Development of Thermotolerance in Mouse Fibroblast LM Cells with Modified Membranes and after Procaine Treatment

Antonius W. T. Konings

Department of Radiopathology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands

ABSTRACT

Mouse fibroblast LM cells have been modified with respect to the content of polyunsaturated fatty acyl (PUFA) chains of the membrane phospholipids. The membranes of the modified cells were enriched in PUFA chains and were more fluid as compared to the normal cells, as judged by fluorescence polarization measurements. The thermosensitivity of the PUFA-substituted cells was enhanced. Thermotolerance in the PUFA-substituted fibroblasts could be induced to the same extent as in the nonsubstituted cells. The thermosensitivity in both the PUFA and the nonsubstituted fibroblasts could be enhanced by the treatment of procaine.

Procaine could inhibit the triggering as well as the induction of thermotolerance. It is supposed that the mechanism of heat sensitization by procaine is different from the mechanism of preventing thermotolerance induction. The clinical implications of this finding are discussed.

INTRODUCTION

Hyperthermia is a promising treatment modality for cancer, especially in combination with radiotherapy. Radioresistant S-phase cells and radioresistant hypoxic cells appear to be selectively killed by heat (5). A problem in fractionated heat treatments is the development of thermotolerance. The mechanism of thermotolerance is not understood as yet, although to some degree protein synthesis seems to be involved because cycloheximide inhibits the appearance of heat resistance (7).

The primary target for heat lethality is not known. Some evidence has been presented for the involvement of membranes in cellular responses to hyperthermia (e.g., see Ref. 13). As early as 1924, Heilbrunn (6) proposed that the physical state of the fats might be related to the extent of cell killing by heat. Later (14), it was postulated that an upper limit of membrane fluidity exists with respect to cellular survival. The experiments of Yatvin (24) support the hypothesis that the fluidity of membranes might be a major factor contributing to the death of cells exposed to hyperthermia. Lepock et al. (12) concluded that there is a lack of correlation between hyperthermic cell killing and membrane fluidity, based on their experiments with the antioxidant compound butylated hydroxytoluene in a V79 Chinese hamster cell system. This conclusion has been heavily criticized, however, in a recent paper by Yatvin et al. (26). For mammalian cells, data are accumulating (Refs. 4 and 8; this study), suggesting a relation between changes in membrane fluidity as introduced by alterations in the growth medium of the cells and thermosensitivity.

The local anaesthetic procaine is a membrane-active drug which has been shown (24, 28) to enhance thermosensitivity. A direct correlation has been established between bacterial hyperthermic survival with anesthetic potency (27). Although cell membranes are well accepted as the site of local anesthetic action (18), the precise working mechanism is not known. Recent studies point to direct anesthetic:protein interactions as well as to direct anesthetic:lipid interactions (3). The latter may lead to disturbed protein configurations in an indirect way (2). Local anesthetics may increase membrane fluidity in model lipid systems (15) and increase the lateral mobility of membrane proteins of lymphocytes (21).

Substances which act on target structures for heat damage may interfere with the development of thermotolerance. It was the aim of this study to investigate the capacity of mouse fibroblasts in vitro to develop thermotolerance when the membranes of the cells were altered into a more fluid state and also to study the effect of procaine when present during the triggering or induction period of thermotolerance development.

MATERIALS AND METHODS

Cell Culture. Mouse fibroblast LM cells (CCL 1.2) from the American Type Culture Collection were adapted to growth in suspension culture in serum-free, lipid-free, and protein-free medium of Higuchi (9) modified to contain 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.4), 1 mg of methyl cellulose per ml, penicillin and streptomycin, and 10 µg of sodium dextrose sulfate per ml (17). The cells were cultured at 36.5°C in serum flasks at 130 rpm in a water bath. Modification of the composition of the cells was achieved as published previously (22).

Hyperthermia. Hyperthermia was performed in precision water baths (±0.05°C) under continuous gentle shaking of 15 ml cell suspensions in 50 ml Erlenmeyer flasks at concentrations of about 3 × 10² cells/ml. Temperatures were determined by thermocouple readings; calibrations were made against a standard precision mercury thermometer. All hyperthermic treatments given were corrected for the time required to reach the desired temperature. The correction applied was calculated from the graphs obtained by continuous temperature readout (10). Cells were made thermotolerant by incubation of 8 min at 44°C followed by 5 h at 37°C.

