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Effect of Osmotic Pressure on Uptake of Chemotherapeutic Agents by Carcinoma Cells[1]

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ABSTRACT

Intraperitoneal chemotherapy has been used to treat cancers which are confined to the abdominal cavity. Several variables which affect drug delivery into tumor cells have been identified, but the effect of osmotic pressure has not been studied. Tumor cell lines were used to evaluate the effect of fluid tonicity on drug uptake. HeLa cells and a murine teratoma cell line were suspended in solutions of tonicities 154, 308, and 616 mosM, each containing the same quantity of 5-fluorouracil, and uptake of the drug was measured at different intervals over 30 min. At all time points the amount of 5-fluorouracil taken up by cells in solutions of 154 mosM was greater than that in 310 mosM solutions, which was greater than the uptake in 616 mosM solutions, each by an average of 40–50%. Incorporation of drug into tumor cells was also assayed *in vivo* using a teratoma cell line propagated i.p. in mice. Tumor cell uptake of doxorubicin was increased to a similar extent when this drug was administered in hypotonic solutions of 154 mosM and was decreased by administration in hypertonic solutions of 465 mosM, as compared to solutions of 310 mosM.

These results demonstrate that the uptake of chemotherapeutic agents into tumor cells is increased significantly when these drugs are infused in solutions of lower osmolalities, a finding which may be exploited in clinical situations.

INTRODUCTION

Intraperitoneal administration of chemotherapeutic agents is gaining acceptance as a treatment for intraabdominal neoplasms. Since the initial report in 1964 (1), many groups have evaluated this form of therapy (2–7). Several variables have been recognized in clinical trials examining the pharmacokinetics of drugs administered i.p. (8–11), but one potentially important variable has been largely ignored: the type of fluid used for drug administration. Infusates used in different studies have included 1.5% peritoneal dialysis solution [345 mosM (12–14)], 1.5% peritoneal dialysis solution with added sodium bicarbonate [390 mosM (15)], normal saline [308 mosM (16–18)], and lactated Ringer's solution [273 mosM (19)]. These solutions span an osmotic range of 108 mosM, the equivalent of approximately 2000 mm Hg. Clinical experience with peritoneal dialysis in patients with renal insufficiency has demonstrated that instillation of nonisotonic solutions into the peritoneal cavity is associated with movement of water into the cavity if hypertonic solutions are used (20) and out of the cavity if hypotonic solutions are used (21). Water movement itself entrains solutes and this phenomenon, known as solvent drag or convective flux (22, 23), is incorporated into equations (24) describing fluxes in a solute, solvent, and membrane system (Appendix A).

The passive transport of drugs across a cell membrane is primarily governed by two factors: diffusive transport down a concentration gradient and convective transport in which the solute, in this case the chemotherapeutic agent, is entrained by the flux of solvent. The toxicity of chemotherapeutic agents limits the quantities that can be infused i.p., but reducing osmolality of the fluid used for drug administration may increase solvent drag into cells. The experiments described here were designed to test this hypothesis.

MATERIALS AND METHODS

Cell Suspensions. HeLa cells were grown in spinner culture in Joklik's medium (Irving Scientific) containing 10% heat-inactivated calf serum, at 37°C with a 5% CO_2 atmosphere. Murine teratoma cells (25) were maintained by serial i.p. passage in 6–7-week-old female C3H mice and were obtained by paracentesis. This tumor arose as a spontaneous mouse teratoma and, when inoculated i.p., resembles human ovarian carcinoma in that it forms small i.p. nodules and ascites. Metastases outside of the peritoneal cavity rarely occur (26).

