Circadian Gating of S Phase in Human Ovarian Cancer

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ABSTRACT

Cell proliferation in 30 patients with ovarian cancer was analyzed using flow cytometry to determine changes in the percentage of cells in S phase. By this measure, proliferation in tumor cells appears to follow a cyclical pattern of peaks and troughs that is out of phase with the circadian rhythm in proliferation of normal tissues. In round-the-clock monitoring of replication stages in tumor cells recovered from I.P. lavage fluid in postsurgery patients, peaks of tumor and nontumor cell DNA synthesis commonly occurred at different times of day. When patients were grouped so that only tumor cell proliferation was being measured, a highly significant 24-h rhythm nearly 12 h out of phase with nontumor cell proliferation was found. This peak in the percentage of S-phase cells occurs most commonly in mid- to late morning and appears to offer an opportunity for timing chemotherapy to coincide with high tumor cell vulnerability and low toxicity to normal tissue.

INTRODUCTION

A sought-after goal in cancer chemotherapy is to exploit the cell cycle-specific nature of many antineoplastics and their generally greater effect on proliferating tissues in order to spare normal tissues while preferentially killing tumor cells. However, attempts to accomplish this by choice of drugs or drug combinations (1) or by manipulation of proliferative activity of the normal tissues or tumors have had little or no success (2-4). This approach has failed in solid tumors when protocols have been based on the notion that cells proliferate at random through any 24-h period.

One intriguing possibility is that of exploiting the natural synchrony of DNA synthesis and cell division that occurs in normal animal tissue as a consequence of the daily gating of proliferation by the circadian clock (5, 6). Clinical efforts in this field were crystallized by the studies of Hrushesky (7), who reported in mice that their large tumor burdens were greater during the circadian stage at which drugs were administered. Chemotherapy given near the optimum circadian stage resulted in fewer dose reductions, fewer missed treatments, fewer infections, and fewer bleeding episodes. Virtually all patients treated chronotherapeutically responded; 81% had complete disappearance of their cancers, and it was found that 66% of all patients so treated remained disease free with a median 60-mo follow-up. The degree to which this phenomenon was dependent on circadian cell kinetics is unknown.

Because of the obvious difficulties in making cell kinetic measurements in human tumors or systemic cells in vivo, it has not been possible to apply our understanding of cell kinetics and chronobiology to anticancer treatment in humans. In the past, cell-kineti analyses to optimize treatment scheduling or drug combinations have been done using transplantable rodent tumors and, in some instances, have been confounded by ignoring the circadian or ultradian rhythmicity that is imposed on the cell kinetic behavior in vivo. Transplantable tumors, passed through hundreds of generations of mice, appear to lose their ability to be rhythmically entrained by their host and often grow entirely randomly or with unpredictable high frequency rhythms in DNA synthesis. In contrast, as we will show here, human ovarian tumors are highly rhythmic and still tightly coupled to the human circadian clock. Knowledge of the timing of these rhythms can therefore provide a cell kinetic basis for the selection of timing of drug administration to improve cancer treatment.

MATERIALS AND METHODS

Clinical Study Parameters. In this study, patient selection and eligibility included only those patients with ovarian adenocarcinoma who underwent surgical implantation of a catheter for I.P. chemotherapy. With informed consent, patients were entered onto the protocol (Institutional Review Board No. 4250) if they were, in the judgment of their attending physician, appropriate candidates for surgical insertion of a catheter at the time of their first or second surgery.

The following data were collected on each patient entering the study: age; sex; performance status; tumor type; histological description; previous or current radiation therapy; previous or current chemotherapy dose(s), schedule, and date(s); all current medications other than chemotherapy agents; monitoring of body temperature; usual time of arising in a.m.; usual bed time in p.m.; meal times; variations in sleep-wake cycle in hospital; date of hospital admission; and date and time of fluid samplings.

Peritoneal washings were collected immediately on ice and transported to the laboratory for analysis of DNA ploidy, relative concentration of tumor-specific antigens, and S-phase fraction. Tumor cells from washings were compared to both fresh and paraffin-embedded tumor and correlated with clinically important characteristics, such as surgical debulking, amount of residual disease, recent chemotherapy, and response to chemotherapy.

