Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 73-81

"THYMINELESS" DEATH IN ANDROGEN-INDEPENDENT PROSTATIC CANCER CELLS

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Received October 3, 1989

The molecular mechanism of "thymineless" death induced by 5-fluoro-deoxyuridine or trifluorothymidine, in androgen-independent rat prostatic adenocarcinoma AT-3 cells was investigated. Fragmentation of genomic DNA into discrete multiples of a nucleosomal unit (i.e. 180bp subunit) and induction of expression of TRPM-2, a programmed cell death-associated gene, temporally correlated with the activation of programmed cell death in this system. In contrast, killing of AT-3 cells by osmotic lysis, or membrane-targeted metabolic inhibitors results in neither the stereotypic DNA fragmentation into nucleosomal oligomers nor the elevation of TRPM-2 mRNA levels but to non-specific biochemical changes characteristic of necrosis. These results suggest that androgen-independent prostatic cancer cells retain a major portion of the programmed cell death cascade which can be activated by non-androgen ablative cytotoxic drugs that induce "thymineless" death.

Eukaryotic cells die by one of two stereotypic processes which are biochemically and morphologically distinct, necrosis and programmed cell death (apoptosis) (1). During necrosis the cell is "murdered" by a hostile microenvironment, without actively participating in the process of cell death. In contrast, programmed cell death occurs when the cell is activated by a specific extracellular signal to commit suicide in a normal microenvironment. Activation of programmed cell death leads to a series of biochemical and morphological events which result in nuclear disintegration and eventual fragmentation of the dying cell into a cluster of membrane-bound apoptotic bodies (2). Irreversible fragmentation of genomic DNA into nucleosomal oligomers (i.e. integer multiples of a 180-base pair subunit), has been widely characterized as an early event in programmed cell death in a number of systems, including cell death in γ-irradiated (3,4) and glucocorticoid-treated rat thymocytes (5,6), in castration-induced involution of rat ventral prostate (7), as well as in cell-mediated cytolysis of target cells (8,9). This DNA fragmentation is a result of activation of a $Ca^{++}Mg^{++}$-dependent endonuclease which selectively hydrolyses DNA at sites

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0006-291X/89 $1.50

located between nucleosomal units, thus resulting in the stereotypic ladder of DNA fragments (10-12). In contrast, during necrosis the DNA is degraded into a continuous spectrum of sizes as a result of simultaneous action of both lysosomal proteases and nucleases, released in cells already dead (13).

Previous studies have demonstrated that castration-induced androgen-withdrawal leads to the activation of programmed death of the androgen-dependent epithelial cells in the rat ventral prostate (7,14). In the present study the androgen-independent Dunning rat prostatic adenocarcinoma cell line AT-3 was used as an in vitro model system to determine whether androgen-independent prostatic cancer cells activate a similar genetic program of cell death in response to non-androgen ablative cytotoxic drugs. 5-fluorodeoxyuridine (5-FdUr) and trifluorothymidine (TFT) are DNA-targeted cytotoxic drugs widely used in cancer chemotherapy (15). Limitation of thymine caused by direct inhibition of thymidylate synthetase by either drug, leads to "thymineless" death, possibly as a result of depletion of intracellular dTTP pool (15). Although a series of studies focused on the mechanism of cytotoxic action of these drugs in mouse FM3A cells have implicated DNA lesions as potential triggers of cell death (16,17), the precise molecular events involved in the activation of "thymineless" death in target-tumor cells have yet to be defined.

In the present study in order to investigate the mode of cell death induced by deoxyribonucleoside triphosphate imbalance (dNTP) in androgen-independent AT-3 prostatic cancer cells, the kinetics and pattern of DNA fragmentation, as well as the temporal pattern of induction of TRPM-2, a gene associated with programmed cell death in a variety of systems, were studied following treatment with 5FdUr or TFT.