Cells were washed once with Hanks' medium (Flow Laboratories) and were then resuspended at a density of 20–30 × 10^6/ml in either NaCl solutions of tonicities 154 mosM (0.5 N), 308 mosM (1 N), or 616 mosM (2 N) or PBS[4] containing NaCl (8 g/liter), KCl (0.2 g/liter), Na_2HPO_4 (1.15 g/liter), and KH_2PO_4 (0.2 g/liter), of tonicities 153 mosM (0.5 N), 306 mosM (1 N), or 612 mosM (2 N). Identical results (see below) were obtained using either NaCl or PBS. Solutions contained 0.6 μg/ml [^{3}H]-5-FU (Amersham) and either [^{14}C]dextran or [^{14}C]sucrose as an extracellular marker. Cells were incubated at 37°C and, at the times indicated, duplicate aliquots of cells were centrifuged through a 9:1 mixture of silicone oil (General Electric Company, Waterford, NY) and light mineral oil (Anderson Laboratories, Fort Worth, TX) for 20 sec at 6000 rpm, using a Savant high speed centrifuge (Savant Instruments, Inc., Farmingdale, NY). Cell pellets were solubilized in 1% sodium dodecyl sulfate and radioactivity was determined using a Packard model 4640 scintillation counter. The quantity of extracellular fluid contained in the cell pellet was determined by the content of ^{14}C, and this was subtracted from the total 5-FU content. Protein in the solubilized pellet was determined by the method of Lowry *et al.* (27) and data were normalized for cell protein. After incubation in NaCl or PBS for 30 min at 37°C, cell viability as assayed by trypan blue exclusion was greater than 95%.

Preliminary experiments indicated maximum uptake of [^{3}H]-5-FU after approximately 5-min suspension in the fluids; therefore, measurements were undertaken at intervals of 30 s (teratoma cells only) and 1, 3, 5, 10, and 30 min for each of the three different tonicities, 0.5 N, 1 N, and 2 N. Covariant analysis of regression of two groups of data was calculated for 0.5 N and 1 N solutions and for 1 N and 2 N solutions.

Inoculated Mice. Six- to 8-week-old female C3H mice were given i.p. injections of 1 × 10^6 teratoma cells. Four days later doxorubicin (Adriamycin; Adria Laboratories, Columbus, OH) was administered i.p., at a dose of 0.1 ml of a 0.1 mg/ml solution/g of body weight, in 0.5 N, 1 N, and 1.5 N sodium chloride solutions. At designated times following administration of the drug solutions, mice were sacrificed using CO_2, the skin was opened, and 5 ml of ice-cold 0.9% sodium chloride were flushed into the peritoneal cavity. Cell-containing fluid was withdrawn and the cells were washed 4 times. Cellular doxorubicin content was assayed fluorimetrically using a modification of the pro-

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[4] The abbreviations used are: PBS, phosphate-buffered saline; 5-FU, 5-fluorouracil.

cedure of Ozols *et al.* (28), and then doxorubicin content was adjusted for cell protein. Assays were carried out in duplicate at each time point and for each tonicity. Control cells which had not been exposed to doxorubicin showed no autofluorescence.

Data from these experiments were converted to percentages, with the amount of doxorubicin taken up in 1 N saline at 30 min standardized to 100%. Student's test was used to compare data obtained at the three time points for 0.5 N and 1 N, 1 N and 1.5 N, and 0.5 N and 1.5 N solutions.

RESULTS

Cell Suspension. From the earliest time points taken after resuspension of cell pellets (30 s to 1 min) and up to 30 min, cells in hypotonic solutions contained a greater quantity of 5-FU than did cells in isotonic or hypertonic solutions (Figs. 1 and 2). The differences between uptake in 0.5 N and 1 N and between 1 N and 2 N solutions were significant ($P < 0.05$) for both cell lines. Cells resuspended in 0.75 N or 1.5 N NaCl solutions contained an intermediate amount of 5-FU (data not shown). The occurrence of cell swelling in hypotonic solutions and cell shrinking in hypertonic solutions was indicated by differences in the volumes of the pellets, which were easily discernible by the naked eye. Although the numbers of cells and the amount of protein in the cell pellets were virtually identical, the volume of the cell pellet was greatest following incubation in 0.5 N solutions and smallest in 2 N solutions.

