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Cell death causes relocalization of photosensitizing fluorescent probes

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ABSTRACT

When cultured cells are treated with fluorescent organelle probes or photosensitizer agents, a characteristic redistribution of fluorescence in cell structures occurs frequently after light irradiation. It is currently assumed that such changes, referred to as relocalizations of the fluorescent compounds, represent an important aspect of the photodynamic process, which is based on the excitation of photosensitizers by light in the presence of oxygen. As cell damage and death result from the oxidative stress induced by photodynamic treatments, we have studied here the redistribution of acridine orange (AO) and 3,3′-dimethyl-oxacarbocyanine ($DiOC_1(3)$) fluorescence after incubation of HeLa cell cultures with these compounds followed by blue light irradiation to achieve lethal effects. The relocalization of dyes from their original labeling sites (AO: lysosomes, $DiOC_1(3)$: mitochondria) to nucleic acid-containing structures (cytoplasm, nuclei and nucleoli) appeared clearly associated with cell death. Therefore, the relocalization phenomenon simply reflects fluorescence changes due to the different affinity of these dyes for living and damaged or dead cells. As fluorescent probes are often photosensitizers, prolonged light exposures using fluorescence microscopy will produce lethal photodynamic effects with relocalization of the fluorescent signal and changes in the cell morphology.

Introduction

It is widely known that during light irradiation, numerous fluorescent probes (FPs) used for selective organelle labeling of cultured cells are also capable of inducing severe photodamage followed by cell death. In these cases, the FP acts as a photosensitizer (PS), which transfers the energy of its excited singlet state to the triplet state, and then to molecular oxygen. Highly reactive oxygen species generated by this process produce oxidative stress and lethal effects on tumor cells, and this biological response is increasingly applied in photodynamic therapy (PDT) of cancer (Villanueva et al., 2003; Stockert et al., 2004; Cañete et al., 2009).

There is a striking difference in the fluorescence of cells when FPs and PSs are used as labeling or staining agents before or after fixation (Stockert, 1974; Horobin et al., 2006). Living cells treated with pyronin Y as FP only show mitochondrial fluorescence, but after continuous microscopic excitation or in cells with damaged plasma membrane, the emission is found in basophilic cytoplasm, nuclei and nucleoli (Cowden and Curtis, 1983), which agrees with the binding affinity of this dye for nucleic acids in fixed cells (Armas-Portela and Stockert, 1979).

In this context, when PSs are used in photosensitization protocols on cell cultures, a characteristic redistribution of fluorescence often occurs after light irradiation, which has been referred to as "relocalization" of the PS. It is currently suggested that this phenomenon represents an important process in cell photosensitization. Upon light irradiation, the PS dye methylene blue was seen to dramatically relocalize from the lysosomes of treated cells to the cytoplasm and then the nucleus (Rück et al., 1992; Walker et al., 2004). In the case of macrocyclic PSs, rapid fluorescence relocalization (sometimes with changes in emission yield) was found during or following light exposure of cells treated with porphyrins (Woodburn et al., 1991, 1992; Rück et al., 1992; Patito et al., 2001; Kessel, 2002; You et al., 2003; Sibrian-Marquez et al., 2007), porphycenes (Kessel and Luo, 1998; Kessel, 2004; Kessel et al., 2005; Mak et al., 2003) and phthalocyanines (Wood et al., 1997; Malik et al., 1997; Ball et al., 1999; Qualls and Thompson, 2001).

Interestingly, this redistribution only occurs after a period of illumination, so it should be considered as a photoinduced relocalization due to the release of PSs from their initial organelle targets following photodamage. In this context, conspicuous morphological changes occur in photosensitized cells showing relocalized PSs. Taking into account the differences between labeling and staining pattern of living and fixed cells, respectively, we have studied here the fluorescence redistribution of the well known photosensitizing dyes acridine orange and 3,3′-dimethyl-oxacarbocyanine after light irradiation at lethal doses.

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Our findings show that the relocalization of these PSs results from the damage and cell death just induced by photosensitization.

Material and methods

HeLa cells were grown on 22 mm square coverslips placed into 35 mm culture dishes using Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin and 1% (v/v) 0.2 M L-glutamine (complete DMEM; all products from Gibco, Paisley, Scotland, UK). Cell cultures were carried out at 37 °C in a humidified atmosphere containing 5% CO_2, and the medium was changed daily.

The photosensitizing and fluorescent probes acridine orange (AO) and 3,3′-dimethyl-oxacarbocyanine ($DiOC_1(3)$) (both from Sigma-Aldrich, St. Louis, MO, USA) were used for labeling of lysosomes and mitochondria, respectively. A small volume (0.1 ml) of 10^{-3} M dye solutions in distilled water was added to 10 ml of complete DMEM (final dye concentration: 10^{-5} M). Cell cultures were incubated in 2 ml of final dye solutions for 1 min ($DiOC_1(3)$) or 2 min (AO). Light irradiation of cell cultures in DMEM was performed after washing with phosphate buffered saline (PBS) or directly (without dye washing) for 25 min using a Reflecta slide projector equipped with a 150 W lamp. The light was filtered through a blue filter ($360 < \lambda < 460$ nm), and 3-cm water layer to absorb heat. The light intensity at the cell culture position was 7 mW/cm^2, as measured with an M8 Spectrum Power energy meter, corresponding to a light dose of 10.5 J/cm^2. After dye treatment, either alone or followed by light irradiation, cells were washed with PBS, mounted in DMEM and observed immediately. In some cases, cells were subjected to formaldehyde vapor fixation to allow permanent mounting (Cañete et al., 2001).

