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SHORT COMMUNICATION

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Photodynamic damage to HeLa cell microtubules induced by thiazine dyes

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Abstract The aim of this study was to analyze possible alterations of the microtubule cytoskeleton of cultured cells subjected to photodynamic treatments with the thiazine dyes methylene blue or toluidine blue. Indirect immunofluorescence labeling of α-tubulin was performed in HeLa cells after 1 or 18 h of incubation with thiazines followed by red-light irradiation for 15 min [leading to surviving fractions (SF) of about 65% (SF_{65}) or 1% (SF_1), respectively]. Untreated control cells showed the normal distribution of interphase microtubules, whereas considerable or severe disorganization of the microtubule network was observed after SF_{65} or SF_1 photodynamic treatments, respectively. A great amount of blebs showing homogeneous fluorescence was also found on the cell surface after SF_1 treatments. Possible mechanisms responsible for the photodamage to microtubules induced by thiazine dyes are briefly discussed.

Key words Microtubules · Photosensitizing agents · Methylene blue · Toluidine blue · Photodynamic therapy

Introduction

Many photosensitizers are currently under investigation for their applicability in the photodynamic therapy (PDT) of cancer. Although the photodynamic action on neoplastic tissues is not yet fully understood, it is accepted that exposure of the sensitizer to suitable light generates the highly reactive singlet oxygen ($^1O_2$), which is the main photoproduct responsible for cell death [28]. Several cell components are possible targets for $^1O_2$, including the plasma membrane [13, 23], nucleus and DNA [26, 27], lysosomes [9, 18, 25], mitochondria [11, 22], and microtubules (MTs) [2, 14, 24].

The increasing success of PDT [7, 21] has promoted the search for photosensitizers more selective and effective than hematoporphyrin-based agents. In this respect, thiazine dyes are promising drugs that show several advantages: (a) well-known physicochemical properties and vital stainability [10, 17]; (b) effective production of $^1O_2$ [3]; and (c) strong absorption of red light [10, 17], which penetrates deeply into the tissues. Photodynamic effects of thiazine dyes have been reported on cell cultures [4–6, 8, 18] and tumors [15, 29]. Taking into account the importance of cytoskeleton components in cell proliferation [1, 19] and the increasing evidence that microtubules (MTs) are specific targets involved in cell photokilling [2, 14, 16, 24], we studied the photodynamic effect of methylene blue (MB) and toluidine blue (TB) on the organization of MTs in cultured cells.

Materials and methods

The human HeLa carcinoma cell line was routinely grown on coverslips placed into 35-mm culture dishes using Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 50 units penicillin/ml, 50 μg streptomycin/ml, and 1% (w/v) L-glutamine (all products were obtained from Imperial Laboratories). Cell cultures were carried out at 37 °C in a humidified atmosphere containing 5% CO_2, and the medium was changed daily.

Stock 0.3-mg/ml solutions of MB (Fluka) and TB (Merck) were prepared in phosphate-buffered saline (PBS), sterilized by filtration through Millipore filters, and kept at –4 °C in a dark environment until use. Nearly confluent cell monolayers were treated for 1 or 18 h with 3-μg/ml solutions of either MB or TB in DMEM supplemented with 1% fetal calf serum. After incubation, the medium with the photosensitizer was removed, cells were washed three times in DMEM, and then new DMEM with 10% serum was added to the cultures.

Irradiation of control and thiazine-treated cells was performed for 15 min using a Kodak slide projector equipped with an Osram 250-W lamp. The light was filtered through a 3-cm water layer (to absorb heat) and a red filter ($\lambda > 600$ nm). The light intensity at the irradiation site was 100 mW/cm² (M8 Spectrum Power Energymeter). Our previous studies [4] had shown that after irradiation the surviving fractions (SF, in percent) of cells treated with MB or TB for 1 h were SF_{60} and SF_{75},

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Table 1 Estimation of the microtubular damage induced by thiazine dyes and red light (*RL* Red light)

Treatment	SF	MT damage[a] at			
		0 h	1 h	4 h	24 h
MB + RL	60%	+	++	++	–
TB + RL	75%	+	++	++	–
MB + RL	1%	+++	+++	+++	+++
TB + RL	1%	+++	+++	+++	+++

[a] The degree of MT photodamage (specifically, disorganization of the interphase MT network) was rated from +++ (highest) to – (none). The MT damage in control cells (lacking either thiazine treatment or red light) was negligible

respectively (about SF_{65}), whereas an SF_1 was found using MB or TB for 18 h. After 18 h of thiazine treatment in the absence of irradiation, values recorded for MB and TB were SF_{95} and SF_{98}, respectively, whereas an SF_{96} was found for irradiated but untreated cells [4].

At variable times after irradiation (0, 1, 4, and 24 h), cells were fixed in methanol at –20 °C for 10 min and hydrated in graded ethanol-PBS solutions. Cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 min and then treated with a 1:1000 dilution of mouse monoclonal anti-α-tubulin antibody (Amersham) for 1 h at 37 °C. After being washed in PBS, cells were stained with a 1:50 dilution of rabbit anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG (Southern Biotechnology Associates) for 1 h at 37 °C, rinsed again in PBS, and mounted in PBS-glycerol (1:4, v/v) containing 25 mg 1,4-diazabicyclo(2.2.2)octane/ml (Sigma). Fluorescence observations and photography were performed with an Olympus photomicroscope equipped with an HBO 100-W mercury lamp and a filter set for blue exciting light (490 nm).