Inoculated Mice. *In vivo* drug uptake was assayed by administering doxorubicin i.p. to tumor-bearing mice, as described in "Materials and Methods." The quantities of doxorubicin taken up by cells correlated inversely with the tonicities of the three solutions containing the drug. The differences were significant at all three time points for 0.5 N and 1.5 N solutions, at 15 and 30 min for 0.5 N and 1 N solutions, and at 15 min for 1 N and 1.5 N solutions (Fig. 3).

These *in vivo* experiments confirmed the effects of solvent drag shown in the experiments with tumor cell suspensions; decreasing the tonicity of the surrounding fluid increased flux of chemotherapeutic agents into tumor cells within the peritoneal cavities of mice.

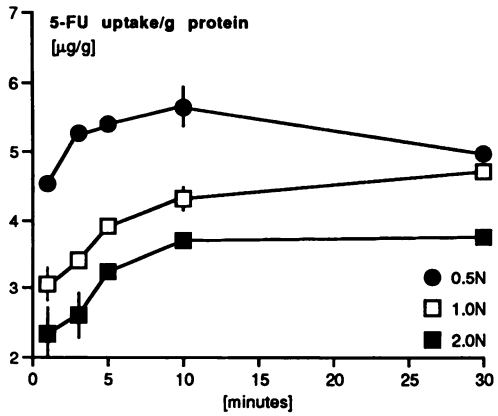

Fig. 1. Effect of fluid tonicity on uptake of 5-FU in HeLa cells. HeLa cells which had been maintained in spinner culture were pelleted by centrifugation. At time 0, cells were resuspended in 0.45% (0.5 N), 0.9% (1 N), or 1.8% (2 N) NaCl containing [^{3}H]-5-FU and [^{14}C]dextran, as described in "Materials and Methods." Cells were incubated at 37°C and, at the times indicated, aliquots of cells (3×10^6 cells in 200 μl) were layered over 1.5 ml oil and centrifuged as described in "Materials and Methods." The amount of extracellular fluid contained in the cell pellet was calculated from the [^{14}C]dextran and was subtracted from the amount of [^{3}H]-5-HU in the pellet. 5-FU uptake was adjusted for cell protein.

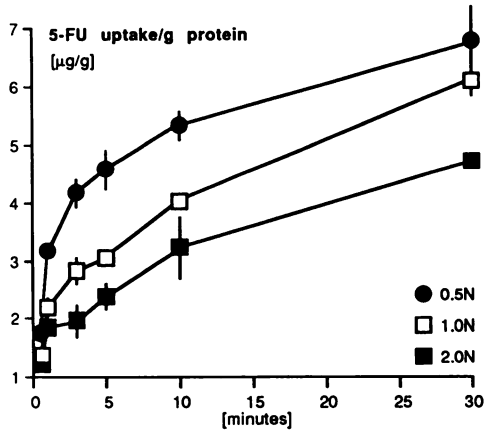

Fig. 2. Effect of fluid tonicity on uptake of 5-FU in a teratoma cell line. Cells were maintained and obtained as described in "Materials and Methods." 5-FU uptake was determined as described for HeLa cells, except that phosphate-buffered saline was used.

DISCUSSION

Cell Suspensions. Studies in our laboratory have previously demonstrated that suspension of cells in hypotonic solutions results in cell swelling (29). As water flows into cells, solutes entrained in the solvent are carried intracellularly and, upon continued incubation, cell volumes return to near normal values by 15–30 min. This process, termed regulatory volume decrease, is mediated by the cellular loss of osmotically active ions (30–32). Cells suspended in hypertonic solutions undergo cell shrinking, which is followed by a return to baseline volume and is termed regulatory volume increase (33). In theory, the increased cellular uptake of drug in a hypotonic solution would be nullified by loss of the drug during regulatory volume decrease, and the reduced solute flux in hypertonic solutions would be compensated for by the uptake of water during regulatory volume increase. If intracellular drug binding sites exist, then the initial increase in solute uptake in hypotonic solutions should be followed by increased drug retention, which was demonstrated in the ovarian carcinoma cell line.

