Effects of Amiloride on Thermosensitivity of Chinese Hamster Cells under Neutral and Acidic pH

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ABSTRACT

The modifying effects of amiloride on the thermosensitivity of Chinese hamster V-79 cells were examined under both neutral (pH 7.3) and acidic (pH 6.6) conditions. Amiloride, a diuretic drug, is known to inhibit the Na+/H+ exchange activity. Under the extracellular pH of 7.3, amiloride (0.1-0.5 mM) enhanced the thermal cell killing powers of 42°C hyperthermia with increasing concentration and exposure time of the drug. The age response of cells to 42°C hyperthermia in the presence or absence of amiloride (0.5 mM) showed that amiloride sensitized cells to heat, especially those at G1-S boundary through S phases. On the other hand, the lowering of extracellular pH to 6.6 enhanced cell killing by 42°C hyperthermia. When cells were exposed to 42°C hyperthermia in the presence of amiloride at pH 6.6, cell survival decreased still more. The thermosensitizing effects of the lowered pH at 6.6 and amiloride appeared to be additive. From these results, it is suggested that the thermosensitization by amiloride is probably due, in part, to the inhibition of cellular Na+/H+ exchange activity. The present study proposes the possibility that amiloride may be useful as a hyperthermic sensitizer in a clinical treatment of cancer.

INTRODUCTION

There exist several evidences supporting the view that hyperthermia is useful as a new modality of cancer treatment (1). The details of the mechanisms of thermal cell killing are not yet clarified. In in vitro studies, however, it has been demonstrated that thermal cell killing is enhanced by lowering medium pH (2-4), that cells in radioresistant S phase are thermosensitive (5-7), or that heat treatment of cells increases the sensitivity to some chemotherapeutic agents (8, 9). The data suggest that the target of thermal cell killing may include the plasma membrane with the cellular pH regulating system and/or chromatin.

A Na+/H+ exchange system in plasma membranes is reportedly a major mechanism for the regulation of internal pH in eukaryotic cells (10, 11). This Na+/H+ antiport system exists in various mammalian cell lines, e.g., Chinese hamster lung fibroblasts (12, 13), BALB/c 3T3 (14), Swiss 3T3 (15), mouse neuroblastoma clone (16), and dog kidney cells (17). Further, a diuretic drug, amiloride, is known to inhibit the Na+/H+ exchange activity (18). In the present study, the modifying effects of amiloride on thermal cell killing were examined under normal and low pH conditions using Chinese hamster V-79 cells.

MATERIALS AND METHODS

Cell Line and Culture Conditions. Chinese hamster V-79 cells were maintained in growth medium, 1 liter of which contained 730 ml of Eagle’s minimum essential medium, 100 ml of NCTC-135 (Difco Laboratories, Detroit, MI), 20 ml of lactalbumin hydrolysate (Difco), and 150 ml of bovine serum. Details of culture conditions have been described elsewhere (19, 20).

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RESULTS

Effects of Amiloride on Cell Growth and Survival. To determine the effects of amiloride on cell growth, exponentially growing cells were exposed to amiloride (0.01 to 0.5 mM) at 37°C for up to 72 h. Cell growth curves were shown in Fig. 1. At concentrations below 0.1 mM, the cell growth rate did not change from the control (data not shown). For the concentrations from 0.1 to 0.3 mM, cell growth delayed with increasing concentration. At the concentrations over 0.3 mM, the cell growth rate decreased significantly. The modifying effects of amiloride on the thermosensitivity of Chinese hamster cells under neutral and acidic pH.

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THERMOSENSITIZATION BY AMILORIDE

Fig. 1. Growth curves of cells exposed to amiloride at 37°C. Arrow, time when the drug was added.

Fig. 2. Hyperthermic treatment time-survival curves of cells exposed to 42°C in the presence or absence of amiloride.

growth was completely inhibited and a decrease in number of cells was observed. On the other hand, on acute exposure of cells to amiloride up to 0.5 mM for 5 h at 37°C, no cell lethality in terms of colony formation was observed and the frequency of S-phase cells after treatment with amiloride was about 60%, a value similar to that observed in the exponentially growing cell population (data not shown).

Thermosensitizing Effect of Amiloride. Cells were exposed to 42°C hyperthermia in the presence of amiloride at concentrations from 0.1 to 0.5 mM at a normal pH of 7.3. Survival curves of cells under these experimental conditions were shown in Fig. 2. When cells were heated in the presence of the drug, cell survival decreased with increasing concentration and exposure time of the drug, as compared with that for 42°C alone.

Cell Cycle Dependency of the Effect of Amiloride and/or 42°C Hyperthermia. The age response of cells to amiloride (0.5 mM) and/or 42°C hyperthermia was examined by HU pretreatment. Exponentially growing cells were synchronized at the G1-S boundary by exposure to HU (2 mM) for 6 h. Such treatment killed cells in S phase and made cells in other phases proceed to G1-S boundary. After the removal of HU, cells were incubated in normal medium for various periods and then further exposed to HU (5 mM), amiloride (0.5 mM), or 42°C hyperthermia with or without amiloride for 2 h.

Age response curves were shown in Fig. 3. The changes in cell survival for the fractionated HU treatments indicate that the cells proceed from G1-S through G2 + M phases after the removal of the initial HU treatment. In the case of amiloride treatment, no cell lethality was observed throughout the phases tested. In contrast, cell survival for 42°C hyperthermia began to decrease in early S phase, reached the minimum at a 3-h interval (middle-S phase), and then increased to a level above the initial one (no interval) after about a 7-h interval (G2 + M phases). When synchronized cells were exposed to 42°C hyperthermia in the presence of amiloride, thermal cell killing was markedly enhanced from G1-S through middle S phase. Such thermosensitizing effect of amiloride was smaller at late S and G2 + M phases.