Cell Survival. To obtain quantitative dose-survival curves, the colony-forming ability of single cells was determined by a cloning technique described previously (22). The cells were plated in Petri dishes of 60 mm (Greiner, Nürtingen, Federal Republic of Germany) containing a layer of 0.5% Noble agar in RPMI 1640 (Flow, Irvine, Scotland). Appropriate dilutions were made from each incubation flask and mixed with 10⁶ feeder cells per plate. The feeder cells were mouse fibroblast LM cells which had been superapeutically irradiated with 100 Gy of X-rays. A set of at least 5 Petri dishes was used for each experimental condition. Each experiment was repeated at least 3 times. The Petri dishes were incubated at 37°C in a humidified incubator with 95% air and 5% CO₂ for about 14 days to obtain countable colonies. Control plating efficiencies were routinely around 60%. The survival curves were normalized to control plating efficiencies determined individually for each experiment.

Biophysical and Biochemical Assays. As an indication of fluidity,
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Chart 1. Effect of PUFA supplementation during cell growth on cell survival after hyperthermia with and without 10 mM procaine. Procaine was present only during the heat treatment and washed out directly thereafter. Points, mean of 4 to 5 determinations; bars, SE.

fluctuation polarization measurements on whole cells and isolated cellular membranes were performed with the aid of the probe DPH as described in previous studies (22, 23). The lipids of cells and of isolated membranes were extracted, and the fatty acid composition was determined by methods described before (22, 23).

RESULTS

Thermosensitivity of PUFA Cells and Effect of Procaine.

When mouse fibroblast LM cells are grown in a serum-free medium supplemented with 5,8,11,14-eicosatetraenoic acid (20:4, arachidonic acid), the amount of PUFA chains in the phospholipids increases from 6.6 to 35.9%, and the polarization value decreases from 0.244 to 0.153 (22). The change in PUFA as well as in fluidity was not restricted to whole cells but also observed in the isolated plasma and nuclear membranes as well as in the mitochondrial and microsomal fractions. The change in PUFA content in the different subcellular membranes was comparable with the changes observed in the phospholipids of the whole cells. The changes in fluidity, however, were less in the individual membranes as compared to the whole cells, probably because of the uptake of DPH in the cytoplasmic compartment of the cells. The actual data on membrane PUFA content and membrane fluidity are documented in a study published previously (23).

The cells with the more fluid membranes (PUFA cells) were more heat sensitive as compared to the cells of the normal parent line as can be seen in Chart 1. It was of interest to know if the local anaesthetic drug procaine could still further sensitize these thermosensitive cells. Addition of 10 mM procaine just prior to the heating of the cells could indeed further sensitize the cells. The extent of sensitization by procaine of the PUFA cells was comparable to that found for normal cells. This enhanced thermosensitivity is expressed on both the level of the shoulder ($D_0$) as well as the level of the slope ($D_0$) of the survival curve.

Development of Thermotolerance in PUFA Cells. Heating mouse fibroblast LM cells during 8 min at 44°C is not lethal to the cells and results in about 100% survival. This treatment, however, triggers the development of thermotolerance during incubation of the cells at 37°C. The fibroblasts with the more fluid membranes were still able to build up a state of thermotolerance after the triggering heat dose. This is shown in Chart 2. The extent of the acquired thermotolerance of the PUFA cells is comparable with the acquisition of thermotolerance seen in the normal cells. At the level of 10% survival, cellular heat resistance is increased by a factor of about 2.3 for both the normal cells and the PUFA cells.

Effect of Procaine on the Development of Thermotolerance.

When 10 mM procaine is present during the 8-min 44°C initiation period (triggering) of thermotolerance development (as normally seen in the control cells) and washed out directly thereafter, the thermotolerance development during the 5 h at 37°C is severely inhibited (Chart 3). The extent of acquired thermotolerance is routinely tested during a second heat treatment as shown previously (Chart 2). As control, experiments with the conditions of 8 min and 37°C in the presence of procaine have been performed. After washing these cells and incubating for 5 h at 37°C, no effect of the former procaine contact could be established in terms of heat sensitivity during the testing heat treatment. This means that the results of the experiments shown in Chart 3 are

The abbreviations used are: DPH, diphenylhexatriene; PUFA, polyunsaturated fatty acid.