Flow Cytometry. Propidium iodine was obtained from Polysciences (Warrington, PA); aHMFG-2, from Unipath (London, England); fluoresceinated goat anti-mouse IgG, from Polysciences; and RNase, from Cooper Biomedical (Philadelphia, PA). Flow cytometric analysis was carried out on a Becton-Dickinson FACS IV with a 2-watt argon laser. The laser was tuned to 488 nm and usually operated at 400 milliwatts of power. All filters were from Diffract Optics (Hudston, MA). Propidium iodine (PI)-stained samples were measured through a 620LP and two 590D emission filters with the PMT set at 440-V plate voltage. Fluoresceinated samples were measured through a 514.5BW and a 560SP emission filter with the PMT set at 550-V. Dual emission samples were measured by splitting the emission with a 50% transmission mirror. Narrow angle scatter of the 485-nm beam was collected through a 1.0-OD. neutral density filter. Data were transmitted to a Hewlett-Packard 9845B computer for analysis.

Peritoneal Washings and Solid Tumors. Irrigations were performed with a balanced electrolyte solution. Cells in ascitic fluid or saline were obtained from patients who were undergoing surgical debulking and implantation of a catheter for I.P. chemotherapy. Irrigations containing tumor cells and tumor fragments in addition to reactive mesothelial

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2 L. E. Scheving, personal communication.

3 The abbreviation used is: aHMFG-2, anti-human milk fat globule protein.
cells and a small percentage of white cells (10) were collected by centrifugation. In bloody tissue samples, 3 to 5 x 10^6 cells were first treated with 0.83% ammonium chloride in 0.01 M KHCO_3, 10^-4 M EDTA (pH 7.3) to lyse RBC. In preparation for flow analysis, minced tumor fragments fixed in high-citrate buffer (10)-0.5 M citrate buffer (pH 2.0) for a minimum of 1 h were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at a setting of 4 for 10 s. Cells were centrifuged and resuspended in Pucks G+ for 10 min, centrifuged again, and washed in Pucks G. Cells were resuspended in isotonie buffered saline at pH 7.2 containing 400 IU of RNAs, incubated for 30 min at 37°C, and centrifuged. Thirty min prior to analysis the cell pellet was resuspended and stained in isotonie buffered saline containing 50 μg/ml of propidium iodide and 0.01% Tween-20. Samples were routinely analyzed within 5 h of removal from high-citrate buffer.

DNA histograms were analyzed for the percentage of S + G2 cells as described previously (10-12). S-Phase Analysis of DNA Histograms. In those patients where the tumor cell subpopulation was completely separated from the normal cells in the DNA histogram (e.g., pentaploid tumors), one-dimensional DNA histograms were resolved using a least squares-best fit of DNA distributions based on the technique of Dean and Jett (11). S phase in this technique is approximated by a broadened polynomial, and the G1 and G2 phases are approximated by normal distribution functions. A Marquardt-type algorithm is used to facilitate convergence.

For the majority of patients, however, the tumor and normal cell populations overlapped, making direct application of the above method impractical. For these samples we made the simplifying assumption that the S-phase region of a particular cell population is a rectangle of constant height (12). With this assumption, an approximation to the proportion of cells in S or S + G2 in each subpopulation could be obtained by analytically separating the populations using an interactive automatic computer graphics method. Best estimates of the position of the G1 and G2 peaks for each population are marked by the user by moving a cursor on the graphics screen, and preliminary best-fitting curves to the data are calculated under these constraints. The data histograms and the current computed best fit are displayed on the computer graphics screen, and the user adjusts the fit by manually shifting the position of the G1 and G2 peaks. A graphical display indicates the current sum of squared deviations, and the positions of the peaks are adjusted by the user until a minimum sum of squares is obtained.

The above methods applied to a series of samples from a single patient give the variation through time of the proportion of tumor and/or normal cells with S or G2 DNA content. This is not, however, an unequivocal representation of variation in cell proliferation, since some noncycling cells may be present in the S or G2 fractions (13, 14). Identification of such cells can only be done by labeling methods, which are difficult to do in a clinical setting. Nevertheless, the variations we have observed must be related in some way to cell proliferation, so we have assumed here that the rise and fall in the fraction of cells with S or G2 DNA content parallels the rise and fall in DNA synthesis activity in the population.

