The Effect of Heparin on the Cytotoxicity and Uptake of Anti-neoplastic Drugs in Cultured Burkitt Lymphoma Cells

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SUMMARY

Preliminary clinical studies have been reported suggesting an increased response rate to a five-drug combination program in lung cancer patients who were anticoagulated with heparin. This study was undertaken to determine the effect of heparin on the cytotoxicity and cellular drug uptake of four antineoplastic agents. Burkitt lymphoma cells (P3J) in continuous culture were incubated with heparin in a concentration of 0.5 unit/ml for 0 or 72 hr prior to the addition of radioisotopically labeled nitrogen mustard, methotrexate, 6-mercaptopurine, or 5-fluorouracil. Cytotoxicity and cellular drug uptake were then determined.

Heparin alone had no effect on cell growth, viability, or DNA synthesis, as measured by thymidine uptake, during the log phase of culture growth over 72 hr. The cytotoxic effect of the four antitumor drugs also was not altered by incubating the cells with heparin for 0 or 72 hr. The uptake of the radioactively labeled agents in heparin-treated cultures was not significantly different from that of control cultures.

INTRODUCTION

Increased tumor response to antineoplastic drugs in patients with lung carcinoma who were anticoagulated with heparin prior to the administration of combination chemotherapy has been described by Elias et al. (8-10). In 2 instances, the patients had displayed resistance to the drug combination prior to heparinization. Other isolated cases of human tumor response to heparin alone or as adjuvant therapy (1, 11, 16) have been reported. Apparent tumor regression resulting from anticoagulant therapy has also been reported in in vivo animal studies (3, 17). Human and animal tumors have been shown to be rich in fibrin and fibrinogen (14). Fibrin is deposited in and around the tumor but particularly at the growing edges (26). This fibrin network appears to be necessary for tumor growth (18). Elias (9) has proposed that heparin may act by preventing further fibrin deposition, allowing the physiological fibrinolytic system to remove the existing peritumor fibrin deposits. This could lead to increased tumor penetration of drugs or increased sensitivity to immune mechanisms.

Both augmentation and inhibition of cellular transport of methotrexate by other antineoplastic drugs and antibiotics have been reported (31). Enhanced antitumor response to alkylating agents complexed to heparin has also been noted with in vivo animal studies (7, 22). Since heparin can affect the surface properties of tumor cells (23, 24) and interfere with a number of enzyme systems (6), this study was undertaken to determine whether the reported enhancement of drug response could be due to a direct effect of heparin on the cellular uptake and cytotoxicity of various chemotherapeutic agents. These studies were performed with Burkitt lymphoma cells grown in cell culture. Cultured Burkitt cells are useful as a model system. Since they are derived from a human neoplastic cell line, they grow to high density in culture media (2 X 10^6 cells/ml), and they are responsive to a number of chemotherapeutic agents. Genetic and cytological studies have verified that Burkitt cell lines grown in culture are derived from the malignant cells of the original neoplasm (12).

MATERIALS AND METHODS

Burkitt lymphoma cells (P3J) obtained from Dr. Charles Shipman, Department of Oral Biology, University of Michigan, were maintained in continuous culture at 37°C in 50% basal Eagle's medium plus 50% McCoy's 5A medium containing 10% fetal calf serum; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. Subcultures of approximately 1 X 10^6 cells/ml (initial concentration) were established for all the experiments except those otherwise indicated. The cells were then allowed to proliferate without refeeding during a 48-hr sampling period. At this initial concentration, control cultures begin to plateau after 10 to 12 hr, but cell number continues to increase up to 48 hr and viability remains above 95%. Cell growth was markedly attenuated in all drug-treated cultures.

Heparin was added to one-half of the cultures, and individual antineoplastic drugs were added to all cultures. Duplicate cultures were assayed for each drug treatment. Radioactively labeled drugs were utilized for the uptake studies. Control cultures without heparin or antineoplastic agents were run simultaneously. For determination of cellular...
drug uptake, 1- or 2-ml samples were withdrawn at fixed time intervals over a 4-hr period and immediately added to 3 volumes of ice-cold 0.9% NaCl solution to halt further drug uptake. The samples were centrifuged and washed a 2nd time with 2 volumes of iced, 0.9% NaCl solution and then were filtered on Metricel filters (0.45-μm pore size). The filters were dried under infrared lighting, placed in counting vials with butyl-2-(4'-r-butylyphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole-toluene scintillation fluid, and counted on a Packard Tri-Carb scintillation counter.

