Effects of Hyperthermia on Cell Surface Charge and Cell Survival in Mastocytoma Cells

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ABSTRACT

Changes in the structure of the surface of mastocytoma cells were induced by hyperthermia and were investigated by means of cell electrophoresis. A decrease in the cell electrophoretic mobility was detected as early as 15 min after treatment at 42°C and progressed more rapidly under hypoxic conditions than under oxic conditions. Subsequent recovery of electrophoretic mobility at 37°C was dependent on the length of heat treatment and oxygenation. The surviving fraction of cells detected by their colony-forming ability and the fraction of electrophoretically recovered cells 24 hr after exposure to hyperthermia showed a good statistical correlation. It was suggested that the mechanism of electrophoretic mobility reduction by heating was the vertical translocation of hyaluronidase-sensitive charge from the peripheral layer into a deeper layer by combined use of specific enzymes and stepwise different ionic strengths. These results suggest the importance of irreparable changes of membrane conformation in the loss of colony-forming ability of heated tumor cells.

INTRODUCTION

The finding that tumor cells are more sensitive to heat treatment than are corresponding normal cells (1) stimulated intensive investigation and practical use of hyperthermia in tumor therapy. Higher heat sensitivity of hypoxic tumor cells which are more resistant to radiation therapy than are oxic cells (5, 19) bears out the usefulness of hyperthermia. The mechanism of tumor cell killing, however, is obscure. The hypothesis that membrane-related damage can lead to cell death (22) is based upon the following findings: (a) change of membrane properties by hyperthermia such as transport of amino acid, ions, and polyamines (4, 9, 25), fluorescence of membrane protein (2), and labilization of lysosomal enzymes (8, 12); (b) modification of heat sensitivity of cells by such membrane-specific agents as amphotericin B and local anesthetics (6, 23, 24); and (c) dependency of thermal sensitivity on the lipid composition of membranes (3, 23). However, it was difficult to indicate an exact correlation between the change of the membrane properties and cell death, because these membrane changes were detected by averaging all the cells, whereas the cell survival on colony formation conforms to the "all or none" phenomenon of individual cells. This discrepancy was overcome by applying cell electrophoresis to detect the membrane change. Since the EPM is measured on individual cells under a phase microscope, the fraction of cells having membrane damage is easily obtained. Using this method, a good statistical correlation was obtained between the fraction of cells having unrepaired membrane changes and the fraction of non-colony-forming cells.

MATERIALS AND METHODS

Cells. Furth's mastocytoma cells, maintained as ascites type by serial transplantation, were used throughout the study as described in a previous paper (10). Cells in the logarithmic phase of growth in the abdominal cavity of C57L x A/Hem Fl mice were extracted with calcium- and magnesium-free phosphate-buffered saline. They were centrifuged and resuspended in Moist's Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% fetal calf serum. The cells were then cultured overnight in a humidified CO2 incubator at the concentration of 3 x 10⁶ cells/ml. This in vitro cultivation before the experiment resulted in a high and uniform EPM of cells. Viability of the cells after overnight incubation was 97 to 99%, and their cloning efficiency ranged from 44 to 102% with an average of 74%.

Treatment of Cells. The mastocytoma cells (2 x 10⁶) were suspended in 2 ml of fresh culture medium, introduced into glass test tubes, and immersed in a water bath at 42°C. Blowing of oxic gas (95% O₂ + 5% CO₂) or hypoxic gas (95% N₂ + 5% CO₂) onto the surface of slanted cell suspensions started 15 min before the heat treatment to saturate the gas phase at room temperature and continued during hyperthermal exposure. The blowing of gas containing 5% CO₂ maintained constant pH in the cell suspension.

After exposure to hyperthermia at 42°C for various durations, the treated cells were divided into 4 groups. Subsequent processes given for each group were: (a) immediate measurement of EPM; (b) measurement of EPM after cultivation at 37°C in a CO₂ incubator for 24 hr at a concentration of 3 x 10⁶ cells/ml; (c) culture in 0.4% agarose for 8 days to test the ability of the cells to form macroscopic colonies; and (d) dye exclusion tests using 0.05% trypan blue at various periods of culture at 37°C in a CO₂ incubator.