MATERIALS AND METHODS

Chemicals

The cytotoxic drugs 5-fluorodeoxyuridine, trifluorothymidine, oubain and iodoacetate, were purchased from Sigma Chemical Co. (St. Louis, MO).

In Vitro Cytotoxicity Assay

AT-3 is a Dunning rat prostatic tumor cell line which is highly anaplastic and androgen-independent (18). This cell line was maintained by serial passage of monolayer cultures in medium consisting of RPMI 1640, containing 10% fetal calf serum, streptomycin (100μg/ml), penicillin (100 Units/ml) and dexamethasone (250nM). Cells were grown in CO_2 at 37°C. Cell death at the end of each treatment period was determined by utilizing a colorimetric assay that is based on the reduction of tetrazolium salt, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan product, by the mitochondrial dehydrogenases present only in living metabolically active cells but not dead cells. This assay was carried out essentially as described by Carmichael et al (19). Following solubilization of formazan crystals in DMSO (100μl/well), the absorbance was read at 540nm using a Titertek Multiscan MC (Flow Laboratories, McLean, VA). Percentage cytotoxicity was determined by the formula (1-Abs.sample/Abs.control) x 100, where Abs. sample is the absorbance of cells in the

treated cultures and Abs. control is the absorbance of cells in the starting untreated control cultures (i.e. at time 0).

Quantitative Analysis of DNA Fragmentation

Monolayers of AT-3 cells (starting density of $10^5/cm^2$) were incubated with medium alone (control) or with sterile distilled water, oubain (10 μM) or iodoacetate (0.7mM) for 30min, 1hr, 3hrs and 6hrs or with 5FdUr (100μM), or TFT (100μM) for 6, 12, 24 and 48hrs. At the end of each incubation period cells were suspended by treatment with 0.25% trypsin solution, collected by centrifugation, pooled and counted. Cells were subsequently disrupted in 0.5% (v/v) Triton-X-100 and 5mM EDTA in PBS for 30 min at 4°C, and the cellular lysates were subjected to centrifugation at 20,000g for 20 min to separate the low molecular weight DNA fragments from intact chromatin (20). DNA in the resulting pellets and supernatants was determined by the diphenylamine reaction as described by Burton (21). The quantity of DNA fragments released in the supernatant was expressed as a percentage of total starting DNA.

Qualitative DNA Analysis

AT-3 cells were incubated with sterile distilled water, oubain, iodoacetate, 5FdUr, or TFT for various periods of time. At the end of each incubation period cells were suspended by trypsin treatment and the cellular pellets were resuspended in 10mM Tris-HCl buffer, pH8.0 containing 0.5% (v/v) Triton-X-100 and 5mM EDTA (approx. 10^7 cells/ml), and cells were lysed with 0.5% SDS in the presence of proteinase K (300μg/ml) (Sigma Chemical Co., St. Louis, MO) (16hrs at 37°C). DNA was extracted from the cellular lysates by sequential extractions with phenol, phenol-chloroform/isoamyl alcohol (24:1) and chloroform/isoamyl alcohol (24:1). DNA was precipitated by addition of two volumes of absolute ethanol, in the presence of 10mM $MgCl_2$ and 0.3M sodium acetate, pH5.2, and overnight incubation at -20°C (22). Precipitated DNA was pelleted by centrifugation at 15,000g for 20min at 4°C, and the final pellet was dried under N_2 and then resuspended in TE buffer (10mM Tris-HCl pH8.0, 1mM EDTA). DNA content was determined spectrophotometrically (A_{260}~50μg DNA/ml) and DNA samples (10μg) were electrophoretically analyzed on 1.6% agarose gels as previously described (7).

