Regression of Human Breast Carcinoma Tumors in Immunodeficient Mice Treated with 9-Nitrocamptothecin: Differential Response of Nontumorigenic and Tumorigenic Human Breast Cells in Vitro^1

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ABSTRACT

We have shown recently that the plant alkaloid camptothecin and its derivatives inhibited growth of human carcinoma and melanoma cells in vitro and induced regression of advanced human malignant melanoma tumors growing in immunodeficient (nude) mice. Here, we have extended these studies to show that the camptothecin derivative 9-nitro-20(S)-camptothecin (9NC) induces complete regression of advanced breast carcinoma tumors growing in nude mice. We also report that 9NC inhibits growth of nontumorigenic and tumorigenic breast cells in vitro. However, flow cytometry studies show that 9NC elicits differential effects on the cell cycle of nontumorigenic and tumorigenic cells. In general, 9NC-treated nontumorigenic cells accumulate slowly at the G2 phase of the cell cycle with no cell death. In contrast, 9NC-treated tumorigenic cells traverse rapidly from G1 to S phase followed by cell death. Removal of 9NC from the cell cultures resulted in most nontumorigenic cells dividing, whereas tumorigenic cells continued to die after removal of 9NC. Taken together, the findings indicate a different response of nontumorigenic and tumorigenic breast cells to 9NC.

INTRODUCTION

The plant alkaloid CPT^1 was first reported to possess anticancer activity in 1966 (1). Subsequently, it was shown that CPT was cytotoxic for L929 murine fibrosarcoma and ME-180 human cervical carcinoma cell lines (2). Flow cytometry studies showed that CPT-treated human leukemia cells exhibit DNA degradation in S phase or are arrested in G1 and G2 phases, depending on whether the leukemia cells are of myeloid or lymphoid lineage (3, 4). We have shown recently that exposure of human U-937 leukemia cells to CPT and its derivatives 9NC and 9AC result in an 80- to 100-fold increase in expression of c-jun and jun-B mRNAs followed by a characteristic degradation of cellular DNA (5). A recent report has shown that indeed CPT induces scissions in DNA strands of U-937 cells and that the drug-treated cells are preferentially arrested at the G2M phase of the cycle (6). Furthermore, CPT induced the expression of differentiation markers in the human myeloid cells U-937, THP-1, and HL-60 (6). It is now documented that CPT interferes with the breakage-reunion process of topoisomerase I by stabilizing the enzyme-DNA "cleavable complexes" and thus interfering with the process of DNA breakage-reunion, an event which leads to fragmentation of nuclear DNA (7). The sensitivity of malignant cells to CPT has been correlated positively with topoisomerase I activity (8-12).

In the 1970s, water-soluble sodium CPT was used to treat cancer patients in clinical trials (13-16), but low antitumor activity and the occurrence of severe toxicity led to its discontinuation. The clinical trials included treatment of advanced cancer patients with sodium-CPT, but the outcome was that there were no clinical benefits (13-16). Recently, a water-soluble derivative of CPT, CPT-11, used in phase II clinical studies on patients with refractory leukemias and lymphomas has exhibited some effectiveness (17). Also, CPT-11 has been used in phase I and phase II studies on patients with advanced non-small cell lung carcinoma (18) and gynecological cancers (19), respectively, but the response rate was low. Ineffectiveness of sodium CPT was observed in our studies with immunodeficient (nude) mice bearing human tumors when the drug was administered i.v. and i.m. (20). In contrast, p.o. and i.m., but not i.v., administration of the water-insoluble CPT prevented growth of human solid tumors in mice, with the degree of effectiveness depending on the mode of administration (20). Administration, p.o., of water-insoluble CPT is currently being tested in an ongoing phase I clinical study on patients with various types of cancer. In other preliminary reports we have shown that CPT and 9AC inhibit the growth of tumors induced by a variety of human xenografts in nude mice (10, 20, 21). We have further shown that CPT derivatives induce regression of a human malignant melanoma tumor grown in nude mice, and this regression is accompanied by degenerative changes in the tumor cells that resemble degenerative changes observed in the melanoma tumor cells treated with CPT derivatives in vitro (21). Finally, no toxic effects were observed in drug-treated nude mice without xenografts and drug-treated melanocytes, the normal counterparts of melanomas, in vitro (21). In this report, we demonstrate that 9NC induces regression of human breast carcinoma tumors grown in nude mice. We also demonstrate that 9NC elicits different responses from nontumorigenic and tumorigenic epithelial cells in vitro. The responses include inhibition of cell proliferation, changes in cellular morphology, and changes in the distribution of cells at various phases of the cell cycle.