Discriminating Tumor and Nontumor Diploid Cells by Ploidy. Fresh tumor cells and tissues were taken intraoperatively and compared with fixed paraffin-embedded biopsy materials using flow cytometry as described previously (15). DNA content and tumor-specific immunofluorescence were assayed in the cells derived from postsurgical i.p. washings. Tumor-specific staining of peritoneal cells. The proportion of blood cells in washings was estimated by examination in the clinical pathology laboratory and by flow cytometry using B- and T-lymphocyte and monocyte/macrophage-specific fluorescent antibodies. In addition, differential counts and cytologies were performed on the saline irrigations 2 to 4 times each day. Cells were tested for Leu-M3 and for B-1 (pan-B-cell marker). A monoclonal antibody with specificities to all members of the normal white cell population (antileukocyte; Becton Dickinson) was routinely used to assay for normal white cells. In this system the antileukocyte antibody stains >90% of peripheral blood leucocytes.

To distinguish between tumor cells and nontumor mesothelial cells present in the abdomen, a fluorescent assay for tumor antigen found in adenocarcinomas was used. αHMFG-2 is reported to be specifically expressed in adenocarcinomas and to discriminate between these cells and mesothelium (16, 17). Cells were stained with a fluorescent antibody against αHMFG-2 (Unipath, London, England), and the proportion of tumor cells in the sample was estimated by counting labeled cells using a fluorescence microscope.

RESULTS

A protocol involving frequent irrigation of the peritoneal cavity by means of an indwelling catheter has permitted us to perform cell kinetic analyses on human tumor cells with a degree of accuracy not previously possible.

In the past 2 yr, 45 patients have been analyzed for DNA index, αHMFG-2 (human milk fat globule) tumor antigen, mean S + G2 phase, and proliferation dynamics; i.e., period of S + G2 waves, amplitude of S + G2 waves, and phase or time...
of day when peak proliferation occurs through one to five 24-h periods. Of these, 30 yielded sufficient sample densities to be included in this analysis. In earlier work, we measured DNA content, tumor-specific immunofluorescence, and white cell-specific immunofluorescence in both flow cytometry and microscopy to identify and quantitate tumor cells in i.p. washes (10, 21). Although some variation was noted in the proportion of tumor cells and reactive mesothelium present in the wash fluid, the composition following surgery tended to be characteristic for each patient. That is, the proportion of tumor cells in all stages of replication remained relatively constant throughout the sampling interval even though the S + G2 fraction varied through 24 h and as a function of the time since surgery. The overall proportion of tumor to normal cells was altered only after chemotherapy (10).

A complete analysis of proliferation following surgery and chemotherapy in one patient is shown in Fig. 1. Fresh tumor samples and paraffin-embedded surgical samples from this patient established that the tumor had a DNA index of 2.42 and was actively proliferating. The tumor represented 15% of cells in i.p. washings (Fig. 1). Approximately 15% of the cells were also aHMFG-2 positive. Round-the-clock analyses were performed for 3 days following surgery and again for 4 days prior to and 1 day following the second course of chemotherapy. Rhythmic changes in S + G2 fraction of both diploid non-tumor and aneuploid tumor cells were followed. The patient’s diploid cells show a rhythmic 24-h rhythm, while the best fit to the tumor cell rhythm is 12 to 13 h. In Fig. 1, one-dimensional DNA histograms of cells collected for 4 days prior to and 1 day following chemotherapy are shown. The diploid (2C/G1, 4C/ G2) nontumor cells and pentaploid (5C/G1, 10C/G2) can be seen most clearly in the 1745-h sample in Fig. 1b. In the hours during and immediately following the administration of Cytoxan (375 mg/m²) and cisplatin (67.5 mg/m²; Fig. 1a, 1740 h Day 4 through 800 h Day 5), cells become very sticky. In the 6 h following treatment (800 to 1400 h Day 5; Fig. 1a), cell numbers return to normal proportions with little change in cell kinetic parameters. However, when the patient returned 3 wk later to begin the third course of chemotherapy, no tumor cells were detectable by flow cytometry (2100 and 930 h, Fig. 1b).

In all but 2 of the 30 round-the-clock analyses assembled in this study, significant (P < 0.05) rhythmic changes in the fraction of cells in S + G2 were detected. Most individuals showed 8- to 12-h or 24-h rhythms in proliferation, and the S + G2 fraction varied from less than 1% to more than 35% (Fig. 2). In an earlier study with a smaller patient population (12 analyses), the population as a whole showed a significant 12-h rhythm in the number of cells in S + G2, when data were pooled and subjected to cosinor and Maximum Entropy Spectral Analysis (10, 21). Maximum proliferation occurred in the evening and again in the mid-morning hours. With these data alone it was not possible to determine whether a fraction of the population of cells was undergoing DNA synthesis twice in each 24 h or whether two distinct populations (or fractions of the same population) were being gated into S phase once in each 24 h but 12 h out of phase. However, with the accumulation of additional analyses, three distinct subgroups of cells found in i.p. washes of patients could be identified based on the DNA index, cytology, and the presence or absence of a tumor-specific antigen.

