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Damaging action of photodynamic treatment in combination with hyperthermia on transmembrane transport in murine L929 fibroblasts

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Photodynamic treatment of murine L929 fibroblasts with hematoporphyrin derivative caused inhibition of the 2-aminoisobutyric acid transport system. This was reflected by an increase in the apparent K_m with a constant V_{max}, indicating impairment of the carrier function rather than a decrease of the number of transport sites. Hyperthermic treatment of these cells resulted in a moderate decrease of the activity of the 2-aminoisobutyric acid transport system. Overall protein synthesis was severely inhibited both by photodynamic treatment and by hyperthermia. Hyperthermia subsequent to photodynamic treatment resulted in an additive inhibition of 2-aminoisobutyric acid transport and of protein synthesis. After photodynamic treatment both 2-aminoisobutyric acid transport and protein synthesis were repaired. The repair of 2-aminoisobutyric acid transport depended on protein synthesis, as shown by the virtually complete blockage of repair by anisomycin. After hyperthermia (either alone or subsequent to photodynamic treatment), no recovery of 2-aminoisobutyric acid transport was observed, although protein synthesis was restored to the initial level. Apparently, hyperthermia subsequent to photodynamic treatment blocks the repair of photodynamically induced damage of this transport system. The experimental results further indicate that protein synthesis is not the rate-determining step for the repair of 2-aminoisobutyric acid transport, although it is necessary in this process. Cell survival was decreased both by photodynamic treatment and by hyperthermia. The combined effects of these two treatments were additive. It is discussed that these results indicate that photodynamic inhibition of 2-aminoisobutyric acid transport is not causally related to loss of clonogenicity, contrary to earlier suggestions.

Introduction

Activation of photosensitizers by light results in photooxidation of proteins, lipids and nucleic acids both in model systems and in intact cells [1–5]. The introduction of photodynamic therapy of tumors with HPD as sensitizer triggered an increasing interest in photodynamically induced cellular damage, ultimately leading to loss of clonogenicity (for reviews, see, for example, Refs. 6, 7). In previous papers it was shown that membrane transport systems and DNA repair systems of murine L929 fibroblasts are very sensitive to photodynamic treatment [8,9], whereas several other parameters, for example, activities of cytosolic, mitochondrial and lysosomal enzymes, are far less sensitive [10].

Despite these and many other studies, a causal relationship between a particular type of photodynamic damage and loss of clonogenicity has not yet been unambiguously established. If a particular type of photodynamic damage is directly involved in loss of cell viability, some predictions can be made. First, it should be expected that, under varying experimental conditions, the type of damage suspected to be responsible for cell death varies in the same manner as the loss of clonogenicity. Moreover, it can be anticipated that the damage responsible for the loss of clonogenicity is not repaired readily during incubation subsequent to the damaging treatment [10,11].

It has been suggested previously that photodynamic damage of amino-acid transport systems may be causally related to cell death [8,12]. These suggestions were based on the observation that these transport systems

Abbreviations: HPD, hematoporphyrin derivative; DPBS, Dulbecco's phosphate-buffered saline.

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are extremely sensitive to photodynamic treatment, but not on the above-mentioned considerations. Therefore, the photodynamically induced inhibition of 2-aminoisobutyric aicd transport of L929 fibroblasts was reinvestigated in this context.

Materials and Methods

NCTC mouse fibroblasts, clone L929 and ATCC number CCL1 were obtained from Flow Laboratories. Tissue culture products and newborn bovine serum were obtained from Gibco. Hematoporphyrin derivative was purchased from Photofrin Medical, Cheektowaga, NY. 2-Amino[1-^{14}C]isobutyric acid, L-[U-^{14}C]leucine, L-[U-^{14}C]serine, L-[U-^{14}C]lysine, L-[U-^{14}C]tyrosine, L-[U-^{14}C]phenylalanine and L-[U-^{14}C]aspartic acid were obtained from Amersham International. Pico-Fluor 30 was from Packard Instruments. Anisomycin was purchased from Sigma. All other chemicals were analytical grade and were used without further purification.

