ABSTRACT

Since cancer cells produce large amounts of lactate via aerobic glycolysis and since an acidic pH has been shown to selectively enhance the cytotoxic effects of hyperthermia, we are examining the influence of cell exposure to drugs which inhibit lactate transport and lower intracellular pH upon cytotoxic effects of hyperthermia. Quercetin, a bioflavonoid that produces lactate transport inhibition, showed no hyperthermic potentiation of cytotoxic effects in HeLa cells under a variety of cultural conditions.

INTRODUCTION

It is well established that exposure to elevated temperatures can produce regression of cancer in animal models and in humans (15); however, the physiological mechanisms involved are somewhat undefined, and the clinical therapeutic index remains low (15, 21). In pursuing the cellular mechanism of heat-induced cytotoxicity, early cell culture studies suggested that cancer cells were more sensitive to heat than were normal cells (4, 11). However, more recent experimental data tend to indicate that there may be very little inherent difference in the intrinsic heat sensitivity between the transformed cell and its normal cell counterpart (14). Many cellular and physiological factors have been identified which can influence the thermosensitivity of mammalian cells, e.g., cellular growth stage, cell cycle phase, extracellular and intracellular pH, and duration of treatment. In contrast, treatment of cells with rutin, a structurally related bioflavonoid that lacks the property of lactate transport inhibition, showed no hyperthermic potentiation.

Quercetin, an Inhibitor of Lactate Transport and a Hyperthermic Sensitizer of HeLa Cells

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MATERIALS AND METHODS

Experiments were carried out with HeLa S-3 cells grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Details of the cell culture procedures including the maintenance, the trypsinization, and the test for contamination of cultures with Mycoplasma were described elsewhere (22). No antifungal agent was used.

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throughout this study. Cell counts were performed with a Model B Coulter Counter.

Cell survival was assayed by colony-forming ability of plated single cells to obtain quantitative dose-survival curves. Details of cloning experiments including colony count have been described elsewhere (23).

Plated monolayer cells were heated to within 0.05% of the desired temperature by totally immersing rubber-stopped glass flasks in a water bath heated by a Haake Model 52 temperature circulator. Water bath temperatures were verified by a National Bureau of Standards thermometer.

The pH of the culture medium was adjusted by varying the CO₂ content of the gas phase within the flasks. The buffering system of Eagle’s minimal essential medium consisted of 26 mM NaHCO₃ at 5% CO₂ for neutral pH of 7.4. To obtain pH of 6.7, for example, the flasks were flushed with the gas mixtures containing 26% CO₂. The pH of the culture medium was monitored throughout the treatment procedures by sealing a combination electrode (Altex combination pH electrode) in a treatment vessel and monitored with a temperature-compensated digital pH meter (Altex Model 3560).

Quercetin was purchased from Sigma Chemical Co., and rutin was obtained through the generosity of Dr. John Johnson and Dr. Efraim Racker of Cornell University, Ithaca, N. Y. The compounds were dissolved in DMSO immediately prior to experiments. DMSO concentrations within the medium did not exceed 0.06%. The gas mixtures were purchased from Matheson Gas Products (East Rutherford, N. J.).

RESULTS

Effect of Quercetin on Cell Proliferation and Survival. Prior to hyperthermia study, experiments were carried out to determine the toxicity of quercetin on HeLa cells. Control cells increased exponentially with a doubling time of about 16 hr. The growth rate of cells exposed to chronic incubation 0.01 mM quercetin was the same as that of the control cells (data not shown). Cells exposed to 0.1 mM quercetin maintained exponential growth, although a lengthening of the doubling time of about 36 hr was observed (Chart 2). 0.2 and 0.4 mM quercetin produced a decrease in cell numbers. On the other hand, exposure of cells to rutin up to 0.2 mM for 72 hr showed no growth-inhibitory effects (data not shown).

Acute exposure of cells to quercetin or rutin on cell survival
was also determined in terms of colony formation of single-plated cells at 37°C. The cell survival curves show no drug-induced cytotoxicity to 0.1 mM after 4-hr incubation under neutral or acidic pH (Chart 3).