Northern Blot Hybridization Analysis

Cytoplasmic RNA from cell pellets was isolated according to the method of Auffrey and Rougeon (23). Total cytoplasmic RNA samples (10μg) from AT-3 cells, untreated, or following treatment with various cytotoxic drugs were size-fractionated on 1.4% agarose/formaldehyde (2.2M) gels, and transferred to Hybond-N membranes (Amersham Corp., Arlington Heights, IL) as previously described (14). DNA probes used for hybridization analysis were kindly provided by Dr. D. Cleveland (Johns Hopkins University, Baltimore, MD), human β-actin (24) and Dr. M. Tenniswood (University of Ottawa, Ontario, Canada), TRPM-2 cDNA (25). Insert-specific DNA was released by appropriate restriction enzyme digestion and inserts were labeled to high specific activity (5~6 x 10^8 dpm/μg of DNA) by oligolabeling, using [α^{32}P] dCTP and the multiprime DNA labeling kit purchased from Amersham. Membranes were hybridized according to the procedure previously described (14). Autoradiography was performed by exposing the filters to Kodak XAR-5 film with intensifying screens at -70°C for 1-3 days. Autoradiographs were quantitated with a scanning densitometer and results were normalized relative to the expression of β-actin transcript.

Statistical Analysis

Numerical values are expressed as the mean±SD. Statistical analyses of significance were made by a one way analysis of variance (ANOVA) using the Kruskal-Wallis test.

RESULTS

In Vitro Cytotoxicity

The time course of AT-3 cytotoxicity induced by various lytic treatments that lead to necrosis is shown on Table 1. Induction of osmotic lysis by

Table 1

Correlation between cytotoxicity and DNA fragmentation during necrosis

Treatment	Period	% Cytotoxicity	% DNA Fragmentation
Osmotic Lysis(+H_2O)	30 min	63.7 ± 0.8	0.4 ± 0.3
	1 hr	70.0 ± 3.1	2.8 ± 0.5
	2 hrs	78.2 ± 0.2	8.7 ± 1.5
	3 hrs	94.4 ± 1.8	24.6 ± 1.0
	6 hrs	100	38.8 ± 2.0
+Oubain (10μM)	30 min	32.7 ± 0.7	0.1 ± 0.2
	1 hr	42.8 ± 1.0	3.6 ± 1.2
	2 hrs	56.4 ± 0.4	8.4 ± 1.3
	3 hrs	79.0 ± 0.3	19.6 ± 1.5
	6 hrs	88.4 ± 0.5	35.7 ± 3.8
+Iodoaceate (0.7mM)	30 min	67.1 ± 2.8	1.4 ± 0.8
	1 hr	78.0 ± 1.2	6.0 ± 1.5
	2 hrs	90.0 ± 0.6	14.7 ± 1.3
	3 hrs	94.5 ± 5.6	22.8 ± 2.5
	6 hrs	100	43.7 ± 1.1

Time course of DNA fragmentation in AT-3 cells during necrosis. Cells were exposed to distilled water, oubain (10μM) or iodoacetate (0.7mM) for various periods of time as indicated. Cytotoxicity determinations after various lytic treatments was based on MTT-metabolic reduction assay as described in "Materials and Methods". The cytotoxicity results are expressed as mean ± SD of eight values. DNA fragmentation values are the mean ± SD of four different experiments.

treatment of AT-3 cells with distilled water leads to cell death at a dramatically rapid rate. Within 30 minutes of exposure to water approximately 64% cytotoxicity is observed (Table 1), and by 3 hours of treatment cell death is essentially completed (i.e. > 94% cytotoxicity). Treatment of cells with cytotoxic drugs that are targeted at the integrity of cell membrane, such as oubain (Na^+K^+ ATPase inhibitor) and iodoacetate, leads to cell death at a comparable rate as osmotic lysis (Table 1). In marked contrast, "thymineless" death induced by 5FdUr (100μM) or trifluorothymidine (100μM) proceeds at a significantly slower rate since over a 24hr-period of cytotoxic treatment with either drug, only 30% of cells are killed (Table 2).

