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Induction of endonuclease-mediated apoptosis in tumor cells by C-nitroso-substituted ligands of poly(ADP-ribose) polymerase

(zinc finger/drug-induced cell death)

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ABSTRACT **6-Nitroso-1,2-benzopyrone and 3-nitrosobenzamide, two C-nitroso compounds that inactivate the eukaryotic nuclear protein poly(ADP-ribose) polymerase [NAD+:poly(adenosine diphosphate D-ribose) ADP-D-ribosyltransferase, ADPRT, EC 2.4.2.30] at one zinc-finger site, completely suppressed the proliferation of leukemic and other malignant human cells and subsequently produced cell death. Tumoricidal concentrations of the drugs were relatively harmless to normal bone marrow progenitor cells and to superoxide formation by neutrophil granulocytes. The cellular mechanism elicited by the C-nitroso compounds consists of apoptosis due to DNA degradation by the nuclear calcium/magnesium-dependent endonuclease. This endonuclease is maintained in a latent form by poly(ADP-ribosyl)ation, but inactivation of ADPRT by C-nitroso drugs derepresses the DNA-degrading activity. ADPRT is thus identified as a critical regulatory enzyme component of a DNA-binding multiprotein system that plays a central function in defining DNA structures in the intact cell.**

In pursuing the biological consequences of the inhibition of poly(ADP-ribose) polymerase [NAD+:poly(adenosine diphosphate D-ribose) ADP-D-ribosyltransferase, ADPRT, EC 2.4.2.30] within intact cells we have developed a group of C-nitroso-substituted molecules that are the oxidation products of previously described ligands containing amino groups (1). These C-nitroso ligands uniquely oxidize one of the zinc fingers of ADPRT, resulting in zinc ion ejection and concomitant inactivation of the poly(ADP-ribosyl)ation activity of ADPRT without abrogating its binding to DNA (1). In the present paper we demonstrate a cytostatic and apoptotic action of two of these ADPRT ligands, 6-nitroso-1,2-benzopyrone (NOBP) and 3-nitrosobenzamide (NOBA),¶ toward malignant human cells. The apoptotic effect was traced to the derepression of a calcium/magnesium-dependent endonuclease that under resting conditions is known to be inhibited by poly(ADP-ribosyl)ation (2).

MATERIALS AND METHODS

Cells and Cell Lines. With the exception of 855-2 (3) and human neutrophils (4), all tumorigenic and nontumorigenic cell lines were obtained from the American Type Culture Collection and were cultured as recommended by the vendor. Rhesus (*Macaca mulatta*) bone marrow cells and human peripheral blood stem cells were prepared according to published procedures (5).

Assays. For cell proliferation tests, cells were cultured in 96-well tissue culture plates at 3×10^3 cells per well (250-μl total volume per well) for 2 hr (or the indicated length of time) in the absence or presence of various concentrations of NOBA or NOBP; thereafter 0.5 μCi of [methyl-^{3}H]thymidine (85 Ci/mmol; 1 Ci = 37 GBq) was added per well and its incorporation into DNA following an 18-hr incubation was determined radiochemically (6). Proliferation was also monitored by direct cell counting. Since both assays were in agreement, the more convenient radiochemical test was used routinely. The tetrazolium reduction assay for cell viability (7), which was originally developed as a test for mitochondrial succinate dehydrogenase (8), was compared with trypan blue uptake and plating efficiency, and on the basis of positive correlation among the three assays, the more quantitative dye reduction method was adopted.

Clonigenic Studies. Human 855-2 and HL-60 leukemic cells were plated at 10^5 per ml and human and rhesus bone marrow mononuclear cells and human peripheral stem cells at 4×10^5 per ml, and assays for colony-forming units were performed as reported (5). A semiquantitative assessment of malignant cells in bone marrow specimens was done by flow cytometry with labeled fluorescein-conjugated antibodies against common acute lymphoblastic leukemia antigen (CALLA, CD10) (9). The fluorometric assay for DNA unwinding (fragmentation) and the assay for calcium/magnesium-dependent endonuclease in isolated nuclei (10) reproduced published methods (11–13). Gel electrophoretic tests for DNA fragmentation were adapted from standard methods (13). Superoxide generation by human neutrophils was quantitated as described (4).