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not caused by a direct action of residual procaine present during the second heat treatment but have to be ascribed specifically to the presence of procaine during the triggering heat treatment. The prevention of thermotolerance development was not complete when 10 mM procaine was used. Complete blocking of the initiation of thermotolerance could be achieved with 20 mM procaine present during the 8-min heating period (not shown). At this high concentration, however, cell survival after this first heat treatment was severely affected (survival < 20%), which was not the case when 10 mM procaine was used (survival > 60%). In Chart 4, it can be seen that thermotolerance development is also inhibited by procaine when the drug is present only during the induction period at 37°C. In this experiment, the cells were heated at 44°C for 8 min, followed by 1 h at 37°C. At this time point, different concentrations of procaine were added to the cells, and the incubation at 37°C was continued for 4 h. Hereafter, the procaine was washed out, and the cells were tested at 44°C for the times indicated in the chart. Thermotolerance development could completely be blocked by 20 mM procaine when this drug was present during the last 4 h of thermotolerance induction at 37°C. As can be seen in the chart, the inhibition of thermotolerance development was drug dose dependent. Procaine was not added directly after the triggering heat dose in order to be sure that, in these experiments, the effect of the drug was studied solely during the induction period and not during a last part of the initiation of thermotolerance. Therefore, 1 h at 37°C in the absence of procaine was allowed, before the drug was added to the cell system. It is our experience (not shown) that, with this cell line, almost no thermotolerance is induced within 1 h after the initiation period. The development starts after about 60 to 90 min, thus, in a period that procaine is present in the system.

DISCUSSION

Membrane Fluidity in Relation to Thermosensitivity and the Acquisition of Thermotolerance. There is no general agreement in the present literature (13, 26) on the importance of membrane fluidity in the mechanism of thermosensitivity and the development of thermotolerance. In this paper, it is shown that fluidizing the membrane of the mouse fibroblasts by an enhanced incorporation of PUFA coincides with an increased thermosensitivity. The PUFA content of the phospholipids in the plasma membrane was raised from 4.7 to 44.3%, while the fluorescent polarization value of DPH in the plasma membrane was decreased from 0.247 to 0.223 (for further details, see Ref. 23). This enhanced membrane fluidity corresponds with an increased thermosensitivity by a factor of about 1.5 at the level of 10% survival (Chart 1). On the other hand, when mouse fibroblasts are made thermotolerant, a decrease in thermosensitivity by a factor of about 2.3 (at the 10% survival level; Chart 2) can be measured without any effect on membrane lipid composition and membrane fluidity (11). These results indicate that, at least for mouse fibroblasts, thermosensitivity is not causally related to membrane lipid composition per se. The fact that the cells with the more fluid membranes were equally capable of developing thermotolerance (Chart 2) supports this idea.

The observation (11) of thermotolerance development without concomitant changes in membrane lipid composition and membrane fluidity leads to the supposition that the underlying molecular process is different from the process of thermal adaptation. It has long been recognized (1, 19, 20) that this acclimation to temperature (homeoviscous adaptation) is often accompanied by changes in membrane lipid composition and membrane fluidity of the cells. The process of homeoviscous adaptation generally takes place over longer periods of time (days, weeks) and involves many cell generations. Thermotolerance development takes place within h and during one cell generation.

Difference between PUFA Substitution and Procaine Treatment in Relation to Thermosensitivity and Development of Thermotolerance. Substitution of cellular membranes with PUFA, resulting in an increased membrane fluidity, as well as treatment of the cells with procaine sensitizes the cells for hyperthermic treatments. The fact that procaine inhibits the development of thermotolerance, in contrast to PUFA substitution, indicates that, in the latter case, the mechanism of action of both treatments is different. Apparently, fluidizing the membranes as such is not sufficient to prevent the development of thermotolerance. It may very well be that the molecular mechanism by which procaine sensitizes the cells for heat damage and the mechanism by which procaine inhibits the development of thermotolerance are essentially different. The first action may be made directly on the plasma membrane or membrane-related structures (cytoskeleton), comparable with the fluidizing action of PUFA substitution (Chart 1). In this case, conformation changes of membrane proteins by PUFA substitution or procaine treatment may be the analogous mechanism of action. The second action of procaine (preventing the state of thermotolerance) may be related to actions on nonmembrane targets. The possibility of the inhibition of heat-shock (membrane) protein synthesis by procaine is an interesting one and currently under investigation.

Prevention of Thermotolerant Development. Nontoxic doses
of the membrane local anaesthetic drug, procaine, can inhibit the in vitro development of thermotolerance. Experiments are in progress to investigate the potential of other local anaesthetics and “membrane-active” drugs in preventing thermotolerance in in vitro cell cultures as well as in tumor-bearing mice.

In vivo experiments with the local anaesthetic, lidocaine, have shown that tumor regression is enhanced after a hyperthermic treatment of mouse tumors in the presence of the drug (16, 25). In these experiments, the serum levels of lidocaine necessary to achieve tumor regression were within the therapeutic range for the control of arrhythmia in humans.

The possibility of preventing thermotolerance development by relatively harmless substances is of clinical importance. More heat doses may be applied during fractionated radiotherapy when thermotolerance is absent. As a consequence, the total radiation dose necessary to sterilize the tumor may be reduced substantially. Further studies will be necessary to verify this possibility.

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