Heparin solutions were prepared within 24 hr of use, and the anticoagulant activity (in units/ml) was assayed by addition of stock solution to normal human plasma and determination of the thrombin clotting time (27). Heparin activity in the cell cultures was assayed by the same method. Antineoplastic drug solutions were mixed just prior to use. Antineoplastic drug concentrations equivalent to the total-body-water concentration in man after equilibration of a single i.v. injection of a clinical dose were used for the cytotoxicity studies. This was calculated by dividing a standard mg/kg dose for each agent: HN2, 0.2 mg/kg; MTX, 0.5 mg/kg; 5-FU, 15 mg/kg; and 6-MP, 2.5 mg/kg; by 700 ml (70% total body water/kg). Additional higher and lower concentrations of HN2 and MTX were also utilized. For MTX and 5-FU, radioactively tagged and unlabeled drugs were mixed to achieve an appropriate concentration for drug-uptake studies. Approximately 64 times the calculated concentration of HN2 and 6 times the calculated concentration of 6-MP were used for the uptake studies because of the low specific activity of the labeled drugs. The concentration of heparin (0.5 unit/ml) used was equivalent to a blood level that would be clinically attainable during anticoagulation in humans (27).

Thymidine incorporation was measured after the cultures were incubated with thymidine-3H, 0.5 μCi/ml, for 4 hr. The reaction was terminated by freezing the cultures at −60°. Cultures were then thawed, and the DNA was precipitated with 5% perchloric acid. Thymidine-3H content was determined by scintillation counting, and the DNA content was assayed by the diphenylamine method of Burton (4).

Cell counts were done by hemocytometer, and cell viabilities were determined by erythrocin B dye exclusion at the time of cell counting.

Drugs used in this study were heparin (Connaught Medical Research Laboratories, Toronto, Canada) in powder form which was dissolved in 0.9% NaCl solution prior to use; 14C-labeled HN2 (specific activity, 3.1 mCi/mmmole, Mallinckrodt Chemical Works, St. Louis, Mo.) dissolved in 0.9% NaCl solution; 5-FU-2-14C (58 mCi/mmmole; Mallinckrodt) in 50% alcohol diluted in 0.9% NaCl solution; thymidine-CH3-3H (20 Ci/mmmole, New England Nuclear, Boston, Mass.) diluted in culture medium; MTX-3H (13.7 Ci/mmmole, Amer sham/Searle, Arlington Heights, Ill.) dissolved in 0.1 N NaOH; and 6-MP-8-14C (2.55 mCi/mmmole; Mallinckrodt) dissolved in 0.1 N NaOH.

Significance was determined by Student’s paired t test where appropriate.

RESULTS AND DISCUSSION

Heparin Effect on Burkitt Cells. To ascertain the effects of heparin alone, paired cultures were incubated with heparin and compared with paired control cultures. The cultures were initiated with 1.2 X 10⁶ cells/ml so that the cells would be in the log phase of growth throughout a 72-hr period of observation. Heparin in a concentration of 0.5 unit/ml had no effect on cell growth (Chart 1). The cell counts were not significantly different at any time point (p > 0.05). Cell viability was in excess of 95% in both control and heparinized cultures throughout the sampling period. A heparin concentration of 100 units/ml also had no effect on cell growth or viability. In a separate experiment, DNA synthesis (measured by a 4-hr thymidine-3H pulse) was determined for control and heparin-treated (0.5 unit/ml) cultures after 48 hr of growth. The amount of thymidine incorporated was not significantly different (p > 0.05) for control and heparin-treated samples. A heparin concentration of 100 units/ml also had no effect on thymidine incorporation.