The uptake of 5-FU followed distinctly different patterns for the two cell lines (Figs. 1 and 2). HeLa cells behave as expected when considering the process of regulatory volume decrease; further uptake of the drug ceases between 10 and 30 min. In contrast, the teratoma cells continue uptake of 5-FU beyond 30

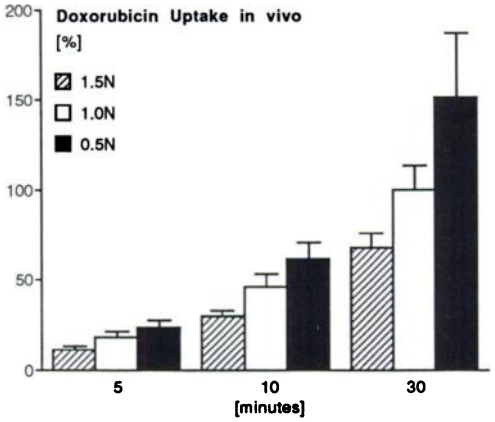

Fig. 3. Effect of fluid tonicity on uptake of i.p. doxorubicin into intrabdominal teratoma cells in mice. Doxorubicin was administered i.p. in solutions of 0.5 N, 1 N, and 1.5 N sodium chloride solution, as described in "Materials and Methods." At the designated time intervals, mice were sacrificed, cell-containing fluid was withdrawn, and doxorubicin uptake was determined fluorometrically. Data from each of four separate experiments were combined by designating as 100% the amount of drug taken up in 1 N sodium chloride at 30 min.

min. When examined microscopically, these cells were discrete (not clumped) and of approximately the same size as the HeLa cells. The observed differences, therefore, would likely arise from either dissimilar membrane permeabilities or intracellular disposition of 5-FU.

Animal Experiments. In the experiments with mice, doxorubicin was used for several reasons. Murine teratoma cells are sensitive to doxorubicin, which can be given to mice without short term toxicity. Doxorubicin is a larger molecular than 5-FU and, as the molecular weight of a solute increases, solvent drag accounts for a greater proportion of the total solute flux (34). Finally, cellular contents of this drug can be assayed using a simple fluorometric method. The increased uptake in hypotonic solutions was statistically significant, but less so than that in the suspensions of tumor cells. Several factors could explain this difference. Firstly, propagation of tumor cells i.p. results in accumulation of ascitic fluid. The experiments were carried out before ascites was clinically evident, but the presence of ascites in any amount will both reduce the drug concentration and adjust the fluid toward isotonicity, obscuring differences between the three experimental groups, as would different amounts of ascites between animals. Secondly, the tonicity of fluid does not remain constant once it is infused i.p., both because of the presence of preexisting i.p. fluid and because of equilibration between the extracellular space, intracellular space, and i.p. space. Finally, it has been shown that i.p. chemotherapy in a volume sufficient to distend the peritoneal space is required to achieve even drug distribution throughout the peritoneal cavity (10). Although the volume of fluid used in our studies distended the peritoneal cavity, it is likely that in some animals a proportion of the tumor cells were sequestered, which would result in a reduction of drug uptake and an increase in the variability between animals.