Chemical Synthesis and Other Methods. Chemical synthesis and tritiation of NOBP and NOBA were performed as reported (14, ¶). The methods for analysis of intracellular drug kinetics, quantitation of intracellular poly-ADP-ribosylation, and quantitative intracellular immunoassays for ADPRT have been published (15, 16). Cellular NAD and ATP levels were measured as reported (14).

RESULTS

Inhibitory Effects on Tumor Proliferation and Tumoricidal Action of C-Nitroso Drugs. Both NOBP (Fig. 1*A*) and NOBA (Fig. 1*B*) suppressed cell proliferation in a drug concentration-dependent manner in human leukemic cell lines repre-

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Abbreviations: ADPRT, ADP-D-ribosyltransferase (polymerizing); NOBP, 6-nitroso-1,2-benzopyrone; NOBA, 3-nitrosobenzamide; CALLA (CD10), common acute lymphoblastic leukemia antigen.

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¶Kun, E., Mendeleyev, J., and Rice, W. G., U.S. Patent pending 07/780,809.

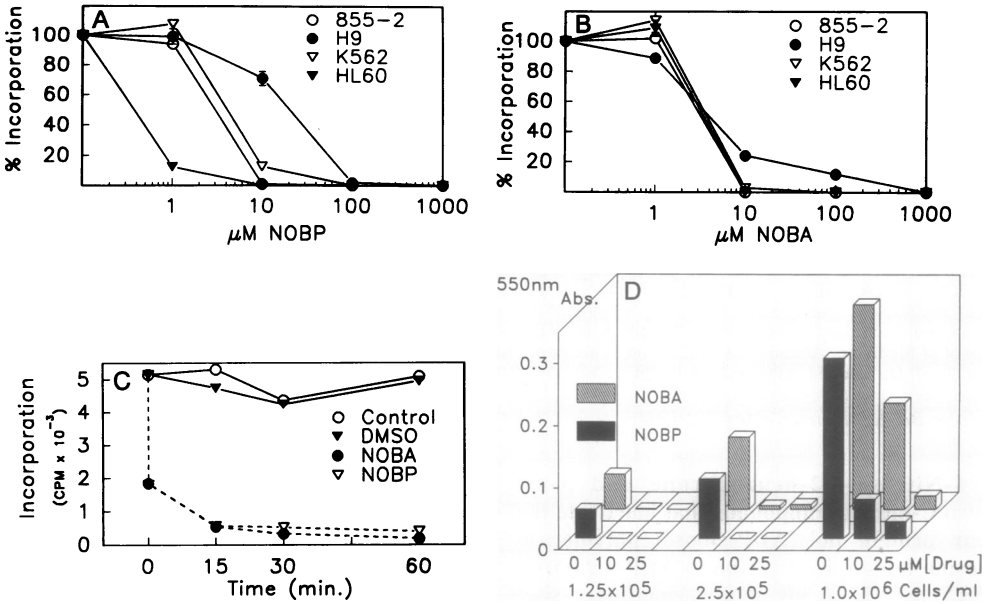

FIG. 1. C-nitroso-substituted ADPRT ligands inhibit human leukemia cell proliferation (*A–C*) and abolish cell viability (*D*). (*A* and *B*) Proliferation was assayed by [^{3}H]thymidine incorporation after treatment of human leukemic cell lines (855-2, H9, HL-60, and K562) with various concentrations of NOBP (*A*) or NOBA (*B*), for 30 min. Results are mean ± SEM from three replicates and values are normalized to the percent of uptake by each cell type in the absence of the drugs. (*C*) [^{3}H]Thymidine incorporation by 855-2 cells was measured after exposure of cells to no drug (control) or to 10 μM NOBP, 10 μM NOBA, or 0.1% dimethyl sulfoxide (DMSO) for the indicated times (mean of three separate experiments with triplicate samples in each experiment). (*D*) Viability of 855-2 cells was measured by the microculture tetrazolium assay after treatment of either 1.25×10^5, 2.5×10^5, or 1.0×10^6 cells per ml with no drug or with 10 μM or 25 μM NOBP or NOBA (mean of three replicates).

