Use of Oxidizing Dyes in Combination with 2-Cyanocinnamic Acid to Enhance Hyperthermic Cytotoxicity in L929 Cells

Hui Wang, Vipin Shah, and Karl W. Lanks

ABSTRACT

Two widely used oxidizing dyes, 2,3,5-triphenyltetrazolium chloride and methylene blue, can greatly potentiate hyperthermic cytotoxicity when administered simultaneously with 2-cyanocinnamic acid. The same compounds are virtually nontoxic to L929 cells if administered alone at 42°C or in combination with 2-cyanocinnamic acid at 37°C. Cytotoxicity was synergistically enhanced by the combined regimens after 3 h of heat exposure. Quercetin, a bioflavonoid known to enhance hyperthermic cytotoxicity, also acts synergistically when administered in combination with 2-cyanocinnamic acid and this effect is apparent after 1 h of heat exposure. Since these compounds do not greatly interfere with pyruvate metabolism at either normal or heat shock temperatures, a mechanism of action based on depletion of NAD(P)H is considered.

INTRODUCTION

Selective killing of tumor cells at elevated temperatures has been the subject of intensive investigation because of the potential applications of hyperthermia in cancer therapy. Effective application will require the development of regimens that enhance the cytotoxicity of hyperthermia, just as have been developed for other therapeutic modalities, e.g., ionizing radiation and chemotherapy. Initial steps in this direction have been taken with studies on the kinetics of thermotolerance induction (1) and the identification of agents that are especially toxic at temperatures used in clinical hyperthermia (2, 3).

Pyruvate availability may be important in heat shock protein induction and hyperthermic cytotoxicity since high levels supplied exogenously in the culture medium enhance heat shock protein induction and protect L929 cells against killing by heat (4). Conversely, killing is potentiated by 2-CC (5), an inhibitor of mitochondrial pyruvate transport (6, 7). The mechanism underlying these effects could involve pyruvate directly through its contribution to the carbon skeleton of a critical metabolic product or indirectly through widespread effects on energy metabolism and redox-sensitive equilibria. Since considerable evidence suggests that oxidative stress is involved in the heat shock response (8), the present study was undertaken to explore the possibility that reducible compounds can potentiate the lethal effects of hyperthermia.

Methylene blue and TTC, two widely used compounds that are susceptible to enzymatic reduction, and quercetin, a bioflavonoid known to enhance hyperthermic cytotoxicity (9), were evaluated for their ability to potentiate hyperthermic cytotoxicity when administered simultaneously with 2-CC. The extent to which these compounds interfere with pyruvate metabolism at both normal or heat shock temperatures was also examined and mechanisms that might explain their effects are considered.

MATERIALS AND METHODS

Cells and Culture Conditions. L929 cells were obtained from MA Bioproducts (Bethesda, MD) and were routinely maintained in semi-supernatant culture in bacteriological polystyrene Petri dishes containing high glucose Dulbecco's modified Eagle's minimum essential medium and 10% newborn calf serum. This unsupplemented medium contained 25 mM glucose, 4 mM glutamine, and 1 mM pyruvate. Cells from the stock cultures were plated in 35-mm plastic tissue culture dishes at a density of 1 x 10^6/cm². One to 2 days later, the monolayers had reached a final density of 2-3 x 10^5/cm². Cultures were washed twice with Dulbecco's phosphate-buffered saline, and 2 ml of the medium of interest were added and changed daily with a final change immediately before exposure to hyperthermia.

Exposure to Hyperthermia. After maintenance for 3 days in the appropriate medium, the following compounds were added with the fresh culture medium immediately before exposure to hyperthermia: 2-CC (Aldrich Chemical Co., Milwaukee, WI) as a 0.2 M aqueous stock solution titrated to pH 7.4 with NaOH; TTC (Sigma Chemical Co., St. Louis, MO) as a 20 mM aqueous stock solution; methylene blue (Fisher Chemical Co., Fair Lawn, NJ) as a 20 mM aqueous stock solution; and quercetin (Sigma) as a 0.2 M stock solution in 50% dimethyl sulfoxide. Cultures were then placed on an aluminum sheet in a water-jacketed CO₂ incubator which had been equilibrated at 42°C. When treated in this manner, continuous monitoring with a thermistor (YSI Instruments Co., Yellow Springs, OH) showed that medium in the dishes reached the indicated temperature within 10 min. Cultures were returned to 37°C after various periods of exposure. Cells were then dispersed with 0.25% trypsin (Sigma), plated at appropriate dilutions, and maintained in DMEM plus 10% newborn calf serum for 7 days. Plates were examined after staining with methylene blue and clones containing more than 10 cells were counted. The surviving fraction was the ratio of the number of clones formed from cultures exposed to hyperthermia divided by the number formed from control cultures maintained in medium of the same composition.