Clinical Applicability. The rationale of i.p. chemotherapy is the direct exposure of malignant cells to drug concentrations 2 log higher than concentrations obtained after i.v. administration. Thus, there is a question as to the clinical value of further increasing intracellular quantities of drug by only 50% when using hypotonic as compared to isotonic solutions. One of the major reasons i.p. chemotherapy fails to cure patients with intraabdominal tumors is the presence of nodules which are too bulky to allow complete tumoricidal penetration of chemotherapeutic agents. Cell membranes demonstrate very restricted diffusibility to passive transport of virtually all solutes, with the result that the outermost layer of cells in a tumor nodule is exposed to drug concentrations 100 or more times minimum tumoricidal levels, but the amounts decrease rapidly at increasing depths in the tumor mass, which has been demonstrated for 5-FU; at approximately 50–60 cell layers deep (600 μm), the concentrations of 5-FU dwindle to 5% of the original (10), so that a several hundredfold pharmacological advantage of the most diffusible chemotherapeutic agent is dissipated by a few millimeters depth of penetration.

Intraperitoneal hypotonic solutions will increase the tumoricidal depth of penetration into tumor nodules of the concurrently administered anticancer agent. The rationale (Appendix B) incorporates three assumptions: that the quantities of drug contained in a 2-liter i.p. infusion are so high that the amount taken up by hypothetical intracellular binding sites can be considered negligible, that the maximum tumoricidal depth of penetration will take place over approximately the same time frame as occurs when using isotonic or hypertonic solutions, and that transport coefficients (Appendix A) are not significantly altered by swelling of the cells. Under these conditions, the model in Appendix B demonstrates that, for any given depth within a mass of tumor cells, more drug accumulates when administered in fluids of lower osmolality.

There is an additional mechanism by which the use of hypotonic solutions may facilitate tumor killing. It has been suggested that bidirectional transport across peritoneal capillaries takes place primarily through intercellular gaps ("pores"), which are not homogeneous and display an increasing diameter from the arteriolar (10 Å) to the venular (40 Å) end (35). This anatomical asymmetry stratifies solute transport (36, 37). The use of hypotonic solutions will ensure some convective flux of the chemotherapeutic agent from the cavity into the afferent arterioles, which certainly will not occur when using isotonic or hypertonic solutions. (Note that capillary hydrostatic pressures of 15–40 mm Hg are insignificant compared to osmotic pressure gradients, which are greater than 1000 mm Hg.) Therefore, vascularized areas of i.p. tumors will be exposed to a "first pass" and a greater concentration of the drug, a potential clinical benefit that is absent when isotonic or hypertonic vehicles are chosen.

Except for their ready availability in 2-liter bags, it is difficult to support the use of hypertonic solutions for i.p. chemotherapy; solvent drag initially retards transport of drugs into cells, hypertonicity *per se* causes peritoneal hyperemia (inflammation) and increased transperitoneal diffusion of drugs (38), hypertonicity and/or low pH temporarily obliterate peritoneal defense mechanisms (39), and dehydrated patients will become more dehydrated. Local toxicity with hypotonic infusions might be more pronounced, especially over the initial 10–15 min when normal cells, like tumor cells, are subjected to osmotic stress and an increase in uptake of a cytotoxic drug. In addition, there will be increased hazards of fluid overload in elderly patients or those with cardiovascular disease. The extent of resistance of normal peritoneal mesothelial and endothelial cells to hypotonic solutions is uncertain. Zelman *et al.* (40) infused alternating hypertonic and hypotonic solutions i.p. in goats, and autopsies revealed no cellular damage until the osmolality was reduced to below 120 mosM.[5]

In a pilot study involving eight patients at this center, we have administered a total of 29 courses of i.p. chemotherapy (*cis*-platinum or 5-FU) in solutions with tonicities ranging from 251 mosM down to 196 mosM, as measured by an osmometer. No intraabdominal symptoms ascribable to hypotonicity were observed, nor did the two patients with cardiovascular disease suffer any related ill effects. Other investigators have used i.p. infusions of 0.45% saline (154 mosM), apparently without ill effect (41, 42). A recent study (43) has suggested that i.p. chemotherapy is potentially curative in patients with ovarian carcinoma who have relapsed or who have had incomplete responses to standard i.v. chemotherapy, a finding which emphasizes the importance of optimizing the administration of i.p. chemotherapy. Lowering the osmolality of the infusion should assist in achieving this goal, a prediction that awaits verification by suitably designed clinical studies.