MATERIALS AND METHODS

Drugs. CPT derivatives were prepared and purified according to published procedures (22). For the in vitro studies, 9NC was suspended in polyethylene glycol (PEG 400; Aldrich, Milwaukee, WI), divided into small aliquots, and stored at -70°C until used. For the in vivo studies, 9NC was used as a fine suspension in cottonseed oil (Sigma). For these studies, the drug suspensions were sterilly prepared shortly before injections in the animals. We have used various CPT derivatives in previous studies (5, 10, 20, 21) but only 9NC throughout this study because of the following practical reasons: (a) the antitumor activity of 9NC in vitro is higher than that of CPT, and about equal to 9AC. Comparison of drug activities was made on the basis of equal drug doses and regimens administered to nude mice xenografted with human tumors; (b) 9NC as a powder or in suspension is more stable than 9AC when exposed to environmental factors including light, oxygen, water, and pH. In the presence of these factors, 9AC degrades more rapidly to products toxic to mice and drug-treated melanocytes, the normal counterparts of melanomas, in vitro (21). In this report, we demonstrate that 9NC induces regression of human breast carcinoma tumors grown in nude mice. We also demonstrate that 9NC elicits different responses from nontumorigenic and tumorigenic epithelial cells in vitro. The responses include inhibition of cell proliferation, changes in cellular morphology, and changes in the distribution of cells at various phases of the cell cycle.

Received 9/11/92; accepted 1/20/93.

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1 This work was supported by funds from The Stehlin Foundation for Cancer Research and The Friends of The Stehlin Foundation.

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3 The abbreviations used are: CPT, camptothecin; 9NC, 9-nitro-20(S)-camptothecin; 9AC, 9-amino-20(S)-camptothecin; PEG, polyethylene glycol; Apo, apoptotic cells.


[CANCER RESEARCH 53, 1577-1582, April 1, 1993]

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Cells and Nude Mice. The breast carcinoma cell lines used in this study were initially tested for ability to induce tumors in nude mice. The cell lines MDA-MB-231, MDA-MB-436, and CLO, with respective doubling times of 48, 44, and 88 h, induced tumors following inoculation in nude mice. The nontumorigenic cell lines included MDA-MB-134, MDA-MB-157, MDA-MB-175, MDA-MB-309, MDA-MB-330, MDA-MB-331, MDA-MB-415, MDA-MB-435, SKBR-3, BT-474, BEC, and PWY, with doubling times ranging from 44 to 86 h. The cell lines coded MDA-MB (24) have been obtained from Dr. R. Cailleau; the lines SKBR-3 and BT-474 were obtained from Dr. D. Slamon; and the lines BEC and PWY were from Colonel A. Leibowitz. CLO cell line was established in this laboratory as a xenograft from a biopsy taken from a patient with breast carcinoma (25). Successively, a CLO cell line was derived from a xenograft also in this laboratory. The majority of these cell lines has been used previously in our laboratory (25). All cells were grown in mycoplasma-free cultures in RPMI 1640 containing 10% fetal calf serum and antibiotics. Cell cultures containing equal number of cells received drug directly from the stock suspension in PEG, followed by gentle agitation of the cultures to ensure even distribution of the material added. Untreated and drug-treated cultures were grown in T25 Falcon flasks (Becton-Dickinson, Oxnard, CA) at 37°C in a humidified CO2 incubator. After the desired periods of treatment, the cells were harvested by trypsinization and counted or studied by flow cytometry, whereas other flasks were stained for microscopy. In the present study, we used Swiss immunodeficient (nu/nu) mice of the NIH-1 high fertility strain, routinely bred and maintained under strict pathogen-free conditions in our laboratory as described (26, 27). The nude mice were 75- to 85-day-old males. No sex-related differences in growth rates have been observed for any of the tumors used.