Pyruvate Oxidation and Lactate Production. These determinations were made simultaneously on monolayer cultures maintained in 25-cm² tissue culture flasks as described previously (10) except that the compounds of interest were added along with the incubation medium. The results are expressed as nmol of substrate converted to product per mg protein per h.

RESULTS

Preliminary experiments showing decolorization of methylene blue and conversion of TTC to a red reaction product established that the dyes entered monolayer L929 cells and were reduced (data not shown). Fig. 1 shows that TTC alone was toxic at 42°C, but TTC was much less so in combination with 2-CC. The degree of sensitization by 2-CC had relatively modest effects. The degree of sensitization by 2-CC was similar to that previously observed in DMEM and is much less than would be seen in medium lacking glutamine and pyruvate (4, 5). Simultaneous exposure to 2-CC and either of the dyes resulted in markedly enhanced cytotoxicity at 42°C and synergistic enhancement, i.e., a response greater than expected from simple additive effects, was readily apparent after 3 h of heating. From Figs. 2 and 3 it can be seen that methylene blue in combination with 500 µM 2-CC was toxic at 37°C, but TTC was much less so in combination with 2-CC. None of the agents exhibited significant toxicity when administered alone at 37°C to cultures maintained in complete medium or in cultures deprived of glucose and pyruvate or glutamine and pyruvate.
ENHANCING HYPERTHERMIC CYTOTOXICITY

Fig. 1. Effect of méthylène blue and TTC combined with 2-CC on the survival of L929 cells. Cultures were maintained in complete DMEM with the indicated agents added to the medium immediately prior to heat shock: DMEM alone (•), 500 µM 2-CC (O), 100 µM TTC (A), 100 µM méthylène blue (B), 500 µM 2-CC + 100 µM TTC (C), 500 µM 2-CC + 100 µM méthylène blue (D). Points, mean cloning efficiencies of 4 to 6 plates in 2 series of cultures heated at 42°C relative to the corresponding untreated controls maintained at 37°C. Bars, SE. Arrows, surviving fraction less than 10⁻³.

Sensitization by méthylène blue and TTC in the presence of 2-CC was dose dependent (Figs. 2 and 3, respectively) and was apparent at 50 µM 2-CC, a concentration that had no effect on survival at 37°C in either combined regimen. Consistent with the results in Fig. 1, only limited sensitization by méthylène blue was seen after 2 h of heat exposure. Quercetin, an agent with a quite different structure and proposed mode of action (11), also sensitized to hyperthermic cytotoxicity in combination with 2-CC (Fig. 4). Synergism was especially apparent after 1 h exposure at 42°C. All of the effects of 2-CC combined with méthylène blue, TTC, or quercetin were reproduced in DMEM containing 10% newborn calf serum (data not shown).

The effects of these agents on pyruvate oxidation were not dramatic (Table 1). Although TTC inhibited pyruvate oxidation 44% with a corresponding increase in lactate synthesis, the other agents singly and in combination inhibited far less. Interestingly, méthylène blue in combination with 2-CC increased lactate synthesis to a greater extent than it inhibited pyruvate oxidation. The dyes did not affect glucose or pyruvate metabolism in a temperature-dependent manner even though clonogenic survival was reduced to 2 to 10% of control values within the time frame of the metabolic measurements. Thus, méthylène blue added to the system containing 2-CC increased lactate production from pyruvate 65% at 37°C and only 55% at 42°C. Quercetin also increased lactate synthesis from pyruvate to a greater extent than it inhibited pyruvate oxidation. This effect of quercetin was not seen with glucose as substrate (Table 2) since, under these conditions, oxidation was inhibited with no change in lactate synthesis. The effects could also not be cor-
ENHANCING HYPERTHERMIC CYTOTOXICITY

Table 1: Effect of sensitizing agents on CO\textsubscript{2} and lactate production from medium pyruvate