Cell Electrophoresis. The EPM of individual cells was measured at 25 ± 0.5°C (S.D.) with a Zeiss cytophoremeter as described in previous papers (14). Each cell was allowed to move 16 μm alternatively in both directions following the reversal of current in a 66.7 mM phosphate buffer (50.1 mM Na₂HPO₄ + 16.5 mM KH₂PO₄, pH 7.3; ionic strength, 0.167) supplemented with 5.4% sorbitol. For measurement of EPM at different ionic strengths, the phosphate buffer was diluted stepwise with water to 53.4, 40, 26.7, 13.3, or 6.7 mM and then supplemented with 5.4% sorbitol to maintain its constant viscosity (15).

To estimate the contributions of each of the glycosaminoglycans to the cellular EPM, enzymatic treatment of cells was performed as described in previous papers (15, 16). The enzymes used were neuraminidase from Vibrio cholerae (20 units/ml; General Biochemicals, Chagrin Falls, Ohio), chondroitinase ABC from Proteus vulgaris (1 unit/ml; Seikagaku Kogyo Co., Tokyo, Japan), or hyaluronidase from Streptomyces hyalurolyticus (10 turbidity-reducing units/ml; Seikagaku Kogyo Co., Tokyo, Japan). The hyaluronidase was homogeneous by Sephadex gel filtration or DEAE-cellulose chromatography, and the contents of protein and sugar were below 0.4 and 0.1 μg, respectively.

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2 To whom requests for reprints should be addressed.
3 The abbreviation used is: EPM, electrophoretic mobility.

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RESULTS

Decrease in EPM. Chart 1 shows the changes in the EPM with duration of treatment at 42°. EPM decreased evidently within 15 min of heat treatment. The EPM reduction was more rapid and profound under the hypoxic condition than under the oxic condition. When EPM was measured after incubation for 24 hr at 37° following the heat treatment, marked recovery in EPM was observed in cells heated under the oxic condition. No recovery was apparent in cells heated under the hypoxic condition.

Chart 2 compares the frequency distribution of EPM of nontreated cells and of cells exposed to 42° for 60 min under the hypoxic condition. The 2 distributions are separated by the boundary of EPM of -0.80 to -0.90 μm·sec⁻¹·V⁻¹·cm⁻¹. Cells for which EPM was greater than -0.90 μm·sec⁻¹·V⁻¹·cm⁻¹ were therefore designated as electrophoretically intact cells. Cells incubated for 24 hr after hypoxic exposure for 60 min showed more reduced EPM than did cells measured immediately after the exposure. No recovery in EPM was observed during the postincubation.

Chart 3 illustrates the timed recovery in the frequency distribution of EPM of cells heated 30 min under the oxic condition. Immediately after heating, the peak of the distribution of EPM was -0.80 to -0.90 μm·sec⁻¹·V⁻¹·cm⁻¹. The main peak returned to -1.0 to -1.1 during the next 24 hr at 37°, which was the same as the peak EPM in nontreated cells. Some fraction of cells retained the EPM below -0.90 μm·sec⁻¹·V⁻¹·cm⁻¹.

Cell Survival and EPM. The fraction of electrophoretically intact cells (EPM > -0.90 μm·sec⁻¹·V⁻¹·cm⁻¹) at 24 hr after treatment was plotted against the duration of hyperthermia in Chart 4 and 5. Both the surviving fraction of cells detected by colony-forming ability and the fraction of intact cells detected by dye exclusion tests are plotted in the same charts. The fraction of intact dye-excluding cells determined 24 hr after the heat treatment was always higher than the fraction of intact cells on the other 2 parameters. It required about 2 days until all of the nonsurviving cells lost their dye exclusion ability. The relationship between the intact fraction of cells on electrophoresis and the surviving fraction on colony formation was tested statistically. The coefficients of correlation (r) were 0.986 (n = 6) for oxic hyperthermia and 0.988 (n = 6) for hypoxic hyperthermia. These results suggest that the heated cells for which EPM did not recover within 24 hr of subsequent incubation lost their colony-forming ability.