senting B-cell-precursor acute lymphoblastic leukemia (855-2 cell line), T-cell-lineage acute lymphoblastic leukemia (H9 cell line), acute promyelocytic leukemia (HL-60 cell line), and chronic myelogenous leukemia (K562 cell line). Proliferation of 855-2 cells at a cell density of 10^5 per ml was inhibited 50% (IC_{50}) by NOBP at 1.68 ± 0.09 μM and by NOBA at 1.96 ± 0.12 μM (mean ± SEM, $n = 3$), either drug at 10 μM always produced 100% suppression of cell proliferation, which occurred without prolonged exposure to the drugs (Fig. 1*C*), and a pulsed addition (>0.5 hr) of either compound at 10 μM irreversibly inhibited the ability of 855-2 cells to proliferate (Fig. 1*D*).

Correlation Between Drug Concentration and Cell Density. As seen from Fig. 1*D* there is a stoichiometry between drug concentration and cell density. It was calculated from drug kinetics (14, 16) and metabolism (17) and on the basis of the known nuclear matrix localization of ADPRT (18, 19) and the number of ADPRT molecules per cell (15) that nitroso drug at ≈400 molecules per molecule of ADPRT produces tumoricidal action. Direct chemical extraction of the drugs by chloroform/methanol (1:1) and NMR analysis in isolated systems (unpublished results) showed no covalent binding of nitroso drugs with nucleic acids; thus the drugs acted with a high degree of specificity on ADPRT.

Tumoricidal Apoptotic Effects of C-Nitroso Drugs. Colony formation of 855-2 and HL-60 cells in semisolid medium was completely abolished by either of the C-nitroso drugs when incubated for 2 hr at 10 μM (Table 1), and in fact no cell structures were detectable after 10 days of culture (Fig. 2). On the other hand, colony formation by rhesus bone marrow cells and human peripheral stem cells, which were equal in size, was only partially depressed without cell killing, as detected by the decrease in the number of colonies, and no effect was observed with normal human bone marrow itself (Table 1). Furthermore, NOBP and NOBA were relatively harmless to the oxidative burst function of normal human neutrophils. Stimulated neutrophils generated 55.9 ± 2.7 nmol of superoxide per hr per 10^5 cells (mean ± SEM, $n = 11$) in the absence of ligands and 34.1 ± 5.0 and 44.4 ± 3.5 nmol per hr per 10^5 cells (mean ± SEM, $n = 11$) in the presence of 10 μM NOBP and NOBA, respectively.

At a cell concentration of 10^6 per ml, exposure to 10, 25, and 100 μM NOBP (Fig. 3) for 1 hr resulted in a dose-dependent and preferential killing of leukemic cells, with

Table 1. Effects of NOBP and NOBA on colony formation by leukemic and normal cells

Source of Cells	*n*	Colony-forming units		
		Untreated	10 μM NOBP	10 μM NOBA
Leukemic cell lines (CFU)				
855-2	8	45.6 ± 5.3	0	0
HL-60	4	31.0 ± 8.1	0	0
Normal progenitor cells (CFU-GM)				
Rhesus bone marrow	6	42.3 ± 4.5	32.0 ± 3.1	32.2 ± 3.1
Human bone marrow	2	64.5 ± 14.5	64.5 ± 2.5	65.0 ± 4.0
Human peripheral blood stem cells	4	30.3 ± 7.0	16.7 ± 5.9	20.2 ± 6.6

Frequencies of colony-forming units (CFU) per plate for 855-2 and HL-60 leukemic cell lines and frequencies of granulocyte/macrophage CFU (CFU-GM) per plate for bone marrow progenitor cells of normal rhesus or human origin or for human peripheral blood stem cells were quantitated without (untreated) or following a 2-hr exposure to 10 μM NOBP or NOBA. Results are expressed as the mean ± SEM from the indicated number (*n*) of preparations.