Xenografts and Drug Treatment. Approximately 2 × 10^6 cells were inoculated in each nude mouse, s.c., as described (28, 29). The day of cell inoculation was designated as Day 0. In studies of inhibition of tumor growth, drug treatment of animals started on Day 1, i.e., 24 h after cell inoculation. In studies of tumor regression, drug treatment started when the tumors reached the sizes indicated in the text or figure legend. The animals were given injections of 9NC as described (20). Briefly, the drug was administered as a fine suspension in cottonseed oil, i.m., twice/week, at doses of 4 mg/kg of body weight. Studies in our laboratory have first established the fact that camptothecins are much more effective as anticancer agents when administered as cells. A suspension of chicken erythrocyte nuclei was provided by Coulter and appropriate solvents decreases their antitumor activity when compared with the weight. Studies in our laboratory have first established the fact that camptothecins are much more effective as anticancer agents when administered as cells. A suspension of chicken erythrocyte nuclei was provided by Coulter and appropriate solvents decreases their antitumor activity when compared with the weight. Studies in our laboratory have first established the fact that camptothecins are much more effective as anticancer agents when administered as cells. 

Cell cycle fitting and computation of percentage of cell fractions at specific stages were based upon the polynomial S phase algorithm developed by Dean and Jett (31), with an interactive, nonlinear least squares fit performed by the method of Marquardt (32).

Results

Inhibition of Tumor Growth Followed by Tumor Regression. Repeated experimentation with the breast cell lines listed in “Cells and Nude Mice” showed that only three lines, i.e., MDA-MB-231, MDA-MB-436, and CLO, were capable of inducing tumors in all inoculated nude mice. The tumors grew at similar rates; i.e., there was no correlation between rates of cell culture and tumor growth. Fig. 1A shows the growth rate of breast carcinoma tumors in nude mice as a function of time. Fig. 1B shows parallel measurements of mouse weight during tumor growth. It is apparent that increases in body weight accompany increases in tumor size. Fig. 1, A and B, shows the results of a study with CLO cells, but similar results were observed with MDA-MB-231 and MDA-MB-43. No tumors developed in nude mice treated with 9NC after the mice were inoculated with MDA-MB-231, MDA-MB-436, and CLO cells. In this study, 9NC treatments started 1 day after cell inoculation and continued twice/week for a total period of 90 days.

In other studies, nude mice with established tumors received treatments with 9NC biweekly and tumor size and body weight were monitored. The tumors regressed completely in all tumor-bearing nude mice (Fig. 1C) with no toxicity observed (Fig. 1D). 9NC treatment was continued after tumor regression for a total period of 90 days. No tumor regrowth or toxic effects (i.e., loss of body weight) were observed once the tumors had regressed.

Growth Inhibition of Cells in Vitro. All cell lines were tested for growth inhibition induced by 9NC in vitro. Control cell cultures did not receive treatment. Only cells detached by trypsinization were counted in this study. Growth inhibition of the nontumorigenic breast cells MDA-MB-134 by 9NC in vitro is shown in Fig. 2 for three different drug concentrations. To demonstrate the effect of 9NC on the rate of proliferation of the cells, the cell number of untreated cultures (Fig. 2A) was plotted at a scale 10% of the scale used to plot the cell number of treated cultures (Fig. 2, B-D). A concentration of 9NC as low as 2.5 nm in the culture partially inhibited cell growth (Fig. 2B), whereas 9NC concentrations of 7.5 and 25 nm not only inhibited cell growth, but also resulted in the detachment of cells from the substrate (Fig. 2, C and D). Cultures treated with 25 nm 9NC contained 0.025% PEG. This PEG concentration had no apparent effect on the rate of cell proliferation. We also examined the effect of 9NC on proliferation of the tumorigenic cells MDA-MB-231. Treatment of these cells with 2.5 nm 9NC completely inhibited their proliferation after 3 days, with a longer period of treatment resulting in cell detachment. Higher 9NC concentrations of 7.5 and 25 nm resulted in more extensive detachment of the nonproliferating MDA-MB-231 cells (Fig. 2, C, D, G, and H).

