the action of the endonuclease in the cell, suggesting that this nuclease is indeed responsible for the DNA cleavage observed.

DISCUSSION

Here, we detected an endonuclease in the lysate of FdUrd-treated FM3A cells but not in the untreated cells. The endonuclease was revealed to be a protein species of a size of

approximately 40 kDa. It causes double strand breaks in naked DNA (both for FM3A DNA and M13mp18 DNA), and the optimum pH is 6.0-6.5. A remarkable characteristic of this enzyme activity is its non-requirement for divalent metal ions. To our knowledge, mammalian endonucleases, except for DNase II, commonly require divalent metal ions for their activities.[11,20,21] The endonuclease we describe here differs from DNase II: DNase II has a pH optimum of 5, is not inhibited by Zn^{2+}, and cleaves DNA forming 3'-phosphoryl termini.[22]

Previously, we have observed that FdUrd treatment of FM3A cells caused dNTP imbalance and an associated double strand breaks in mature DNA.[5] The double-strand break-causing nature of the endonuclease described here is consistent with these observations.[5] The DNA fragmentations were also found when FM3A cells were treated with deoxyadenosine,[23] 2-chlorodeoxyadenosine[10] or α,α-bis(2-hydroxy-6-isopropyltropon-3-yl)-4-methoxytoluene,[24] compounds that can cause dNTP imbalance in the cell. Based on these observations, we speculate that the dNTP imbalance would induce this unique endonuclease to cause nuclear DNA breaks and subsequent cell death. We have observed recently that in the process of the dNTP imbalance death, mRNA levels of nuclear proto-oncogenes, c-*fos*, c-*jun* and c-*myc*, are increased (Kakutani, et al., submitted); this may be another example of dNTP imbalance-stimulated gene expressions.

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