Cell culture conditions, incubation with 10 μg HPD/ml followed by illumination of the cell layers and determination of the porphyrin content of the cells, have been described previously [8,10]. Illumination was performed under carefully controlled conditions, so that the observed damage was caused by irradiation only and not by other effects, like heat. Hyperthermic treatment was performed by placing a culture dish containing a monolayer of cells covered with 2 ml DPBS in a temperature-controlled waterbath (44.0 $\pm$ 0.1°C). To evaluate repair of 2-aminoisobutyric acid transport, cells were incubated in culture medium at 37°C in a CO_2-incubator for periods up to 26 h following the damaging treatment. Amino-acid uptake was determined by incubating the cell layer with 2.5 μM substrate in DPBS for 5 min at room temperature on a shaker in total darkness [9]. Uptake of all amino acids studied was linear with time up to at least 30 min.

To measure the rate of protein synthesis, the cells were incubated in a culture medium comprising 0.5 μCi L-[U-^{14}C]leucine and unlabeled L-leucine to a final concentration of 0.02 mM (2 h, 37°C, 5% CO_2). At the end of the incubation period, extracellular leucine was removed by washing the cell layer three times with 4 ml of ice-cold DPBS. The cells were scraped off in water and NaOH was added to a final concentration of 1 M. To determine the amount of L-leucine incorporated into proteins, the method of Grollmann was used [13].

The protein concentration was determined according to Lowry et al. [14]. Lipid peroxidation was assayed by measuring thiobarbituric acid-reactive products at 532 nm [15]. Clonogenicity was measured by inoculating treated cells in culture dishes with a diameter of 60 mm to a density of 20 cells/cm^2. After 7–8 days in a CO_2 incubator the colonies were fixed in methanol/acetic acid (3:1, v/v). Colonies were stained by incubation with 3% Giemsa in 10 mM phosphate buffer (pH 7.0). At least three culture dishes were counted in each experiment. For all measurements presented in this paper values were within the range of $\pm$8%, with two to four independent experiments for each datum point.

Results

In control experiments it appeared that illumination of cells without HPD or treatment with HPD in the dark did not have any effect on the parameters (including the hyperthermia effects) described in this study.

Incubation during 1 h with 10 μg of HPD/ml DPBS resulted in an intracellular porphyrin concentration of 1.1 μg/mg protein [10]. The influence of washing with DPBS, illumination, hyperthermic treatment and hyperthermic treatment subsequent to washing or illumination on the porphyrin content of cells is shown in Fig. 1. Washing for 30 min with DPBS in the dark at room temperature, or illumination, did not result in a change of the intracellular porphyrin concentration. The cells rapidly lost 30% of the accumulated porphyrins, however, during the first 10 min of hyperthermic treatment, whereas longer times at 44°C resulted in a more gradual further loss (Fig. 1). Hyperthermic treatment subsequent to washing or illumination resulted in similar curves to those obtained after hyperthermia alone (not shown).

Illumination of HPD-containing cells for periods up to 30 min did not cause detectable lipid peroxidation. Exposure of the cells to 30 mM H_2O_2 during 10 min, on

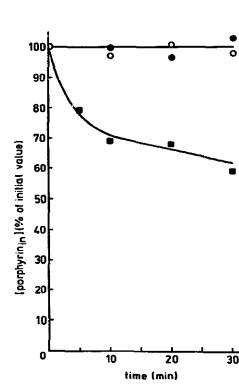

Fig. 1. Influence of washing with DPBS, illumination and hyperthermia on the intracellular porphyrin content of L929 fibroblasts, after incubation with HPD. ○, washing with DPBS at room temperature; ●, illumination; ■, hyperthermia.

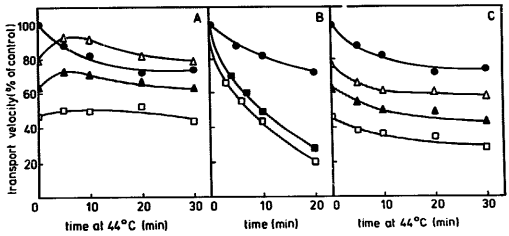

Fig. 2. Influence of photodynamic treatment, hyperthermia and the combination of these treatments on the 2-aminoisobutyric acid transport velocity into L929 cells. (A) Hyperthermia followed by photodynamic treatment. ●, hyperthermia alone; △, hyperthermia followed by 3 min of illumination; ▲, hyperthermia followed by 6 min of illumination; □, hyperthermia followed by 10 min of illumination. (B) ●, hyperthermia alone; ■, photodynamic treatment alone; □, illumination during hyperthermia. (C) Hyperthermia subsequent to photodynamic treatment. ●, hyperthermia alone; △, 3 min of illumination followed by hyperthermia; ▲, 6 min of illumination followed by hyperthermia; □, 10 min of illumination followed by hyperthermia.