Fig. 1. Treatment of nude mice bearing human breast carcinomas with 9NC. Ten nude mice were xenografted with CLO breast cells. Five xenografted mice received no further treatment and served as a control for tumor growth (A) and body weight (B). The remaining five mice were allowed to develop tumors; then treatment with 9NC started, and regression of tumor size (C) and body weight (D) were monitored. Tumor size was measured in mm^2 x 10^3, and body weight was measured in g. The graphs indicate the mean values of tumor size and body weight and include the highest and lowest values measured (vertical bars).

9 H. R. Hinz, P. Pantazis, and B. C. Giovanella, unpublished data.
REGRESSION OF BREAST CARCINOMAS BY CAMPTOTHECINS

Fig. 2. Growth of human breast carcinoma cells treated with 9NC in vitro. Cultures were seeded with MDA-MB-134 and MDA-MB-231 breast carcinoma cells at $1 \times 10^5$ cells/flask. The culture medium was replaced with fresh medium 24 h after cell seeding, and 9NC was added at the same time to final concentrations of 2.5 nM (B and F), 7.5 nM (C and G), and 25 nM (D and H). Control cell cultures received PEG 400 without 9NC (A and E). Cell counting started 24 h (Day 1) after addition of the drug to the cultures and continued every 48 h. Trypsin-removed cells were counted with a hemocytometer. Cells of 3 flasks were counted for each period of treatment and the average cell number was plotted as a function of time. Vertical bars, extreme values measured. A-D, nontumorigenic MDA-MB-134 cells; E-H, tumorigenic MDA-MB-231 cells.

indicating that MDA-MB-231 cells are more sensitive than MDA-MB-134 cells to increased concentrations of 9NC.

Microscopy of 9NC-treated Cells. The effect of 9NC on the nontumorigenic MDA-MB-134 and tumorigenic MDA-MB-231 cells as a function of period of treatment was monitored by direct observation of stained cells. Unlike MDA-MB-231 cells, untreated MDA-MB-134 cells grow attached in clusters. Briefly, treatment of both cell lines with 9NC for 24 h resulted in enlargement of these cells and their nuclei, and this enlargement continued as 9NC treatment continued up to 72 h. Concomitantly, the nucleoli became larger and more heterochromatic. No further enlargement of the cells, nuclei, and nucleoli was observed in cultures treated with 9NC for 5 days, but vacuolization appeared in the cytoplasm of many cells after 6–7 days of treatment. Also, the longer the period of 9NC treatment, the less the uptake of methylene blue by the cells.