The effect of heparin on tumor cells in vivo and in vitro has been studied by numerous investigators and the results have been reviewed by Miller and Ketcham (20) and by Regelson (28). Depending upon the cell culture system, animal model, or heparin concentration used, the direct effects of heparin have varied from no effect to alterations in cell function including mitotic inhibition, altered DNA synthesis, and changes in cell-surface properties. In addition, Norrby (25) has reported a variable or absent heparin effect on duration of lag phase, outgrowth from small inocula, survival time, mitotic time, and cell multiplication in cultured cells, depending upon the heparin concentration used and cell line studied. In the

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The abbreviations used are: HN2, mechlorethamine (NSC 762); MTX, methotrexate (NSC 740); 5-FU, 5-fluorouracil (NSC 19893); 6-MP, 6-mercaptopurine (NSC 755).

A concentration of alcohol equal to the final concentration in drug treated cultures, had no effect on Burkitt cell growth in control cultures.
Burkitt cell system used in the present study, heparin had no effect on cell growth, viability, or DNA synthesis.

Heparin activity was determined by thrombin clotting time in cell cultures over 144 hr and was compared with heparin activity in culture medium alone over the same time period. Activity did not change in the cell-free medium, but decreased 33% between 48 and 72 hr in the cell cultures. Thereafter, the activity remained stable. For experiments requiring 72 hr of incubation with heparin, additional heparinized medium was added at 48 hr of incubation to maintain the heparin activity at 0.5 unit/ml.

Montes de Oca and Dietrich (21) found that heparin was slowly incorporated into cells after a delay of about 24 hr and deposited in the cytoplasm as granules. Peak incorporation occurred by 72 hr. The drop in heparin activity observed in our studies is consistent with their observation, although the number of cells killed in this time period varied with agents and concentrations. The simultaneous addition of heparin, 0.5 unit/ml, did not alter the cell-killing effect of concentrations of HN2 from $5 \times 10^{-7}$ M to $10^{-4}$ M. Neither incubation of the Burkitt cells with this heparin concentration for 72 hr prior to HN2 addition nor increasing the heparin concentration to 100 units/ml had any effect. Similar results were obtained with MTX, 5-FU, and 6-MP.

Chart 2 illustrates the cellular uptake curves for HN2$^{14}$C ($10^{-4}$ M) in control cells and cells exposed to heparin, 0.5 unit/ml, added immediately prior to the addition of HN2. Mean HN2 uptake in the heparin-containing cultures was 10% lower than that of controls, when the difference in uptake was averaged over the time points sampled. However, the difference was not significant ($p > 0.05$) at any of the time points or with summation of the samples. Uptake of HN2$^{14}$C ($10^{-4}$ M) in the presence of heparin, 100 units/ml, that was added simultaneously also averaged 10% lower than control, and when the cells were incubated with heparin, 0.5 unit/ml, for 72 hr prior to the addition of HN2, the uptake averaged 15% lower than control. However, these differences were also not statistically different. Conceivably, cell surface alterations produced by heparin could decrease membrane binding and intracellular incorporation of antineoplastic drugs, but any decrease in uptake that may have occurred did not appear to be biologically important, since the cytotoxicity of HN2 in 3 concentrations was unaffected. Cellular uptakes of antineoplastic drugs in the presence or absence of heparin were

The effect of heparin on the cytotoxicity of various antineoplastic drugs. Table 1 summarizes the cytotoxic effect of the antineoplastic drug addition by the number of viable cells/ml just prior to antineoplastic drug addition.