Microscopic observations and photography were performed with an inverted CKX31 microscope and a BX-UCB epifluorescence microscope (both from Olympus, Tokyo, Japan) equipped with an HBO 100 W mercury lamp and the filter sets for blue (460–490 nm, $DiOC_1(3)$) and blue plus green (460–490 nm+ 510–550 nm, AO) exciting light. Photographs were obtained with a digital camera (Olympus DP70) over a time period not more prolonged than 10 min. They were processed using Adobe Photoshop CS2 software (Adobe Systems, San Jose, CA). Image processing and analysis (IPA) procedures were performed with the public domain ImageJ 1.42 software (http://rsb.info.nih.gov/ij/). Pseudocolor options were used to break the continuous bright gradient of photomicrographs. The isolated red or green signals were converted to gray images and then visualized using a selected option from the lookup table of the IPA software. Conversion of original images to equidensity areas showing several levels in the black–white scale was also made using the Adobe Photoshop software. Convolved images (ImageJ) were obtained by means of the corresponding convolve filter (a matrix of 25 points (5 columns and 5 rows), the central point with a[+25] value and the remaining peripheral points with a[−1] value).

Thiazolyl blue (MTT, Sigma-Aldrich) was used for the assessment of cell survival 24 h after photosensitization treatments. A stock solution (1 mg/ml) in PBS was prepared immediately prior to use. Solutions (100 μl) were added to each culture dish (containing 2 ml of complete medium; final MTT concentration: 47.6 μg/ml). After 3 h incubation, formazan precipitates were dissolved in 1.5 ml DMSO and the absorbance was measured at 540 nm with a plate reader (SpectraFluor, Tecan, Männedorf, Switzerland). Cell survival was expressed as the percentage of absorption of treated cells in comparison with that of

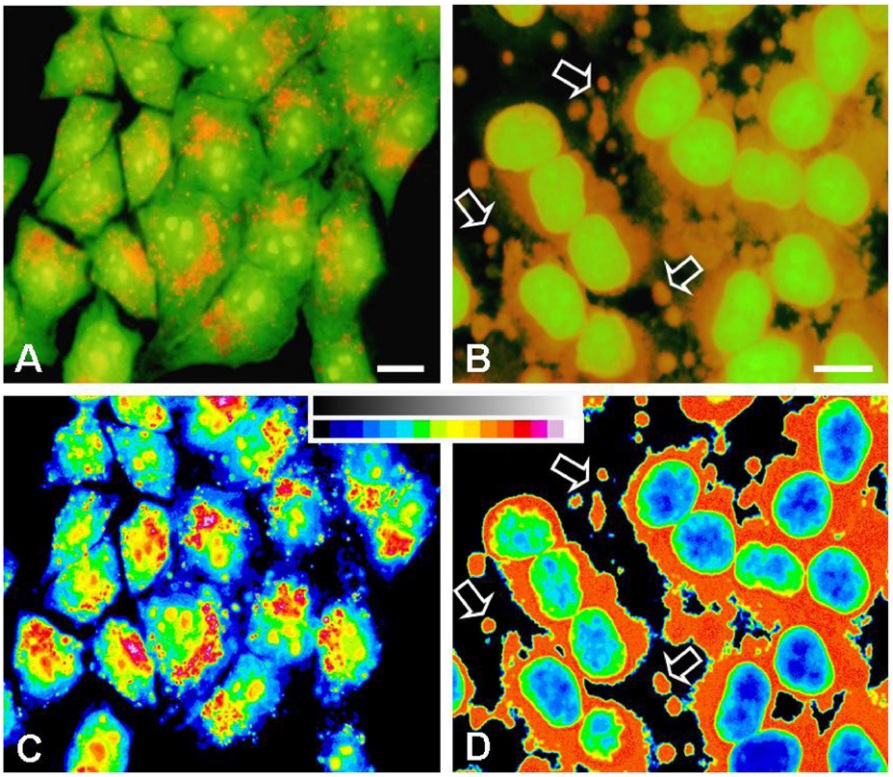

Fig. 1. Fluorescence micrographs of HeLa cells subjected to AO labeling. (A) Shows cells exposed to AO with no subsequent irradiation. (B) Shows the results of AO labeling followed by blue light irradiation, with membranous bubbles arrowed. The corresponding pseudocolor images (C, D, respectively) were generated after isolation of the red signal from the original pictures, conversion to gray images, and displayed with the 16 colors option from the lookup table of the ImageJ software (inset: gray-color code). Bars = 20 μm.