Changes in the Cell Cycle of Nontumorigenic and Tumorigenic Cells Treated with 9NC in Vitro. The present and earlier studies (25) have shown that only a small percentage of human breast carcinomas xenografted in nude mice produced tumors. To understand better the differences in tumorigenic ability of the breast carcinoma-derived cells, we studied changes of the cell cycle in cell cultures treated with 9NC for various periods of time. This study was pursued by flow cytometry to quantitate the relative DNA content in the cells. Histograms of this analysis are shown in Fig. 3. Percentages of cell fractions at specific stages of the cell cycle were calculated from the histograms of Fig. 3 and then plotted as a function of period of 9NC treatment (Fig. 4). The major cell fraction, of about 65% of the untreated MDA-MB-134 cells, is at G0G1 (Figs. 3A and 4). Smaller cell fractions of about 13 and 20% are at S and G2M, respectively. Furthermore, computer analysis indicated the presence of a small fraction of about 2% of the total untreated cell population that consisted of cells with DNA content less than 2n, i.e., less than the DNA content of cells at G0G1 (Fig. 4). The region corresponding to the cells with reduced DNA content cannot readily be observed in the histogram (Fig. 3A). However, it has been reported that this region of the histogram consists of apoptotic cells, or cells that have entered the process of programmed death (33–35). This histogram region has been
Changes in Cell Cycle Induced by 9NC Are Reversible in Non-tumorigenic and Irreversible in Tumorigenic Cells. We also used flow cytometry to investigate whether the effect of 9NC on distribution of MDA-MB-134 and MDA-MB-231 cell cycle is reversible. The results of one representative experiment are shown in Fig. 5 and Table 1. In this experiment, the cells were treated with 9NC for 120 h; then the medium was replaced with 9NC-free medium, and the cells remained in this medium for 120 h. In agreement with the observations reported above, the histograms of DNA content show that the majority of untreated MDA-MB-134 (Fig. 5A) and MDA-MB-231 (Fig. 5D) cells are at G0G1, with smaller fractions at S and A0. Furthermore, cells treated with 9NC for 120 h exhibit a redistribution of their cell cycle as indicated by the increased fractions of cells at G2M and/or S and A0 in both MDA-MB-134 (Fig. 5B and C) and MDA-MB-231 (Fig. 5E) cells, again in agreement with the pattern observed in Fig. 3. However, removal of 9NC from the cultures for 120 h resulted in different patterns of distribution of cell cycle phases in the two cell lines. The predominant fraction in the MDA-MB-134 cell culture became that in G0G1, while a dramatic decrease in the G2M fraction and an increased S fraction were observed (Fig. 5C; Table 1). Also, there was an increase in the A0 fraction. Additionally, reverse G0G1 accumulation of MDA-MB-134 and other nontumorigenic cells, upon removal of 9NC, was followed by resumption of growth of the cells within 8–10 days. In fact, the resumed growth rate was similar to that of the untreated cells. In contrast, removal of 9NC from cultures of MDA-MB-231 cells did not result in changes similar to those observed in MDA-MB-134 cells, but the cells continued to accumulate at A0 (Fig. 5D; Table 1). Similar experiments with 9NC-treated cells followed by removal of 9NC from the cell medium showed that a 9NC treatment period up to 72 h efficiently results in continuous accumulation of MDA-MB-231 cells at A0.

TABLE 1

<table>
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<tr>
<th>Cells/treatment</th>
<th>% of cells in each phase*</th>
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<tr>
<td></td>
<td>G0G1</td>
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<tr>
<td>MDA-MB-134</td>
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<tr>
<td>Untreated</td>
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</tr>
<tr>
<td>+9NC</td>
<td>25.1</td>
</tr>
<tr>
<td>+9NC/-9NC</td>
<td>32.7</td>
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<tr>
<td>MDA-MB-231</td>
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<tr>
<td>Untreated</td>
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</tr>
<tr>
<td>+9NC</td>
<td>18.7</td>
</tr>
<tr>
<td>+9NC/-9NC</td>
<td>16.0</td>
</tr>
</tbody>
</table>

* Percentages were estimated by computer analysis of the DNA content of histograms shown in Fig. 5. The figures were rounded to the first decimal number.