Effect of Amiloride on Thermal Response under Low pH. Cells were exposed to 42°C hyperthermia under pH 7.3 or 6.6 in the presence or absence of amiloride (0.3 mM). The survival curves were shown in Fig. 4. In the treatments with amiloride under pH 6.6 at 37°C for up to 5 h, no or less cell lethality (more than 90% survival) was observed. When cells were exposed to pH 7.3.
42°C hyperthermia under pH 6.6, cell survival decreased as compared with that for heating under pH 7.3. When the 42°C hyperthermic treatment at pH 6.6 was done in the presence of amiloride (0.3 mM), cell survival decreased still more. Thus, cells treated with amiloride under pH 6.6 became markedly thermosensitive.

To characterize the thermosensitizing effect of amiloride, cells were exposed to 42°C hyperthermia for 2 or 3 h in the medium containing 0.1 to 0.5 mM concentrations of the drug at pH 6.6. The surviving fraction ratios for these treatments, and those under pH 7.3 (data from Fig. 2) in comparison, are shown in Fig. 5. With the surviving fraction ratio, the smaller the value, the larger was the extent of the thermosensitizing effect. At both pH 7.3 and pH 6.6, the surviving fraction ratio decreased with increasing concentrations of amiloride. Over the range of the drug concentration examined, the surviving fraction ratio was lower at pH 6.6 than at pH 7.3, and the concentration-response curve at pH 6.6 paralleled the curve at pH 7.3.

**DISCUSSION**

Thermosensitization of cells by amiloride was observed in the simultaneous treatment with the drug and 42°C hyperthermia at a normal pH of 7.3 (Fig. 2). This hyperthermic potentiation by amiloride was dependent on the drug concentration and the duration of treatment. Amiloride is known as a potent inhibitor for the Na⁺/H⁺ exchange system in mammalian cells (12, 14). It has also been shown that this Na⁺/H⁺ exchange agent contributes to the regulation of intracellular pH (10, 11). On the other hand, thermal cell killing is markedly enhanced under acidic conditions (2–4). The thermosensitizing effect of amiloride may thus be, in part, due to the inhibition of Na⁺/H⁺ exchange activity, which results in the decrease in intracellular pH.

Thermotolerance is induced during heat treatment at relatively low temperatures (below 42.5°C) (1). In the present study (Fig. 2), the control survival curve of cells exposed to 42°C hyperthermia included the tail region, indicating that thermotolerance was induced during heat treatment. When cells were exposed to hyperthermia in the presence of amiloride, the tail region of the survival curves disappeared with increasing concentrations of the drug (Fig. 2). A similar effect was observed when cells were exposed to hyperthermia under low pH (Fig. 4). These results suggest that amiloride may inhibit the thermotolerance induction of cells during heating.

Hyperthermia selectively killed S-phase cells (Fig. 3). This result confirmed the findings reported previously (5–7). Hyperthermia reportedly increases nuclear protein content and changes association of protein and DNA (22–24). Chromosome aberrations induced by hyperthermia were observed only when cells in S phase were exposed to hyperthermia (25). These findings suggest that the structure of chromatin including DNA may become sensitive to heat during the DNA synthetic phase. From the synchronization experiments (Fig. 3), amiloride was found to sensitize cells to heat mainly in G1-S through middle S phase. Thus, amiloride appeared to induce direct or indirect enhancement of cellular heat damages in chromatin. Under the present conditions of acute treatment with amiloride, neither cell lethality nor change in S-phase cell frequency was observed. Therefore, it is unlikely that the thermosensitive S-phase cells selectively survive during the treatment with amiloride.

Some membrane active agents, e.g., local anesthetics, enhance thermal cell killing (26, 27), and further hyperthermia changes the membrane fluidity (28) and permeability (8, 29) of some drugs. These findings support the idea that one of the primary cellular targets of hyperthermia may be in plasma membranes. In addition to the inhibition of Na⁺/H⁺ exchange, amiloride was recently reported to inhibit protein kinase activity of the epidermal growth factor and insulin receptors in the plasma membrane (30). Hence, amiloride supposedly act on the plasma membrane to make cells sensitive to heat. Besides the effects on plasma membranes, inhibitory effects of this drug (0.4 mM) on RNA and protein syntheses are reported (31). Thus, amiloride may have multiple toxic effects in the enhancement of thermal cell killing.

Low pH sensitized cells to heat (Fig. 4) consistent with the findings reported (2–4). Under pH 6.6, amiloride further reduced the cell survival for 42°C hyperthermia. Thus, the thermosensitizing effect of amiloride was observed not only under neutral pH but also under acidic pH, where effects of low pH and amiloride appeared to be additive (Fig. 5). From these results, the synergistic interaction of these two factors, i.e., the extracellular low pH and amiloride, with 42°C hyperthermia apparently occurs through independent processes. However, since thermosensitization by the acidic condition reportedly increases with lowering of the extracellular pH from about 7.0 to 6.1 (4), a hypothesis, that both factors sensitizes cells to heat through the lowering of intracellular pH, is not contradicted.

The difference in thermosensitivity between normal and cancer (or transformed) cells is now considered not to be of significance (32). When hyperthermia is applied to cancer therapy, the relatively low pH usually seen in malignant tissues may favor the effectiveness of the hyperthermic treatment. Further, in this study, amiloride was shown to have a thermosensitizing effect under acidic pH as well as under normal pH. Thus, amiloride may be useful as a hyperthermic sensitizer in the clinical treatment of cancer. The mechanism(s) for the thermosensitization of amiloride should be further investigated.

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**REFERENCES**


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