Appendix A

The principles of nonequilibrium thermodynamics are used to develop equations in terms of solute flux (J_s), volume flux (J_v), transmembrane hydrostatic pressure (ΔP_m), and osmotic pressure differential across a membrane ($\Delta\pi_s$). The "practical transport equations" describe

[5] A. Zelman, personal communication.

a single solute in water migrating through a membrane.

$$J_v = L_p(\Delta P_m - \sigma\Delta\pi_s) \quad (A)$$

$$J_s = C_s(1 - \sigma)J_v + \omega\Delta\pi_s \quad (B)$$

where C_s is the average concentration of the solute, L_p is the hydraulic permeability, σ is the reflection coefficient, and ω is the solute permeability (sieving) coefficient.

When $J_v = 0$,

$$J_s = D_s/\Delta x \cdot \Delta C_s = \omega\Delta\pi_s \quad (C)$$

where D_s is the diffusion coefficient and Δx is the membrane thickness.

Eq. B then becomes:

$$J_s = D_s/\Delta x \cdot \Delta C_s + C_s(1 - \sigma)J_v \quad (D)$$

Relating to membrane permeability, P

$$J_s = P \cdot \Delta C_s \text{ (diffusive flux)} + C_s(1 - \sigma)J_v \text{ (convective flux)} \quad (E)$$

Appendix B

A tumor nodule is represented as a series of n concentric spheres of decreasing surface areas (A_1, A_2, A_3--A_n), each separated by a single layer of cells, and the nodule lies in a (relatively) large volume of infused i.p. fluid containing a chemotherapeutic agent at concentration C_s.

The mass transport (m) of the agent across each successive sphere/unit time may be identified.

When i.p. fluid is isotonic ($J_v = 0$)

$$\dot{m}_1 = A_1P(\bar{C}_s - \bar{C}_{s_1}) = D_{d_1}$$

$$\dot{m}_2 = A_2P(\bar{C}_{s_1} - \bar{C}_{s_2}) = D_{d_2}$$

$$\dot{m}_n = A_nP(\bar{C}_{s_{n-1}} - \bar{C}_{s_n}) = D_{d_n}$$

and

$$C_s > C_{s_1} > C_{s_2} > --C_{s_n}$$

Thus the total amount of drug (D_x) delivered into the nodule over a specified (unit) time interval may be expressed as:

$$D_x = D_{d_1} + D_{d_2} + D_{d_3} + --D_{d_n} = D_{\text{diffusive}}$$

and

$$D_{d_1} > D_{d_2} > D_{d_3} > --D_{d_n}$$

When cells are immersed in nonisotonic solutions, the reflection coefficient (σ) across their membranes for all solutes falls in the range $1 > \sigma > 0$, so that there is a sequential changing osmolality across successive spheres of cells prior to reaching equilibrium.

When i.p. fluid is hypertonic there is efflux of water from cells ($J_v < 0$) and

$$\dot{m}_1 = A_1P(\bar{C}_s - \bar{C}_{s_1}) - A_1(1 - \sigma)\bar{C}_s|J_v|$$

$$\dot{m}_2 = D_{d_2} - D_{c_2}$$

$$\dot{m}_n = D_{d_n} - D_{c_n}$$

i.e.

$$D_x = D_{\text{diffusive}} - D_{\text{convective}}$$

Similarly, when i.p. fluid is hypotonic ($J_v > 0$)

$$D_x = D_{\text{diffusive}} + D_{\text{convective}}$$

So that for both the total and the depth related delivery of drug

$$D_{x_{\text{hypotonic}}} > D_{x_{\text{isotonic}}} > D_{x_{\text{hypertonic}}}$$

$$D_{n_{\text{hypotonic}}} > D_{n_{\text{isotonic}}} > D_{n_{\text{hypertonic}}}$$

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