The histograms of DNA content show that the majority of untreated MDA-MB-134 (Fig. 5A) and MDA-MB-231 (Fig. 5D) cells are at G0G1, with smaller fractions at S and A0. Furthermore, cells treated with 9NC for 120 h exhibit a redistribution of their cell cycle as indicated by the increased fractions of cells at G2M and/or S and A0 in both MDA-MB-134 (Fig. 5B and C) and MDA-MB-231 (Fig. 5E) cells, again in agreement with the pattern observed in Fig. 3. However, removal of 9NC from the cultures for 120 h resulted in different patterns of distribution of cell cycle phases in the two cell lines. The predominant fraction in the MDA-MB-134 cell culture became that in G0G1, while a dramatic decrease in the G2M fraction and an increased S fraction were observed (Fig. 5C; Table 1). Also, there was an increase in the A0 fraction. Additionally, reverse G0G1 accumulation of MDA-MB-134 and other nontumorigenic cells, upon removal of 9NC, was followed by resumption of growth of the cells within 8–10 days. In fact, the resumed growth rate was similar to that of the untreated cells. In contrast, removal of 9NC from cultures of MDA-MB-231 cells did not result in changes similar to those observed in MDA-MB-134 cells, but the cells continued to accumulate at A0 (Fig. 5D; Table 1). Similar experiments with 9NC-treated cells followed by removal of 9NC from the cell medium showed that a 9NC treatment period up to 72 h efficiently results in continuous accumulation of MDA-MB-231 cells at A0.

DISCUSSION

We have shown previously that CPT and its derivatives inhibit the growth of a wide variety of human tumors xenografted in nude mice (10, 20, 21). In this report, we have extended these studies to show that the CPT derivative 9NC not only inhibits growth but also induces regression of advanced human breast carcinoma tumors grown in nude mice. Remarkably, 9NC induced regression of breast carcinoma tumors in nude mice without toxic effects on these animals. We have shown recently that 9NC at doses used in this study has no toxic effects in normal nude mice (21). Taken together, these results indicate that 9NC exerts a selective toxic action on malignant cells.

To understand better the mechanism involved in the ability of 9NC to inhibit growth of breast carcinomas (20) and further induce regression of advanced tumors (this study) in nude mice, we monitored cellular responses to 9NC treatment in vitro. To reach meaningful conclusions, we used breast cell lines that have exhibited different abilities to induce tumors in nude mice. All these cell lines were derived from breast adenocarcinoma. However, they differ in their abilities to produce tumors after inoculation in nude mice (reviewed in Ref. 36). Initially, we observed that 9NC had different effects on the proliferation of the nontumorigenic and tumorigenic cells in vitro. 9NC concentrations that partially inhibited proliferation of nontumorigenic and tumorigenic cells in vitro, respectively.
origenic cells completely inhibited proliferation of the tumorigenic cells. Furthermore, continuous presence of 20 nM 9NC in cultures of the tumorigenic cells resulted in detachment of these cells from their substrate, whereas only 9NC concentrations of 20 nM or above resulted in detachment of nontumorigenic cells from their substrates. Our comparative studies were made on cell lines treated with 20 nM 9NC. This 9NC concentration is several-fold lower than the concentrations of CPT used by other investigators in studies in vitro (2-4, 6, 34, 35). It is of interest that 9NC-induced inhibition of the breast cells does not correlate with growth rate in vitro and is accompanied or followed by dramatic increases in the size of these cells, their nuclei, and nucleoli. These increases were similar in both nontumorigenic and tumorigenic cells. Similar increases have been observed in 9NC- and 9AC-treated human melanoma cells but not the normal counterparts of melanomas, the melanocytes (21). Taken together, these findings suggest that size increase is a common response of breast carcinoma cells to 9NC, regardless of the ability of these carcinoma cells to induce tumors in nude mice. However, a striking difference was observed in the distribution of cell cycle phases in cultures of nontumorigenic and tumorigenic breast carcinoma cells treated with 9NC. This drug induced slow progression of the nontumorigenic cells in the S phase, and their subsequent arrest at G2M with only a small percentage of apoptotic (A0) cells generated after prolonged periods of 9NC treatment. In contrast, 9NC initially induced a rapid shift of the tumorigenic cells from G0 to S and G2M with a subsequent increase in the A0 fraction of 9NC-treated cells. As a result, all tumorigenic cells were induced to die following treatment with 9NC, and induction of cell death continued even after removal of 9NC from the environment. With regard to these findings, recent reports have shown that CPT specifically arrests human lymphocytic and myelogenous leukemia cells at G2M and S phase, respectively (3, 4, 6). However, we cannot directly compare our result with the results of those studies because of the different experimental conditions. Our 9NC was prepared and purified in our laboratory. We would like to caution other investigators that commercially available CPT preparations contain impurities which may be toxic in vivo and in vitro.5