Effect of Quercetin on Cell Survival following Hyperthermia. Charts 4 and 5 show cell survival curves as a function of exposure time at 41 and 42°C under various pH and drug concentrations. It is apparent that quercetin increases the cytotoxic effect of hyperthermia. Although present at 41°C, the potentiating effect of quercetin on hyperthermia-induced cell killing is far more pronounced at 42°C and is particularly evident under acidic conditions. It should be noted that the control flasks (no exposure to the compound) were similarly treated with the drug solubilized with DMSO in the culture medium at <0.1 μg/ml final concentration; within the range studied, DMSO produced no enhancement of hyperthermic cytotoxicity. In contrast to the results with quercetin, treatment of HeLa cells with rutin provided no hyperthermic potentiation under either neutral or acidic pH (Chart 6).

DISCUSSION

The data presented demonstrate that an inhibitor of lactate transport potentiated the cytotoxic effects of hyperthermia. Quercetin, a bioflavonoid compound that has the property of lactate transport inhibition, was a potent hyperthermic sensitizer in HeLa cells. Rutin, a structurally related bioflavonoid that lacks the activity of lactate transport inhibition, showed no hyperthermic potentiation. The magnitude of the hyperthermic potentiation by quercetin was found to be dependent on the drug concentration, medium pH as set by CO2 tension, temperature, and the duration of treatment.

Although the data are consistent with the idea that quercetin is a hyperthermic sensitizer because of its capacity to produce intracellular lactate retention with a resultant depression of intracellular pH, other possible mechanisms can be proposed. Furthermore, there are complexities in the experimental design that will require further study to clarify the cause and effect relationships involved. For purposes of a mechanistic discussion, 2 explanations can be offered to account for the observed hyperthermic potentiation produced by quercetin.

(a) Hyperthermic sensitization resulted from drug-induced lactate accumulation and intracellular acidifications with a resultant perturbation of the energy state of the cells. The increased effect under low pH conditions resulted from a low initial intracellular pH that was lowered further by drug effects carrying it to extreme values with cytotoxic consequences.

(b) Quercetin may have multiple (unknown) toxic effects beyond its known inhibition of lactate transport; these could be of greater importance to the observed effect than impairment of lactate efflux. The increased toxicity under acidic and hyperthermic conditions could have resulted from increased cell uptake of a toxic drug under nonphysiological conditions.

The above 2 possibilities are not mutually exclusive; an increased cell uptake of quercetin could also increase its inhibitory effects on lactate efflux.

The following considerations support a proposed correlation between the hyperthermic sensitization produced by quercetin and the effect of the drug on lactate transport. The concentrations of quercetin used in the present experiments are within the range of those found by Belt et al. (2) to inhibit lactate efflux, produce intracellular acidification, and inhibit glycolysis in Ehrlich ascites cells. They are also comparable to those found by Soulina et al. (29) to inhibit growth of L1210 leukemia cells in cell culture. Johnson et al. (20) obtained a very similar metabolic effect on lactate efflux, intracellular acidification, and inhibition of glycolysis using IBCLA, a synthetic anhydride of lactic acid that appears to have a great specificity for inhibition of the lactate transporter in the plasma membrane.

The degree of intracellular acidification produced by quercetin and IBCLA in Ehrlich ascites tumor cells in zwitterionic buffers of pH 7.3 was between 6.4 and 6.5. This was sufficient to inhibit rates of glycolysis in Ehrlich ascites tumor cells with the pH set
by an acidic external buffer and tributyltin added as an equili-
brating agent (2). Similarly, Dickson and Oswald (7) observed
reduced rates of glycolysis accompanying a reduced intracellular
pH in a rat mammary tumor cell line using a Krebs-Ringer
phosphate-bicarbonate buffer system to set both the medium
and the intracellular pH. It should be noted, however, that there
presently exist no data on the effect of the initial medium pH or
intracellular pH on the degree of intracellular acidification that
would be produced by quercetin treatment. Moreover, extrapo-
lation from glycolysis experiments in cultured L1210 and P388
leukemia cells suggests that the immediate inhibitory impact of
quercetin will be lessened in bicarbonate-based buffer systems
(29). This is in accord with our data presented in Charts 4 and
5, wherein the major hyperthermic enhancement is seen after 2
to 4 hr of quercetin exposure.