Results

The organization of MTs in control cells and in cells subjected to conditions for SF_{65} and SF_1 was studied either immediately or at 1, 4, and 24 h after light exposure (Table 1). Control cells (lacking thiazine treatment, irradiation, or both) showed the bright yellow-green fluorescence characteristic of FITC decorating MTs. The normal network of MTs, often irradiating from the perinuclear (centriolar) area toward the cell edges, was found in interphase cells (Fig. 1a), whereas mitotic cells showed well-organized spindle MTs.

Immunolabeling results of treatment with the two thiazine dyes were very similar. However, the photodynamic damage to MTs was dependent on the incubation period as well as on the elapsed time after irradiation. Treatments for SF_{65} (1 h of dye incubation and 15 min of red light) perturbed the organization of MTs, the FITC fluorescence appearing scarcely structured and often being confined to the perinuclear region. The maximal effect on interphase MTs was observed at 1 and 4 h after irradiation, most cells showing a round shape. Few mitotic cells were found immediately or at 1 h after an SF_{65} treatment, and a slight accumulation of metaphases was observed after 4 h. Recovery to the normal interphase MT network and amount of mitotic cells was evident after 24 h.

Treatments for SF_1 (18 h of dye incubation and 15 min of red light) immediately induced a severe alteration in MTs. Most interphase cells appeared collapsed, no MT network could be detected, and only some perinuclear regions showed considerable but diffuse FITC fluorescence. A great amount of blebs (small cytoplasmic evaginations on the cell surface) appeared with homogeneous fluorescence (Fig. 1b), possibly due to depolymerization of MTs. Weak and diffuse fluorescence as well as no mitotic cell was also seen at 1, 4, and 24 h after SF_1 treatments.

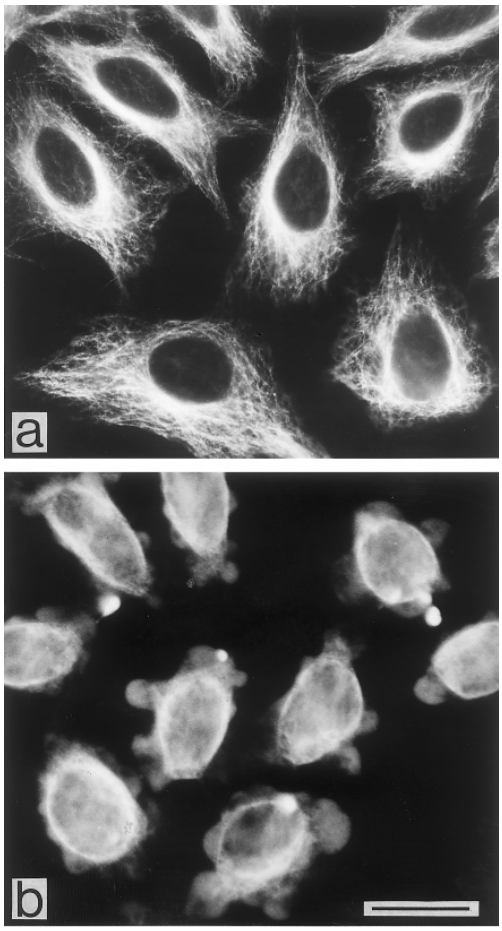

Fig. 1a, b Immunofluorescence labeling of α-tubulin in **a** control HeLa cells showing the normal network of interphase MTs and **b** cells subjected to an SF_1 photodynamic treatment with TB followed by immediate immunoprocessing. Disorganization of the MT network and occurrence of blebs filled with α-tubulin are clearly visible in **b**. Bar = 20 μm

Discussion

MTs are highly dynamic and drug-sensitive structures involved in the determination of cell shape, cytoplasm organization, organelle motility, and chromosome segregation [1]. Given that some antitumor drugs (e.g., vinca alkaloids, taxol) induce specific alterations in MTs [19], these cytoskeleton elements could be also important targets for photoinactivation of tumor cells in PDT. Alterations in cell shape and the formation of blebs [2, 14] can be

attributed to the photodamaging of cytoskeleton MTs, in particular those linked to the plasma membrane [12]. Immunodetection of α-tubulin shows that MB and TB induce severe photodamage to MTs in HeLa cells, leading to a reversible or irreversible disorganization of the interphase MT network after SF_{65} or SF_1 treatments, respectively, the latter also producing massive blebbing.

Although several studies on MT photosensitization have been carried out [2, 14, 16, 24], its precise cellular mechanism is not yet well known. The close proximity of MTs to other organelles (lysosomes, mitochondria, endoplasmic reticulum) in which sensitizers accumulate [4, 9, 16] has been purported to be responsible for MT photodamage [16]. However, since the action radius of $^1O_2$ produced by photodynamic effects is much shorter than 100 nm [20], damage to MTs that is due only to their proximity to other photosensitized organelles is unlikely, and other possible mechanisms for MT photodamage must be also taken into account.

In this respect, it is known that tubulins have hydrophobic binding sites for small aromatic ligands such as guanosine triphosphate (GTP), colchicine and derivatives, vinca alkaloids, griseofulvin, taxol, podophyllotoxin, and chlorpromazine [19]. Interestingly, chlorpromazine shares with MB and TB an aromatic phenothiazine ring, and it is therefore tempting to speculate that hydrophobic interactions of MB and TB with tubulins could be involved in MT photodamage. In addition, photosensitization of MTs could also be induced by thiazine dyes either free in the cytosol or bound to RNA in ribosomes. Although further studies are required to elucidate the precise mechanism responsible for cell death, disorganization of the MT cytoskeleton seems to be an important event in the photoinactivation of tumor cells by thiazine dyes.

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