Flow cytometry studies have shown that the A0 fraction consists of cells with DNA content less than 2n, or apoptotic cells (36-38). The cardinal features described for programmed cell death, or apoptosis, are reviewed in Ref. 39. Initiation of apoptosis has been associated with a rise in cytosolic calcium (40), induction of mRNAs coding for c-fos, c-myc, and certain heat-shock protein (41), and degradation of internucleosomal DNA, perhaps by a calcium-magnesium-dependent specific endonuclease (39). We have shown recently that low concentrations of CPT derivatives, i.e., 1.25 to 12.5 nM, temporarily induce expression of the early response genes, c-jun, jun-B, and c-fos, in human U937 leukemia cells (5). Induction of expression of these genes is regulated partly at the transcriptional level and correlates with internucleosomal DNA fragmentation (5), a characteristic of programmed cell death, or apoptosis (39). However, it is still unknown how these mRNAs correlate with apoptosis.

Although topoisomerase I has been implicated in the cytotoxic action of CPT and its derivatives on malignant cells (see "Introduction"), it remains unclear how these agents distinguish between human tumorigenic and nontumorigenic cells (Ref. 39 and this study). Recently, it has been demonstrated that induction of high levels of MYC protein correlates with apoptosis in normal fibroblasts, whereas low levels or absence of MYC protein prevent programmed cell death (42). Therefore, it is possible that 9NC induces synthesis of high levels of MYC protein in tumorigenic cells, whereas 9NC-treated nontumorigenic cells synthesize low levels of, or no, MYC protein. Also recently, it has been shown that wild type p53 protein, which regulates the cell cycle, protects cellular DNA from damage, whereas absence of wild type p53 or presence of mutated p53 allows proliferation of cells with damaged DNA (43). However, it is not known whether p53 is directly or indirectly involved in the G1-S checkpoint of the cell cycle that is suggested to be responsible for the genomic instability of cancer cells (reviewed in Ref. 44). Therefore, it is possible that wild type p53 protein protects nonmalignant cells from the cytotoxic action of 9NC, while the absence of wild type p53 or presence of mutated p53 in tumorigenic cells confers sensitivity to 9NC-induced toxicity. Current studies in our laboratory are directed toward investigating whether levels of MYC protein and presence of wild type or mutated p53 protein(s) correlate with ability of 9NC to elicit programmed cell death in tumorigenic, but not nontumorigenic, human breast epithelial cells.

One major feature of the response of the tumorigenic cells to 9NC is the biphasic mode of progression of these cells through the phases of the cell cycle, until they ultimately accumulate at A0 (Fig. 4). One hypothesis for this bimodality is that the population of tumorigenic cells consists of two subpopulations of cells that differ in their sensitivity to 9NC. We have no experimental findings supporting this hypothesis, but flow cytometry studies described in a recent report have shown that treatment with the antiestrogen tamoxifen reveals the existence of subpopulations of breast carcinoma cells with differential response to the hormone (45).

Finally, another interesting finding was that 9NC removal allows nontumorigenic cells to reenter the cell cycle after a lag period and resume sustained proliferation. In contrast, tumorigenic cells do not resume proliferation but continue to die after removal of 9NC. Therefore, 9NC need not be continuously present to result in death of the tumorigenic breast cells. These observations may be important for the use of CPT and its derivatives in the treatment of breast cancer patients.

ACKNOWLEDGMENTS

We thank A. DeJesus for technical assistance, P. Nelson for photography, and K. Lee for typing the manuscript.

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