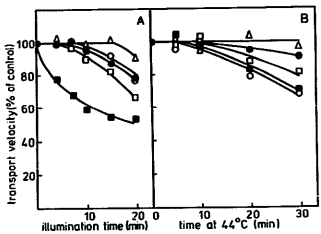

Fig. 3. Photodynamic (A) and hyperthermic (B) effects on the transport velocity of several amino acids. ●, L-tyrosine; ○, L-phenylalanine; ■, L-serine; □, L-lysine; △, L-aspartic acid.

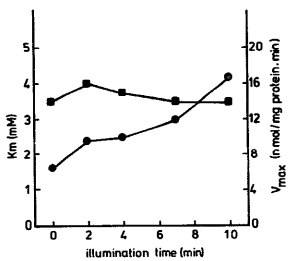

Fig. 4. Effect of illumination on the kinetic parameters of 2-aminoisobutyric acid transport in HPD-loaded L929 fibroblasts. ■, V_{max}; ●, K_m.

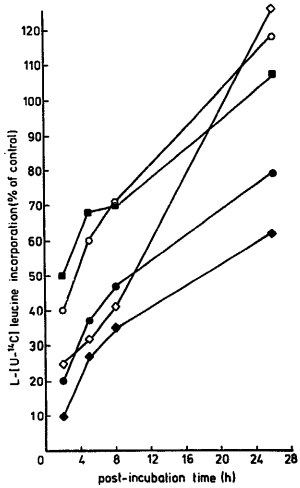

Fig. 5. Repair of protein synthesis (L-leucine incorporation into protein) after photodynamic treatment, hyperthermia and hyperthermia subsequent to photodynamic treatment. ○, 10 min of illumination; ●, 20 min of illumination; ■, 10 min hyperthermia; ◇, 10 min hyperthermia subsequent to 10 min of illumination; ◆, 10 min hyperthermia subsequent to 20 min of illumination.

the other hand, resulted in pronounced accumulation of thiobarbituric acid-reactive products (0.315 A_{532} units/mg protein).

2-Aminoisobutyric acid influx was linear with time up to 40 min and the substrate was accumulated in L929 fibroblasts, indicating energy-dependent, active transport [8]. The influence of illumination, hyperthermia and the combination of these two treatments on the transport velocity into HPD-containing cells is shown in Fig. 2. Photodynamic treatment of the cells resulted in a strong inhibition of transport, whereas hyperthermia caused a 25–30% decrease of the transport velocity. Hyperthermic treatment before illumination caused an inhibition that was clearly less than additive (Fig. 2A). When hyperthermia was applied during or after illumination, however, an additive effect was observed (Figs. 2B and 2C).

The effect of photodynamic treatment and hyperthermia on several other amino-acid transport velocities is depicted in Fig. 3. Transmembrane transport of serine appeared to be most susceptible to photodynamic treatment (Fig. 3A), whereas the other amino-acid transport systems were considerably less sensitive. A 30 min hyperthermic treatment resulted in inhibition of amino-acid transport velocities by 0–30% for the different amino acids (Fig. 3B).

Uptake of 2-aminoisobutyric acid always showed saturation kinetics, indicating that passive diffusion was negligible. Kinetic analysis further revealed that the inhibition of 2-aminoisobutyric acid transport by photodynamic treatment was caused by an increase of the apparent K_m value, whereas V_{max} remained unchanged at 14 nmol/mg protein per min (Fig. 4). The K_m value increased gradually during 10 min of illumination from 1.6 to 4.2 mM.

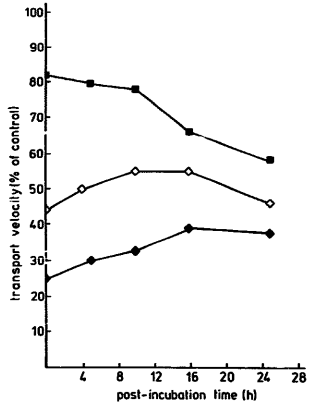

Fig. 7. Repair of 2-aminoisobutyric acid transport after hyperthermia alone, and after hyperthermia subsequent to photodynamic treatment. ■, 10 min hyperthermia; ◇, 10 min hyperthermia, subsequent to 10 min of illumination; ◆, 10 min hyperthermia, subsequent to 20 min of illumination.

