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Detecting photosensitizing molecules at transmission electron microscopy by fluorescence photoconversion of diaminobenzidine

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Photodynamic therapy (PDT) is an effective and moderately invasive therapy based on the action of photosensitizers (PSs). These compounds exert their cytotoxic effect when excited by light at proper wavelengths [1] and this is due to their capability to preferentially dissipate the adsorbed energy through photochemical processes rather than by fluorescence emission. As a consequence of these photochemical processes, oxidizing chemical species are produced (singlet oxygen, free radicals or reactive oxygen species) which can injure cell structures, and induce either necrosis or other regulated forms of cell death [2].

For the cytotoxic effect to be induced, PSs must effectively enter the cell and localize to sensitive subcellular sites: in fact, the oxidizing chemical species which are produced upon irradiation of PS-loaded cells are very unstable and can only affect closely surrounding molecules. Localization of the photoactive molecules at a fine subcellular resolution is therefore crucial for detecting the target organelles and, consequently, for understanding the subcellular bases of damage and death of photosensitized cells.

The aim of the present investigation was to directly visualize PS molecules at transmission electron microscopy (TEM) exploiting the fluorescence properties of the photoactive molecules to obtain the photoconversion of diaminobenzidine (DAB) into an electrondense product.

Human HeLa cells were grown in 25 cm^2 flasks in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, in a humidified air atmosphere containing 5% CO_2. The cells were seeded onto glass coverslips 24 hr before being incubated for different times (5 to 60 min) in the dark with either 10^{-5} M Rose Bengal acetate (RB-Ac) or 10^{-6} M Hypocrellin B acetate (HypB-Ac): these molecules have been obtained by addition of two acetate groups to the PSs, RB and HypB, with the aim to increase their capability to enter the plasma membrane [3]. These modified compounds cannot serve, *per se*, as PSs (since the photophysical and photochemical properties of the native PSs are quenched), but they behave as fluorogenic substrates (FSs): after entering the cell, the acetate groups are removed by cell esterases, and the native molecules are restored with their fluorescence and photosensitizing properties.

At the concentrations used, these FSs are non-cytotoxic for cultured HeLa cells under dark conditions, while inducing massive cell death after light irradiation [4,5]. At the end of the incubation times, slides were removed from the medium, either to be immediately observed as fresh preparations by conventional or confocal fluorescence microscopy, or to be processed for TEM (see below). To investigate the phototoxic effects of either PS, some FS-loaded cell samples were submitted to light emission diode (LED) irradiation (FRAEN Company, Milan, Italy), using a 530 ± 15 nm LED for RB, and a 480 ± 15 nm LED for HypB; the total dose was 1.6 J/cm^2, with a uniform light fluency of 20 mW/cm^2 at the surface of the coverslips with the cells. In the attempt to detect early cell photodamage, cells were collected 5 to 30 min post irradiation (P-I).

For TEM, RB-Ac- or HypB-Ac-loaded HeLa cells on coverslips were fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4° C for 1h, washed and incubated with 3,3' diaminobenzidine (DAB) (20 mg/10 ml in 0.05 M Tris HCl, pH 7.6) under simultaneous irradiation with two 8W Osram Blacklite 350 lamps for 2 h (these lamps have two emission peaks of high intensity at 550 and 580 nm, thus being suitable for RB or HypB excitation); the cells were then post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide at room temperature for 1 h, dehydrated with acetone and embedded in Epon resin. As controls, some samples were processed as described above but omitting both DAB incubation and exposure to light. For better visualization of the reaction product, ultrathin sections were weakly stained with 2.5% aqueous solution of uranyl acetate for 2 min. The same procedures of fixation and embedding reported above

were used for HeLa cell samples treated with RB-Ac or HypB-Ac and submitted to light irradiation. The thin sections from these samples were stained with 4.7% aqueous solution of uranyl acetate for 5 min and Reynolds lead citrate for 2 min, to visualize the occurrence of organelle photodamage.

After short incubation times (2 to 5 min) with either FSs, brightly fluorescing spots were detected, initially near the plasma membrane and then in clusters close to the nucleus; for longer incubation times (30 min onwards) a weak diffuse fluorescence was also observed in the cytoplasm. Confocal microscopy demonstrated that photoactive molecules were never present in the nucleus even for long incubation times (up 60 min).

As for the distribution pattern of the reaction products and the organelles involved, comparable results were obtained at TEM for HeLa cells loaded with either FSs. At 5 min incubation, a fine granular reaction product was observed at the surface of invaginations of the plasma membrane as well as in vesicles which were often found near the plasmalemma; this proves that the FSs are already converted to the native PS molecules by membrane-bound esterases, and supports endocytosis as the main mechanism for the internalization of these FSs. At 15 to 30 min incubation times, most of the DAB reaction product was observed in multivesicular bodies and residual bodies, and for longer incubation times DAB positivity was also found in the cytosol, consistent with the evidence of diffuse fluorescence at light microscopy. On the contrary, DAB-photoconversion product was never found inside the nucleus.

After 5 to 15 min P-I of FS-laded cells, ruptures of the plasma membrane and of the limiting membranes of residual and multivescicular bodies were found. At 30 min P-I, also the microfilaments and the microtubules were found to undergo clustering and fragmentation, thus demonstrating the presence and action of photoactive molecules in the cytosol.

Since the pioneering paper by Maranto [6], a variety of fluorochromes proved to be DAB photo-convertible into reaction products for light and electron microscopy, and especially in recent years, this procedure has been used in correlative microscopy to investigate at the high spatial resolution of TEM the subcellular localization of specific fluorescently-labelled molecules [7,8]. The present report demonstrates that this procedure may be suitable to convert the fluorescence emission of a PS into an electron dense product.

The ultrastructural localization of photoactive molecules we have observed is fully consistent with the multiorganelle photodamage described after irradiation in culture of RB-Ac- or HypB-Ac-loaded cells [5,6]: both membrane-bounded organelles as well as cytosolic structures such as microtubules and microfilaments undergo massive photodamage eventually leading to necrotic or apoptotic cell death, depending on the FS concentration used.

The efficacy of PDT basically depends on the accumulation of photoactive molecules at specific subcellular sites where photodamage may trigger different forms of either regulated or catastrophic cell death [4,9]. DAB photoconversion promises to be an appropriate tool for directly visualizing the PS molecules at high resolution in single cells: this would allow to elucidate the mode of intracellular penetration and the dynamics of cytoplasmic redistribution and organelle targeting. It is worth noting that DAB deposits do localize in the very close proximity of the sites where photoactive molecules elicited the production of reactive oxygen species upon light irradiation: actually, the half-life of oxidizing chemical species such as singlet oxygen and superoxide or hydroxyl radicals is very short (from 1 ns to 1 □s) and their mobility extends to 1 to 30 nm only [10].

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