Subgroups were defined as follows: (a) patients with tumors (as determined from biopsy or surgical specimen) in which no tumor cells were detectable in the i.p. washes and which consequently could be analyzed for proliferation of the nontumor
diploid mesothelial cells; (b) those with diploid tumors that could not be separated from the proliferating nontumor cells by DNA content alone but only by using a second criterion, either scatter (size) or the presence of a tumor antigen; and (c) those with aneuploid tumor populations detectable in the washings. Patients were assigned to Group 1 (nontumor diploid cells) if the cytology and tumor cell-specific immunofluorescence were negative. The presumption then was that any cell proliferation seen in this diploid population could be attributed to somatic cells, presumably mesothelium.

When these three populations are analyzed separately for proliferation rhythms, Group 1, diploid nontumor, displays a significant 24-h rhythm with a peak in proliferation between 9 and 10 p.m. (Fig. 2, a and b; Table 1) at the time of day previously associated with epithelial cell proliferation in humans (9). Group 3, those with detectable aneuploid tumor in the washings, also shows a significant 24-h rhythm, but with a peak in S + G2 fraction between 11 and 12 a.m. (Fig. 2, e and f). In Group 2, the diploid tumor population, which may also contain any proliferating nontumor cells, shows a rhythm with a 24-h component that peaks at 1445 h, a time that is not significantly different from the aneuploid tumor peak (Fig. 2, c and d). It appears that tumor cells are still tightly coupled to the host circadian clock, but they proliferate about 12 h out-of-phase with the normal cells. It should be noted, however, that in a few individuals the tumor cells showed two peaks in proliferation, usually with peaks in S + G2 at both noon and midnight.

In those individuals with aneuploid or diploid tumors in whom no detectable tumor was found in the i.p. washes (Group 1), survival in a median 18 mo follow-up was considerably better than it was in patients with aneuploid tumor cells with
detectable tumor cells in the i.p. washes (Group 3, Table 1). This finding suggests that the ability of the tumor to release proliferating cells into the peritoneum may correlate with poor prognosis for the patient independently of aneuploidy alone, which is also a negative factor in survival (15).

DISCUSSION

Our data show two high-amplitude sleep-wake synchronized peaks in DNA synthesis in cells from peritoneal washes from ovarian cancer patients. One, associated with diploid non tumor cells in the abdominal washes, is maximal in the evening hours (10 to 11 p.m.) and may be coincident with or identical to the peak in normal cell proliferation (Fig. 3). We emphasize at this point that we do not know if there will be a fixed-phase relationship between these presumed mesothelial cells and the proliferation of the normal non tumor epithelium or of any dose-limiting tissue, but we do note that, in the one careful analysis of human skin epithelial proliferation performed by Scheving (9), peak mitotic index occurred at 3 a.m., 5 to 6 h after the peak in S + G2 reported here. In aneuploid cells in the abdomen, peak DNA synthesis occurs most commonly in the late morning hours and is maximally out-of-phase with reported human systemic cell proliferation. Therefore, it may offer a clear window of time in which to treat the tumor specifically at very high doses with cell cycle-specific antineoplastic drugs.

It has been recognized that relative dosage intensity expressed as mg of drug/m2/day may be the single most important determinant of treatment outcome, and that the common practice of dose deescalation and deferring treatment because of mild to moderate drug toxicities may be dramatically decreasing survival statistics in patients with advanced cancer (1). Attempts to increase relative chemotherapeutic dose intensity based on cell kinetic manipulation confront the difficult problem of anticipating and correcting for perturbations of the cell cycle caused by the drugs themselves. Circadian variation in cell proliferation, on the other hand, being coupled to a broad spectrum of circadian rhythmicity in the body, may remain relatively stable even as treatment continues. If this proves to be correct, selection of the timing of drug treatment based on circadian data should be more effective, and certainly more practically applicable, than other cell kinetic methods for improving drug effectiveness.

REFERENCES

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