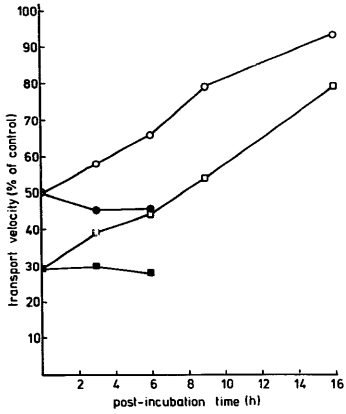

Fig. 6. Repair of 2-aminoisobutyric acid transport in the presence (closed symbols) or absence (open symbols) of 1.25 μM anisomycin, subsequent to photodynamic treatment. ○, ●, 10 min of illumination; □, ■, 20 min of illumination.

Overall protein synthesis was determined by measuring leucine incorporation. This incorporation was inhibited after illumination and after hyperthermic treatment of the cells (Fig. 5). Illumination periods of 10 and 20 min resulted in an inhibition by 60 and 80%, respectively, whereas hyperthermia during 10 min caused a decrease by about 50%. Hyperthermia subsequent to photodynamic treatment resulted in an additive effect (Fig. 5).

Incubation subsequent to photodynamic treatment (with illumination times up to 20 min) resulted in complete recovery of the 2-aminoisobutyric acid transport system (Fig. 6). The velocity of recovery appeared to be independent of the illumination time. Anisomycin (1.25 μM) completely blocked repair of the transport system (Fig. 6). Overall protein synthesis was inhibited 95% at this anisomycin concentration. In the presence of anisomycin, only relatively short incubation periods could be used, as anisomycin caused detachment of the cells from the bottom of the dishes after about 6–8 h.

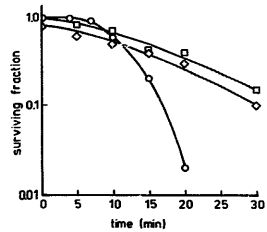

Fig. 8. Influence of photodynamic treatment, hyperthermia and hyperthermia, subsequent to photodynamic treatment, on the survival of L929 cells, ○, photodynamic treatment; □, hyperthermia; ◇, 8 min of photodynamic treatment followed by hyperthermia.

After hyperthermia (either alone, or subsequent to photodynamic treatment) no, or only a very limited, repair of 2-aminoisobutyric acid transport occurred (Fig. 7).

Under all experimental conditions, the overall protein synthesis recovered after damaging treatments, as shown in Fig. 5. The velocity of recovery after photodynamic treatment appeared to be independent of the illumination time.

Cell survival after various treatments of L929 cells is shown in Fig. 8. Illumination of HPD-containing cells during 8 min with subsequent hyperthermia resulted in a survival curve parallel to the survival curve after hyperthermia alone (Fig. 8).

Discussion

In the photodynamic action of HPD on L929 fibroblasts, inhibition of active membrane transport systems [8], DNA repair systems [9] and protein synthesis (this paper) appeared to be early events, in contrast to, for example, inactivation of intracellular enzymes, which occurred only after considerably longer illumination periods [10]. A detailed study of the mechanism of inactivation of these highly susceptible parameters and of their possible repair seemed appropriate in trying to elucidate the contribution of these early events to loss of clonogenicity.

It has been suggested that photodynamic inhibition of transmembrane amino-acid transport might be responsible for loss of clonogenicity [8,12]. This seems a priori plausible, as amino-acid transport systems are extremely sensitive to photodynamic damage and their inhibition might hamper, by substrate depletion, de novo protein synthesis and thus repair of photodynamically induced damage. The results presented in this paper indicate, however, that inhibition of amino-acid transport cannot be responsible for the strong inhibition of protein synthesis in L929 cells. Under the present experimental conditions, the inhibition of amino-acid transport was always much less that the inhibition of protein synthesis (Figs. 3 and 5).

Transport of 2-aminoisobutyric acid proceeds via the Na^+-dependent active 'A' transport system [16]. This transport system is much more sensitive to photodynamic treatment with HPD as sensitizer than the other amino-acid transport systems (Figs. 2B and 3), which were chosen because they represent the most abundant transport systems in eukaryotic cells [17]. Although Na^+-dependent transport systems and the membrane Na^+/K^+-ATPase are interdependent [18], the inactivation of the amino-acid transport system cannot be attributed to inhibition of the ATPase. This is indicated by the observation that preincubation with ouabain (1 mM), which completely abolished the ATPase activity, did not affect 2-aminoisobutyric acid transport in L929 fibroblasts [19].