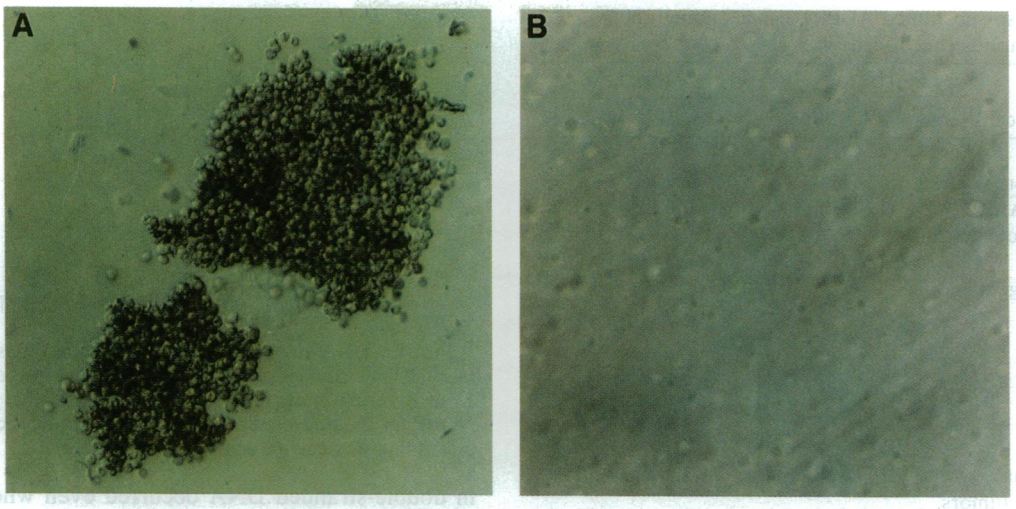

FIG. 2. Inhibition of colony formation of leukemic cells by C-nitroso-substituted ADPRT ligands. Cells were exposed to drugs for 2 hr, and then the drug was washed away. Colonies of HL-60 cells were visualized by high-power magnification and grown either without (*A*) or after a 2-hr exposure to 10 μM NOBP (*B*) for 10 days when colony formation was assayed. The same results were obtained with 10 μM NOBA and HL-60 cells and with 855-2 cells and 10 μM NOBP or NOBA. (×105.)

significant preservation of normal cells. Similar results were observed with NOBA (data not shown). There is some uncertainty regarding the exclusive identification of the malignant cell population by the CALLA antibody (9), since there may be malignant cells present that do not carry this marker but are killed by the drugs (Fig. 3*D*). The tumoricidal action of the C-nitroso drugs was not limited to human leukemias, and an additional 13 human tumor cell lines (Table 2) also exhibited varying sensitivities. In preliminary experiments the propagation of L1210 murine leukemia (Table 2) was also tested *in vivo*, and results showed that NOBA, injected intraperitoneally at a dose of 2 mg/kg twice a day for 6 consecutive days, causing no toxic effects, prolonged the life of BDF mice from 10 days (untreated) to 35 days (end of observation), thus exerting a highly significant *in vivo* chemotherapeutic response [J. Atiba, L. S. Slater and M. Wetzel (University of California, Irvine), J.M. and E.K. (Octamer Research Foundation), unpublished results].

Mechanism of Apoptosis. Three types of internally complementary, but distinct, experimental results indicated that the C-nitroso drugs induced an endonuclease-mediated DNA breakdown in tumor cells, leading to apoptosis. Incubation of 855-2 cells (0.5×10^6 per ml) with 20 μM NOBP for 18 hr resulted in a selective tripling of the calcium/magnesium-dependent endonuclease activity in isolated nuclei (Table 3). Fluorometric assay of DNA strand breaks (11) demonstrated a time- and drug concentration-dependent rate of unwinding of double-stranded DNA (Fig. 4). There was only a negligible difference in NAD content between untreated control cells (289.9 ± 12.0 pmol per 10^6 cells) and drug-treated cells, which exhibited 20% unwinding (241.5 ± 24.4 pmol per 10^6 cells, mean ± SD, $n = 3$). Simultaneously, cellular ATP of 855-2 cells (1748 ± 325 pmol per 10^6 cells) decreased by only 20–25% after treatment with 20 μM NOBP when assayed at the pre-apoptotic stage (1281 ± 166 pmol per 10^6 cells, mean ± SD, $n = 3$).