Kinetics of DNA Fragmentation

Background levels of DNA fragmentation for the control cell cultures (i.e. medium alone) were approximately 2% of total cellular DNA. When AT-3 cells were exposed to water, oubain (10μM), or iodoacetate (0.7mM), there was no significant increase in DNA fragmentation within the first 30 min of cytotoxic treatment, although all three treatments resulted in substantial cytotoxicity by this time (Table 1). By 6hrs of treatment, at a time when cell death is essentially completed for all treatments, approximately 40% of the cellular DNA has been fragmented, indicating that DNA fragmentation occurs subsequent to massive cell death, a phenomenon characteristic of necrosis. When cells

Table 2

Correlation between cytotoxicity and DNA fragmentation during "thymineless" death

Treatment	Period	% Cytotoxicity	% DNA Fragmentation
+5-FdUr (100μM)	6 hrs	0.6 ± 0.1	9.2 ± 1.4
	12 hrs	1.6 ± 0.1	13.8 ± 1.5
	18 hrs	7.5 ± 0.1	22.5 ± 1.5
	24 hrs	26.6 ± 0.5	44.1 ± 1.4
	48 hrs	59.4 ± 0.3	86.8 ± 2.3
+TFT (100μM)	6 hrs	1.0 ± 0.1	5.8 ± 0.9
	12 hrs	1.5 ± 0.1	11.3 ± 0.4
	18 hrs	8.2 ± 0.3	28.0 ± 1.3
	24 hrs	27.1 ± 1.0	45.4 ± 3.5
	48 hrs	57.5 ± 1.8	90.6 ± 2.4

Cytotoxicity results are expressed as mean ± SD of eight values. DNA fragmentation values shown are the mean ± SD of four different experiments.

were incubated with 5FdUr or TFT, the kinetics of DNA fragmentation proceed at a considerable faster rate in relation to cytotoxicity (Table 2). As early as 6hrs of induction of "thymineless" death, cells begin to fragment their DNA even though no measurable signs of cytotoxicity were observed at this time. By 24hrs of treatment, a substantial increase in DNA fragmentation is observed (approx. 45%) and after 48 hrs of exposure to either drug, the entire cell population has almost all of its DNA fragmented, although cell death is not completed (i.e. approx. 60% cytotoxicity). This process of DNA fragmentation following induction of "thymineless" death by non-androgen ablative cytotoxic drugs is essentially an early event, that precedes the first signs of cell death by several hours (Table 2), suggesting that the cells are undergoing programmed cell death as opposed to necrosis.

Qualitative Analysis of DNA

Electrophoretic analysis of total cellular DNA isolated from untreated control AT-3 cells revealed high molecular weight DNA (Fig. 1, lane 1). Comparative analysis of DNA from AT-3 cells exposed to distilled water(osmotic lysis), oubain or iodoacetate for 3 hrs revealed a smear of degraded DNA (Fig. 1, lanes 2, 3 and 4 respectively), indicating random continuous degradation of cellular DNA, a process which characterizes necrosis. In marked contrast, electrophoretic analysis of DNA extracted from cells treated with 5FdUr or TFT for 24hrs (i.e. during "thymineless" state), demonstrated extensive fragmentation of DNA into a distinctive ladder of nucleosomal oligomers (Fig. 1, lanes 5 and 6 respectively), indicating cleavage of chromosomal DNA at internucleosomal loci.

Expression of TRPM-2 Gene

Recent studies have demonstrated that the rat ventral prostate expresses certain androgen-repressed mRNA sequences that are specifically induced after