Photodynamically induced inhibition of 2-aminoisobutyric acid transport is characterized by an increased apparent K_m and a constant apparent V_{max} (Fig. 4). The increased apparent K_m cannot simply be interpreted as a decrease of affinity of the carrier for the substrate, as the apparent K_m of cotransport systems is a composite constant containing, besides the affinity constant for the substrate, several other terms [20–22]. The unchanged apparent V_{max} suggests that photodynamic treatment does not completely inactivate carrier molecules, as this would most probably have been reflected by a decrease of the apparent V_{max} [20].

Inactivation of transport systems by oxidative stress may be caused by peroxidation of phospholipids, essential for enzyme integrity [23]. Such a mechanism is highly unlikely in the case of photodynamic inhibition of the 2-aminoisobutyric acid transport system in L929 cells as no generation of thiobarbituric acid-reactive products was found. The lack of accumulation of such products cannot be attributed to fast secondary reactions of these products because a pronounced generation of thiobarbituric acid-reactive products was observed during exposure of the cells to H_2O_2. Therefore, the inactivation of the transport system is most likely caused by direct photooxidation of susceptible amino-acid residues in the carrier molecule. The results shown in Fig. 6 are in accordance with such a mechanism of inhibition: the repair of transport activity after the damaging treatment depended on de novo protein synthesis, as demonstrated by the effect of anisomycin. On the other hand, protein synthesis was not the rate-determining step for repair of 2-aminoisobutyric acid transport. Another step, which is inhibited to a greater extent than protein synthesis, is necessary. This is indicated by the following observations. The repair rate was always about 3% per h (Fig. 6), irrespective of the illumination time, despite the fact that the primary inhibition of protein synthesis increased strongly with

increasing illumination periods. Further, the repair rate was rather constant over the entire incubation period (up to 16 h, Fig. 6), although the rate of protein synthesis increased strongly during that period (Fig. 5).

As compared to photodynamic treatment, hyperthermia had similar effects on 2-aminoisobutyric acid transport, protein synthesis and cell survival, with one important difference: the inhibition of 2-aminoisobutyric acid transport was not repaired. (Fig. 7). This lack of repair cannot be ascribed to inhibition of protein synthesis, as protein synthesis was always only partly inhibited and, moreover, was repaired. Thus, the inability of 2-aminoisobutyric acid transport to recover after hyperthermia is apparently caused by malfunctioning of a step beyond protein synthesis and this step must have been irreversibly damaged during heat treatment.

When hyperthermia was performed during or after photodynamic treatment, an additive effect was observed (Figs. 2, 5, 7 and 8). With hyperthermia prior to photodynamic treatment the effect of the two treatments taken together was less than additive (Fig. 2). This can presumably be explained by the rapid loss of porphyrins during hyperthermia (Fig. 1).

The combined effect of photodynamic treatment with subsequent hyperthermia on cell survival was additive (Fig. 8), contrary to the results with NHIK 3025 cells [24] and CHO-K1 cells (Boegheim, unpublished data), in which these two modalities of treatment were clearly synergistic. This indicates that the ultimate cause of reproductive cell death after photodynamic treatment, hyperthermia, or a combination of these two, may be different in different cell types.

Further, the results described strongly suggest that, although the 2-aminoisobutyric acid transport system and protein synthesis are very sensitive to photodynamic treatment, their inhibition does not play a crucial role in loss of clonogenicity of L929 fibroblasts. First, it seems highly unlikely that loss of clonogenicity would be causally related to damage that is fully repaired, like 2-aminoisobutyric acid transport and protein synthesis (Figs. 5 and 6). Further, in the case of 2-aminoisobutyric acid transport, with hyperthermia subsequent to photodynamic treatment, the primary effects on the transport system are additive (Fig. 7). However, the damage caused by photodynamic treatment alone was repaired, whereas the damage caused by photodynamic treatment with subsequent hyperthermia was not repaired. Therefore, if photodynamic inhibition of the 2-aminoisobutyric acid transport system were crucial for cell death, it should be expected that the combined effects of photodynamic treatment and hyperthermia would be synergistic with respect to loss of clonogenicity, which is clearly not the case.

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