Mechanism of EPM Change. In order to determine which acidic sugars of cell surface are responsible for EPM in the mastocytoma cells, measurement of EPM was carried out after the removal of 3 kinds of sugars from the cell surface by their specific enzymes. Table 1 indicates the substantial decrease in EPM of nontreated cells by neuraminidase and hyaluronidase, suggesting the presence of neuraminic acid and hyaluronic acid on the cell surface. Hyaluronidase, however, had no effects on EPM in the heated cells. There are 2 possible explanations for these results. One is the loss of negatively charged hyaluronidase-sensitive substance from the cell surface by the heat treatment, and the other is the dislocation of these negative charges from the periphery into a deeper layer so as not to contribute to EPM. The latter possibility was examined by measuring EPM using buffer solutions of lower ionic strength which allowed the charges of the deeper layer to contribute to EPM.
Membrane Change by Hyperthermia

Chart 4. Comparison of the fraction of intact cells detected by 3 different methods after different durations of heat treatment under oxic conditions. Trypan blue exclusion ability (○) and the EPM (●) were determined after 24 hr of subsequent incubation at 37°. The number of macroscopic colonies in 0.4% soft agarose was counted after 8 days of incubation at 37° (×). The coefficient of correlation (r) between the values on EPM and on colony formation was 0.986 (n = 6).

Chart 5. Comparison of the fraction of intact cells detected by 3 different methods after hyperthermal exposure under hypoxic conditions. The symbols are identical with those in Chart 4. The coefficient of correlation (r) between the values on EPM and on colony formation was 0.988 (n = 6).

Chart 6 indicates that digestion with hyaluronidase caused EPM reduction of similar magnitude at every ionic strength in nonheated cells. This result suggests the presence of hyaluronic acid only in the most peripheral layer. Decrease in EPM in heated cells by digestion with hyaluronidase was detected only at ionic strengths lower than 0.067. This result suggests the presence of hyaluronic acid only in the deeper layer. At low ionic strengths, 0.017 and 0.033, heated cells and nonheated cells showed similar EPM with or without the enzyme treatment. EPM of heated cells and EPM of the enzyme-treated nonheated cells showed the same values at the higher ionic strengths. These results suggest that hyaluronidase-sensitive negative charges are lost from the most peripheral layer but translocated to the deeper layers in the heated cells.

**DISCUSSION**

The decrease in EPM of heated cells, first observed by Redman et al. (13), was explained as a topographic change of charged groups and correlated statistically with the cell survival in this experiment. The increase in EPM with decreasing ionic strength has been generally observed (7). As the decay of potential with distance is less rapid in low-ionic strength solutions, the ionized groups embedded more deeply in the surface material exert their greater influence at lower ionic strength. The thickness of ion atmosphere is given according to Debye-Hückel's equation as 3.06 × (ionic strength)^-1/2 Å. If we use the thickness as an approximate estimation of the electrophoretic plane of shear (15), charged groups within the surface layer of 7.5-, 9.7-, or 17-Å thickness determine the EPM at the ionic strength of 0.167, 0.100, or 0.033, respectively. Detection of hyaluronidase-sensitive charges at every ionic strength in nonheated cells but detection of these charges only at ionic strengths below 0.100 in heated cells suggests the dislocation of these charges from the most peripheral layer.
of hyaluronic acid from the most peripheral layer of 7.5 Å into the deeper layer from 9.7 to 17 Å by heating.

Changes in the organization and biosynthesis of hyaluronic acid in respect to proliferation and killing of cells have been reported in some experimental systems (16). These are translocation of hyaluronic acid of cell surface in relation to cell killing after X-irradiation (17) and the stimulation of T-lymphocytes by lectins (18). Production of hyaluronic acid markedly changes accompanying the differentiation of chondrocytes (21) and the cell cycle of cultured mammalian cells (20). Biological roles of hyaluronate containing glycocalyx, however, are still unknown.

REFERENCES

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