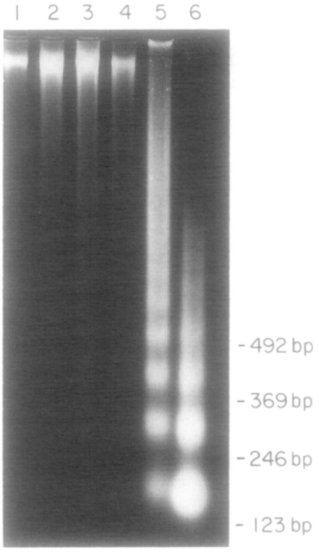

Figure 1. Electrophoretic analysis of DNA during necrosis or "thymineless" death. DNA extracted from AT-3 cells after various treatments was electrophoretically analyzed on 1.6% agarose gels, as described in "Materials and Methods". Lane 1 represents DNA from untreated control AT-3 cells. DNA was extracted from AT-3 cells after a 3hr-incubation with distilled water (lane 2), oubain (10μM) (lane 3), or iodoacetate (0.7mM) (lane 4), or after a 24hr-incubation with 5FdUr (100μM) or TFT (100μM) (lanes 5 and 6 respectively). Approximately 10μg of DNA was loaded per well. Size and location of molecular weight marker (123bp-DNA ladder) is shown on the right.

castration-induced androgen deprivation (14,25,26). To determine whether androgen-independent prostatic cancer cells activate a similar genetic program of cell death in response to non-androgen ablative cytotoxic drugs, the expression of one of these genes designated TRPM-2 (for testosterone repressed prostatic message-2) was studied during "thymineless" death of AT-3 cells. As shown in Fig. 2, TRPM-2 transcripts are constitutively expressed in untreated control cells at relatively low levels. Non-specific cellular lysis of AT-3 cells induced by osmotic lysis, oubain or iodoacetate, did not lead to the induction of TRPM-2 gene above the constitutive level of expression at any time during the time course of cytotoxicity studied (Fig. 2). In marked contrast, induction of "thymineless death" with either 5FdUr or TFT results in a dramatic increase in the expression of TRPM-2 transcript (approx. 10-fold) within the first 6hrs of exposure to the drugs (Fig. 2). By 24hrs of cytotoxic treatment however, when cell death is substantially enhanced (Table 2), TRPM-2 mRNA levels decrease to constitutive expression levels, demonstrating a temporal correlation between the expression of TRPM-2 transcripts and the activation of "thymineless" cell death in androgen-independent prostatic cancer cells.