There is a direct parallel between C-nitroso drug-induced DNA fragmentation and the apoptosis sensitivity of the malignant phenotype, as determined by DNA degradation (Fig. 5). Lanes 1–3 show the migration of DNA extracted from 855-2 cells compared with the DNA extracted from NOBP-treated 855-2 cells (10 and 20 μM); lanes 4–6 show the

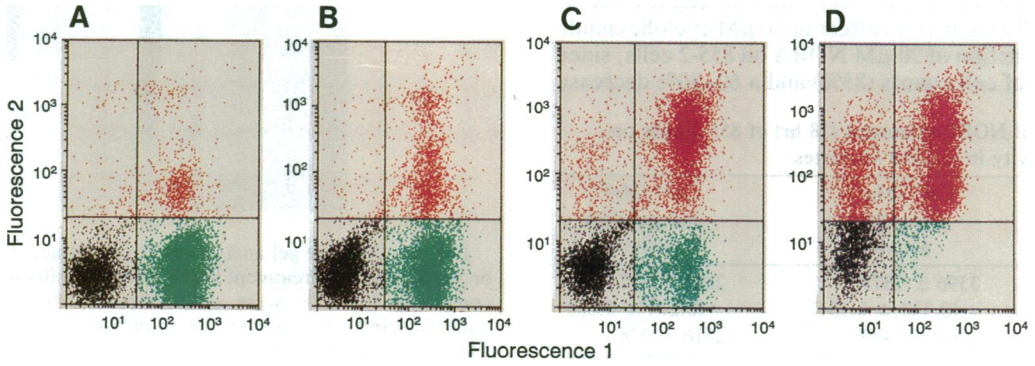

FIG. 3. Selective killing of human malignant cells by C-nitroso-substituted ADPRT ligands. Bone marrow cells were harvested from a patient diagnosed to be afflicted with acute lymphoblastic leukemia. Cells were then exposed to antibodies against CALLA (CD10) according to a published method (9) and incubated for 1 hr without drug (*A*) or with 10 μM (*B*), 25 μM (*C*), or 100 μM (*D*) NOBP. The cells were washed by centrifugation and leukemic cells were analyzed by the dual-wavelength flow cytometer quantitating fluorescein-conjugated anti-CALLA; dead cells were assayed by their incorporation of propidium iodide. The lower left quadrant of each graph depicts the normal population of bone marrow cells that express no CALLA surface antigen (light scattering; black dots); the lower right quadrant identifies the population of CALLA-positive leukemic cells (green fluorescence; green dots). Cells shown in the upper quadrants represent the dead cells that have incorporated propidium iodide (red fluorescence; red dots). The leukemic cells in the lower right quadrant move into the upper right quadrant (dead leukemic cells) after treatment with the ligands, but the majority of normal cells remain viable and stay within the lower left quadrants. The fraction of CALLA-positive cells in the bone marrow was 75%.

Table 2. C-nitroso-substituted ADPRT ligands inhibit the proliferation of various cancer cells

Types of cancer	Cell line	IC_{50}, μM NOBP	IC_{50}, μM NOBA
Leukemias			
B-cell-precursor ALL	855-2	1.7	2.0
T-cell-lineage ALL	H9	20.0	1.3
Acute promyelocytic leukemia	HL-60	0.3	1.2
Chronic myelogenous leukemia	K562	4.0	3.7
Brain tumors			
Astrocytoma–glioblastoma	CRL7712	6.1	9.0
Glioblastoma	D32	4.0	4.3
Glioblastoma	D37	4.4	5.9
Meduloblastoma	HTB186	3.5	3.1
Neuroectodermal tumors			
Neuroblastoma	IMR-132	0.8	1.0
Malignant melanoma	Malme-3M	6.5	9.0
Breast tumors			
Carcinoma	MDA-MB-453	4.5	5.1
Adenocarcinoma	MDA-MB-468	1.2	1.3
Lung carcinoma	A549	37.5	36.4
Colon carcinoma	SW620	7.5	16.2
Fibrosarcoma	HT-1080	8.1	7.6
Wilms tumor	G-401	3.8	3.9
Retinoblastoma	WERI-Rb-1	1.8	3.7
Murine leukemia	L1210	0.4	1.8