<table>
<thead>
<tr>
<th>Addition</th>
<th>Rate of labeled product formation (nmol [1-\textsuperscript{14}C]pyruvate converted/mg protein/h)</th>
<th>37°C</th>
<th>42°C</th>
<th>37°C</th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (DMEM only)</td>
<td></td>
<td>180</td>
<td>160</td>
<td>420</td>
<td>440</td>
</tr>
<tr>
<td>+ 0.5 mM 2-CC</td>
<td></td>
<td>160</td>
<td>140</td>
<td>400</td>
<td>560</td>
</tr>
<tr>
<td>+ 0.1 mM methylene blue</td>
<td></td>
<td>190</td>
<td>180</td>
<td>420</td>
<td>550</td>
</tr>
<tr>
<td>+ 2-CC/methylene blue</td>
<td></td>
<td>170</td>
<td>140</td>
<td>660</td>
<td>870</td>
</tr>
<tr>
<td>+ 0.1 mM TTC</td>
<td></td>
<td>100</td>
<td>100</td>
<td>510</td>
<td>630</td>
</tr>
<tr>
<td>+ 2-CC/TTC</td>
<td></td>
<td>100</td>
<td>110</td>
<td>460</td>
<td>510</td>
</tr>
<tr>
<td>+ 0.1 mM quercetin</td>
<td></td>
<td>150</td>
<td>150</td>
<td>770</td>
<td>890</td>
</tr>
<tr>
<td>+ 2-CC/quercetin</td>
<td></td>
<td>150</td>
<td>140</td>
<td>430</td>
<td>660</td>
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</tbody>
</table>

* Mean of duplicates with SE ±20% or less.

Table 2: Effect of sensitizing agents on CO\textsubscript{2} and lactate production from glucose

<table>
<thead>
<tr>
<th>Addition</th>
<th>Rate of labeled product formation (nmol [1-\textsuperscript{14}C]glucose converted/mg protein/h)</th>
<th>37°C</th>
<th>42°C</th>
<th>37°C</th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
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<td>12.4</td>
<td>15.6</td>
<td>5.8</td>
<td>5.5</td>
</tr>
<tr>
<td>+ 0.5 mM 2-CC</td>
<td></td>
<td>4.9</td>
<td>9.5</td>
<td>12.1</td>
<td>13.3</td>
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<tr>
<td>+ 0.1 mM quercetin</td>
<td></td>
<td>7.3</td>
<td>12.5</td>
<td>5.7</td>
<td>5.6</td>
</tr>
<tr>
<td>+ 2-CC/quercetin</td>
<td></td>
<td>3.2</td>
<td>7.7</td>
<td>7.7</td>
<td>16.0</td>
</tr>
</tbody>
</table>

* DMEM lacking glutamine and pyruvate.
# Mean of duplicates with SE ±20% or less.

related with hyperthermic cytotoxicity since addition of quercetin to the system containing 2-CC inhibited glucose oxidation only slightly more than 2-CC alone.

DISCUSSION

This study demonstrates that either methylene blue or TTC, two relatively innocuous dyes, markedly enhances hyperthermic cytotoxicity when administered in combination with 2-CC, an inhibitor of mitochondrial pyruvate transport. The effect is seen in the presence of serum and does not require energy source restriction. One of the combined regimens, i.e., methylene blue plus 2-CC, uses concentrations of the compounds tolerated by patients (12) and intact tissues (6), respectively; therefore further studies at the whole animal level appear feasible.

Glucose and pyruvate metabolism were examined to determine whether heat shock potentiated metabolic effects of the chemical agents. We found that although the dyes potentiated killing by hyperthermia they did not alter pyruvate or glucose metabolism in a corresponding temperature-dependent manner. Therefore, increased hyperthermic cytotoxicity in the presence of these agents does not appear to be due to enhanced action at elevated temperatures. Nevertheless, the relatively small number of cells accounting for the precipitous decrease in clonogenic survival seen with prolonged heating could exhibit important metabolic changes not reflected in the bulk of the cell population.

Quercetin, alone or in combination with 2-CC, also sensitized L929 cells to killing by hyperthermia. This bioflavonoid which requires an adequate supply of reducing equivalents, appears to be involved in the heat shock response. Determination of reduced nucleotide levels under the conditions described in this study should help to define the mechanism by which 2-CC and oxidizing dyes enhance hyperthermic cytotoxicity.

REFERENCES

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