The magnitude of the increased cell kill produced by quercetin,
aacidic pH, and progressive temperature elevations is impressive
(Charts 3 to 6). It invites consideration of the possibility that
hyperthermia and acidic pH may sensitize the cells to the toxic
effects of the drug, rather than the converse that has been
examined heretofore. A simple model that could explain this
would be an increased cellular uptake of the compound under
extreme conditions of pH and temperature. This has been ob-
erved with regard to the cellular uptake of Adriamycin (16) and
misonidazole (3). As noted above, the consequences of in-
creased uptake could be a more profound inhibitory effect on
transmembrane movement of lactate or toxic effects on other,
as yet unknown, metabolic targets.

The present studies may have solidified the concept that
inhibitors of lactate transport may have cytotoxic effects as first
suggested by Johnson et al. (20); however, further detailed study
will be required to establish the precise mechanism and to link it
with the general case of hyperthermic sensitization by acidifica-
tion. An increased cytotoxic effect of hyperthermic treatment in
the presence of acidified culture medium has been reported by
multiple laboratatories (9, 10, 17, 27). The magnitude of the sen-
sitizing effects at specific pH and temperature values varies with
differing cell lines and medium conditions; however, a pro-
nounced effect is usually seen between a pH (in the culture
medium) of 6.5 and 6.8. The acidic medium conditions used in
the above experiments have included nonbicarbonate buffers
(17, 27), where a pH differential is presumed to exist at the ini-
tiation of the experiment, and, as used in the present studies,
bicarbonate-based buffers (10, 17), where the initial intracellu-
lar and extracellular pH values are essentially identical by reason
of the free diffusibility of CO2 (7, 28). Enhanced hyperthermic cell
kill has been observed in both nonbicarbonate and bicarbonate-
based buffer systems, although the observed effect may be
greater in the nonbicarbonate systems (17).

We have chosen to use a bicarbonate-based buffer system with
the pH set by the pCO2 over the culture medium, because this
more closely approximates conditions existing in vivo in
tumor-bearing animals or patients, i.e., normal pCO2 in most
tissues and high pCO2 in hypoxic tumors. It is reasoned that
drug effects in a bicarbonate-based buffer system are more likely
to be physiologically meaningful than in an artificial buffer system.
The great interest in the relationship between an acidic external
pH and increased sensitivity to hyperthermia resides at least in
part in the perception that it is relevant to clinical cancer. The
microenvironment of large tumors may be characterized by
sluggish blood flow, leading to chronic hypoxia and, in some
measured systems, to a low extracellular pH (1, 9, 33). The pH
of interstitial fluid of a variety of rodent and human tumors has
been studied extensively and found to be below pH 7 (1, 13, 18,
32). The measured pH can be further reduced by glucose infusion
(19, 26). Although the measurement techniques may still not be
free from artifact, the repeated confirmation of the observation
with progressively smaller and less traumatic pH probes gives
increasing credibility to the concept. Even in the absence of
hypoxia, the high aerobic glycolytic rates characteristic of many
tumor cell varieties could provide a source for lactate.

The concept of hyperglycemia-induced tumor acidification has
been extended into in vivo therapy experiments; Urano et al. (31)
have demonstrated that glucose administration does increase
the response to hyperthermia of a transplanted murine fibrosar-
coma. The effect correlated with glucose dosage and increased
with increasing tumor size.

Because hyperthermia alone has produced only marginally
useful results, its combined use with radiation and chemotherapy
is receiving extensive study (5, 6, 25). The most frequent ap-
proaches have been to combine it with conventional cytotoxic
agents (16, 26) or electron-affinic radiosensitizers (3, 12). The
present studies support the concept that inhibitors of lactate
transport may have potential utility as hyperthermic sensitizers,
provided their effects can be produced in vivo without undue
toxicity.

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