Various human tumor cells and a murine leukemia cell line (L1210) were tested for their dose-responsive sensitivities to NOBP and NOBA as described in the legend to Fig. 1. The IC_{50} values were determined from triplicates of each drug concentration within a single screening experiment for each cancer cell type and represent added drug concentration. ALL, acute lymphoblastic leukemia.

same comparison for 855-2 cells treated with 10 μM (lane 5) and 20 μM (lane 6) NOBA. In contrast, a nonmalignant cell line (Vero green monkey kidney cells) treated with 20 and 40 μM NOBA did not respond with DNA degradation (lanes 7–9). We compared two other nonmalignant cell lines (3T3 mouse fibroblasts, MRC-5 human lung fibroblasts) with drug-susceptible tumor lines and consistently found the C-nitroso drug-induced apoptosis to be apparently specific for tumor cells (compare also with Fig. 3 *B* and *C*), and 10 μM drugs that induced apoptosis in malignant cells had no effect on nonmalignant cells. There was no effect of 20 μM cycloheximide on the apoptotic action of 20 μM NOBA on 855-2 cells, since both a decrease of cell counts (85%) and a 60–80% decrease

Table 3. Effect of NOBP treatment (18 hr) of 855-2 cells on endonuclease activity in nuclear sonicates

Treatment of cells	Ca^{2+}/Mg^{2+}-dependent activity	Ca^{2+}/Mg^{2+}-independent activity
None	3396 ± 1877 (0.332 ± 0.184)	2801 ± 697 (0.274 ± 0.068)
20 μM NOBP	10680 ± 4407 (1.047 ± 0.432)	2516 ± 678 (0.247 ± 0.066)

Endonuclease assays were performed (12) with sonicates of nuclei prepared (10) from 855-2 cells (90 μg of DNA per assay system). Numbers represent cpm in supernatants (7.9 mCi/mg) after acid precipitation; numbers in parentheses show "units" (12) (1 unit is defined as 10% of total DNA radioactivity in the assay system). Calcium/magnesium-dependent activity was the difference between the total activity and the ion-independent activity (right column), which was obtained upon incubation with EGTA and EDTA in the absence of added calcium or magnesium ions. Experiments were performed in triplicate, and results are expressed as mean ± SD.

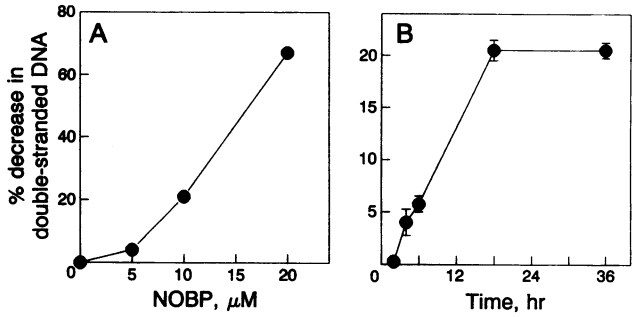

FIG. 4. DNA unwinding in 855-2 cells in response to NOBP. Cells (8×10^6) at a density of 0.2×10^6 per ml were exposed to NOBP for 18 hr (overnight) at the concentrations indicated (*A*) or to 10 μM NOBP for the times indicated (*B*). The fluorescence assay for DNA unwinding was carried out as described (11).

in double-stranded DNA occurred even when protein synthesis was completely blocked. Cycloheximide, as would be expected, by itself inhibited cell proliferation by 70% but had no apoptotic action.