### Table 1

<table>
<thead>
<tr>
<th>Anti-neoplastic drug given</th>
<th>Concentration (M)</th>
<th>No. of drug-treated cultures</th>
<th>Time of heparin addition</th>
<th>Viability ratio $^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HN2</td>
<td>$10^{-4}$</td>
<td>8</td>
<td>None</td>
<td>$2.08 \pm 0.17^{b}$</td>
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<tr>
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<td>$10^{-4}$</td>
<td>10</td>
<td>0 time</td>
<td>$0.53 \pm 0.08$</td>
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<tr>
<td>HN2</td>
<td>$10^{-4}$</td>
<td>4</td>
<td>-72 hr</td>
<td>$0.49 \pm 0.01$</td>
</tr>
<tr>
<td>HN2</td>
<td>$10^{-4}$</td>
<td>4</td>
<td>-72 hr</td>
<td>$0.51 \pm 0.08$</td>
</tr>
<tr>
<td>HN2</td>
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<td>2</td>
<td>0 time</td>
<td>$0.55 \pm 0.01$</td>
</tr>
<tr>
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<td>$10^{-4}$</td>
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<td>$1.01 \pm 0.04$</td>
</tr>
<tr>
<td>HN2</td>
<td>$5 \times 10^{-7}$</td>
<td>2</td>
<td>0 time</td>
<td>$0.94 \pm 0.07$</td>
</tr>
<tr>
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<td>None</td>
<td>$1.24 \pm 0.02$</td>
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<tr>
<td>MTX</td>
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<td>None</td>
<td>$0.78 \pm 0.06$</td>
</tr>
<tr>
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<td>0 time</td>
<td>$0.79 \pm 0.06$</td>
</tr>
<tr>
<td>MTX</td>
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<td>-72 hr</td>
<td>$0.73 \pm 0.07$</td>
</tr>
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<td>0 time</td>
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</tr>
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<td>MTX</td>
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<td>None</td>
<td>$1.15 \pm 0.04$</td>
</tr>
<tr>
<td>MTX</td>
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<td>2</td>
<td>0 time</td>
<td>$1.12 \pm 0.04$</td>
</tr>
<tr>
<td>5-FU</td>
<td>$1.6 \times 10^{-4}$</td>
<td>2</td>
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<td>$0.09 \pm 0.05$</td>
</tr>
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<td>5-FU</td>
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<td>2</td>
<td>0 time</td>
<td>$0.12 \pm 0.04$</td>
</tr>
<tr>
<td>6-MP</td>
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<td>2</td>
<td>None</td>
<td>$1.00 \pm 0.16$</td>
</tr>
<tr>
<td>6-MP</td>
<td>$2 \times 10^{-4}$</td>
<td>2</td>
<td>-72 hr</td>
<td>$1.00 \pm 0.08$</td>
</tr>
</tbody>
</table>

$^{a}$ In all cases of drug treatment, the differences in the viability ratio between heparin and non-heparin-treated cultures was not significantly different ($p > 0.05$).

$^{b}$ Mean ± S.D.

$^{c}$ One hundred units/ml added.

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**Chart 2.** The effect of heparin, 0.5 unit/ml, on the cellular uptake of HN2$^{14}$C ($10^{-4}$ M). Uptake is expressed as cpm since cell number was equal in all cultures. Each point represents the mean of paired cultures, 2 samples/culture, ± range; ●, HN2$^{14}$C ($10^{-4}$ M); ○, HN2$^{14}$C ($10^{-4}$ M) plus heparin, 0.5 unit/ml, added simultaneously.
determined from curves similar to those shown in Chart 2. These data are summarized in Table 2. Heparin did not significantly affect the cytotoxicity or cellular uptake of the 4 antineoplastic drugs studied when added simultaneously to the cultures or when the cells were exposed to heparin for 72 hr prior to the addition of the chemotherapeutic agent. These studies demonstrate that (a) heparin alone has no effect on the growth and viability of Burkitt lymphoma cells in culture and (b) sensitivity of these cells to cytotoxic drugs in culture is not increased by either simultaneous or prior exposure to heparin. Studies by numerous investigators (2, 20, 18, 30) have demonstrated that heparin can decrease or prevent metastases from i.v.-injected neoplastic cells or spontaneously metastasizing tumors by inhibiting the fibrin deposition necessary for endothelial implantation (30). However, incubation of tumor cells with heparin prior to implantation does not affect growth potential (13). Similar effects have been noted for coumarin and warfarin (2, 20) as well as fibrinolysins (5). Human studies have also suggested a lower mortality rate (19) or increased response to chemotherapeutic agents (29) in patients receiving anticoagulants p.o. Although these agents have anticoagulant properties in common, it has not been demonstrated that interference with the clotting mechanism is important for the response of established tumors (2). Warfarin has been shown to have some biological effects in addition to anticoagulation. Depletion of rRNA in murine hepatoma cells in vitro and perturbation of the cell cycle in cultured human glial cells has been observed (15).

It is hazardous to extrapolate from in vitro data obtained from a single cell type to complex interactions between tumor and host that occur in vivo. Additional studies in vitro with other neoplastic cell types and in vivo with animal tumor models will be required to investigate further the potential adjuvant effects of heparin therapy.

ACKNOWLEDGMENTS

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