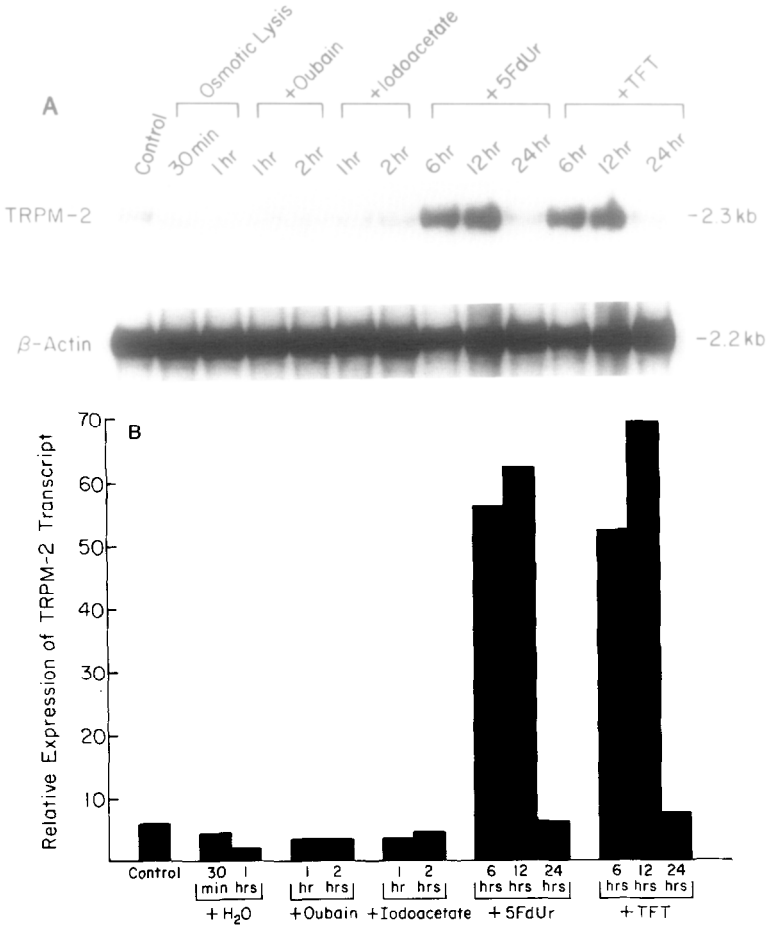

Figure 2. Northern blot hybridization analysis of RNA from androgen-independent AT-3 prostatic tumor cells untreated, during necrosis or "thymineless" death, to TRPM-2 cDNA probe. Total cytoplasmic RNA (10μg/well), isolated from AT-3 cells untreated, after exposure to distilled water (30 mins and 1hr), oubain (1hr and 2hr), or iodoacetate (1hr and 2hrs), or following treatment with 5FdUr or TFT for 6, 12 and 24 hrs, was size-fractionated on an agarose gel (1.4%) and blotted onto nylon membranes as described in "Materials and Methods". The blot was initially hybridized with a ^{32}P-labeled TRPM-2 cDNA probe and subsequently with a ^{32}P-labeled β-actin probe (A). Molecular size in kilobases is indicated on the right. The relative expression of TRPM-2 transcripts as determined from densitometric analysis of the autoradiographic transcript bands is shown in (B).

DISCUSSION

The results presented in this study demonstrate that treatment of androgen-independent AT-3 prostatic cancer cells in vitro with non-androgen ablative agents which induce "thymineless" death, results in the activation of temporally distinct biochemical events characteristic of programmed cell death, i.e. fragmentation of target cell DNA into nucleosomal oligomers, possibly as a result of activation of a Ca^{2+} Mg^{2+}-dependent endonuclease, and an increase in the expression of TRPM-2 gene, both molecular events occuring very early in the process. This lytic cascade is similar to that

observed in the death of the androgen-dependent prostate cells following castration-induced androgen withdrawal (7,14), suggesting that although androgen-independent prostatic cancer cells do not activate the genetic program of cell death in response to androgen deprivation, they still retain the major portion of the biochemical cascade that leads to programmed cell death, which can be activated by specific chemotherapeutic drugs that induce "thymineless" death. In marked contrast, during necrosis induced by osmotic lysis, or interference with the biochemical integrity of the cell membrane (i.e. exposure to oubain or iodoacetate), the majority of cellular DNA is ultimately degraded into a continuous spectrum of sizes. This is a non-specific and essentially late phenomenon, that comprises the result of activation of lysosomal proteases that destroy the histones and expose the entire length of DNA to random attack by nucleases. Furthermore, the present studies suggest that enhanced expression of TRPM-2 transcripts does not merely comprise a non-specific molecular change that occurs during any type of cell death (i.e. both necrosis and apoptosis) but is rather a specific molecular event occurring early in the biochemical cascade that leads to programmed cell death. TRPM-2 gene, originally identified as an androgen-repressed message in the involuting rat ventral prostate (25), has been subsequently demonstrated to be induced in other systems of programmed cell death (27,28). Although the function of its protein product has yet to be defined, recent evidence implying high sequence homology of cDNA encoding TRPM-2 with mRNA encoding a sulfated glycoprotein 2 (26), similar to the major glycoprotein (SGP-2) expressed by the Sertoli cells of the testis (29), points to a potential role of TRPM-2 gene in the regulation of cell membrane function.

The present findings are compatible with previous studies implicating single and double-strand DNA breaks as the underlying mechanism of drug-induced "thymineless" death in a variety of target cells (16,17,30,31).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the gifted help of Barbara A. Lee in the completion of the manuscript. This study has been supported by NIH grant CA 15416.

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