DISCUSSION

Dexamethasone-induced apoptosis of thymus cells is inhibited by cycloheximide, indicating its dependence on protein synthesis (20), whereas this is not the case in C-nitroso drug-induced tumor cell killing, which occurs by the derepression of the calcium/magnesium-dependent specific endonuclease. Nonspecific fragmentation of DNA by carcinogens (21) or by ionizing radiation (22, 23) is known to cause cell death that is passively mediated by ADPRT, because ADPRT undergoes extreme external activation by nicked DNA and acts as a cellular NAD-depleting system. However,

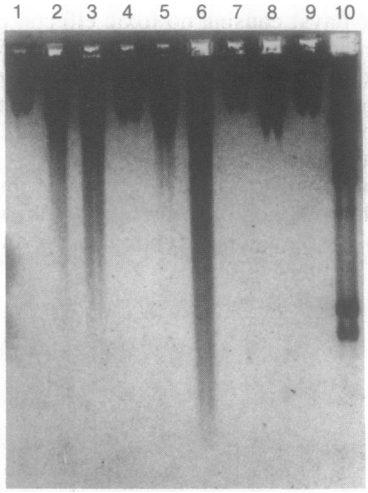

FIG. 5. Agarose gel analysis of DNA extracted from 855-2 cells or Vero cells after treatment for 18 hr with C-nitroso drugs. Samples containing 10^6 cells were sedimented, washed with phosphate-buffered saline, suspended in 100 μl of buffer (150 mM NaCl/25 mM EDTA) containing proteinase K (100 μg/ml) and 0.2% SDS, incubated for 3 hr at 40°C, extracted twice with phenol/chloroform and once with chloroform, and further incubated for 1 hr at 40°C in the presence of RNase A (0.5 mg/ml). Aliquots corresponding to 0.2×10^6 cells were loaded into gel slots. The 1.2% agarose gel was electrophoresed for 3 hr at 50 V and stained with ethidium bromide. Lanes 1 and 4, untreated 855-2 cells; lanes 2 and 3, 855-2 cells treated with 10 and 20 μM NOBP, respectively; lanes 5 and 6, 855-2 cells treated with 10 and 20 μM NOBA, respectively; lane 7, untreated Vero cells; lanes 8 and 9, Vero cells treated with 20 and 40 μM NOBA, respectively; lane 10, φX174 DNA cleaved with *Hae* III to give size standards.

such passive involvement of ADPRT in the direct DNA damage-induced nonselective cell killing is qualitatively different from the selective calcium/magnesium-dependent endonucleolytic cleavage of certain, apparently tumor cell type-dependent DNAs, producing selective apoptosis. The C-nitroso drugs do not directly damage DNA, but they block the polymerase activity of ADPRT, as apparent from the absence of any immediate drastic decrease in cellular NAD, which by contrast is common to all cell death caused by external DNA damage (22, 23).

Our results suggest that it is feasible to develop C-nitroso ligands as potential chemotherapeutic drugs that in their molecular mode of action differ from any of the commonly used anticancer drugs. Topoisomerase II-inhibitory drugs are also known to induce endonuclease-mediated apoptosis (24), but this mechanism significantly differs from the C-nitroso drug-initiated derepression of the endonuclease. The selective cytotoxicity of C-nitroso drugs on malignant cells has been directly determined by selective cell killing (Figs. 2 and 3). In contrast, the action of these drugs on nonmalignant cells, even though partially suppressing the proliferation of some cell types (Table 1), is not cytocidal since no DNA fragmentation is observed in nonmalignant cells (Fig. 5). Tumor cell apoptosis can be explained by the derepression of the calcium/magnesium-activated endonuclease (Table 3), but the partial and cell type-dependent suppression of proliferation (Table 1) of nonmalignant cells probably involves other cellular mechanisms that require further studies.

We presume that the tumor cell specificity of the cytocidal action of C-nitroso drugs may depend on at least two mechanisms: (*i*) the (probably) malignant cell type-specific molecular vicinity of ADPRT and the calcium/magnesium-activated endonuclease within the chromatin, permitting an inhibitory interaction [by way of poly(ADP-ribosyl)ation; see ref. 2] and (*ii*) the special structural features of DNA itself in malignant cells (e.g., amplified oncogenes; see ref. 25). The unusual affinity of ADPRT for certain internal DNA regions (26, 27) may provide a guidance system to ADPRT-associated endonuclease molecules to promote an attack on certain DNA structures following the inactivation of the catalytic function of ADPRT by C-nitroso drugs.

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