Photobleaching of Fluorescent Probe in Microfluorometry and Detection of Active Oxygen Species

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It is important to understand the cause of photobleaching and to know how to protect from the bleaching the fluorescent DNA probes stained cells from bleaching in microscopy. We examined the protective effect of sodium azide (NaN₃) from photobleaching on the micrometer and the detection of active oxygen species produced in the probe solution by photoirradiation using an electron spin resonance (ESR) with a trapping agent. It was found that the bleaching of several probes was protected 11-99% by adding 1 mM NaN₃ as a scavenger for singlet oxygen. The NaN₃-protecting efficiency for photobleaching on the microscope corresponded to the typical ESR signal intensity with the spin trapping agent for singlet oxygen. The OH radical produced in the probe-photosensitization was also detected by the ESR measurement with the another trapping agent. From the results, it was found that there were two types (predominantly, singlet oxygen or radicals) of active oxygen species produced at the probe-photoreactions. It was considered that active oxygen species produced from these photoreactions oxidized the probes to decrease the fluorescence intensity of the DNA probes used in the microscopy, and that the NaN₃ protected the photobleaching considerably without selfquenching of fluorescence.

Key words: Photobleaching, Microscopy, Active oxygen species, ESR, Photosensitizing reaction

I. Introduction

Although we have studied the quantitative detection of nuclear DNA contents [2, 3] in order to analyse the relative DNA ploidity patterns and DNA damage by fluorescent DNA probes or the analysis of Feulgen hydrolysis curves [1, 6, 9], the cause of photobleaching of the fluorescent DNA probes during microfluorometry has been unclear for a long time. The photobleaching of the fluorescent DNA probes stained cells on the microfluorometer has been a large problem for the correct measurements of the DNA contents of the cell because it tends to underestimate the DNA contents from the decreasing fluorescence intensity by bleaching. Furthermore, it is a problem for confocal laser fluoromicrometry that the photobleaching phenomena were also observed at the second scanning of the laser beam, especially, on viable cells or tissues stained by a fluorescent probe, for example, a free calcium ion fluorescent probe and a photosensitizer for photodynamic therapy of cancer.

We examined the protective effect of sodium azide (NaN₃) for photobleaching and the detection of active oxygen species produced in the probe solution after photoirradiation by means of an electron spin resonance (ESR) instrument using trapping agents in order to develop a method for understanding the cause of photobleaching and to avoid photobleaching during microfluorometry.

II. Materials and Methods

Measurement of the photobleaching rate on a fluorescence cytophotometer

Acridine orange (AO) propidium iodide (PI), para-rose aniline (Feulgen) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were used as fluorescent probes to stain the nuclear DNA of cells. The cells of tumor tissues of rats liver (AH-130), a human melanoma cultivated cells

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The fluorescence intensity of a stained cell was measured at the 1 or 2 sec intervals by the cytophotometers. 1 mM NaN₃ was added to the sample of the sealed solution under N₂-gas saturated condition as a scavenger for singlet oxygen. There were no quenching of the fluorescence of each probe solutions quantitatively when the 1 mM NaN₃ was added to them. 1 mM 2-mercaptoethylamine HCl was already contained in the case of sealed solutions of the DAPI samples.

**Detection of the free radical species produced in photosensitization by ESR and a spin trapping reagent**

2,2,6,6-tetramethyl-4-piperidone hydrochloride (TMPD) and 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) were used as a spin trapping reagent for singlet oxygen and OH radical species, respectively. The ESR apparatus was REX-3XR model (JEOL Ltd. Tokyo Japan). The relative yield of these radicals was normalized and was estimated from the ESR signal intensities of two types (TMPD-NO and DMPO-OH) from the calibration curve of the known concentration dependence of a standard nitroxide, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yl-oxy. The light source used for the photoirradiation in ESR measurement was an 1kW Hg-lamp. The intensity of monochromatic light through a monochromater (Shimadzu Inco. Ltd., Kyoto, Japan) was 4 mW/cm². The pH in the ESR sample solution was buffered at 8.0 controlled with 100 mM PBS.

**III. Results and Discussion**

**Photobleaching of the fluorescence intensity of probes stained in nuclear DNA on a fluorescence cytophotometer**

Fig. 1 shows that relative AO-fluorescence intensity decreased exponentially during irradiation time at

![Fluorescence decay curves of 5 μg/ml acridine orange (AO) stained AH-130 cells after 2N-HCl treatment at 30°C for 30 min.](image)

**Fig. 1**

![Percentage (% of Type I (radicals) and Type II (singlet oxygen) in each probe dye from the data of ESR measurement using spin trapping reagents (DEMPO and TMPD), respectively.](image)

**Fig. 2**

<table>
<thead>
<tr>
<th>Dyes</th>
<th>Slope (sec⁻¹)</th>
<th>Slope (sec⁻¹) N₂gas + 10⁻⁴ M NaN₃</th>
<th>Restraint (%)</th>
<th>Microscope (Model)</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO (F530)</td>
<td>1.470 × 10⁻³</td>
<td>6.791 × 10⁻⁴</td>
<td>(-53.8)</td>
<td>BH2-QRFL</td>
<td>AH-130</td>
</tr>
<tr>
<td>(F650)</td>
<td>2.954 × 10⁻³</td>
<td>8.865 × 10⁻⁴</td>
<td>(-70.0)</td>
<td>BH2-QRFL</td>
<td>AH-130</td>
</tr>
<tr>
<td>(F650)</td>
<td>1.237</td>
<td>1.287 × 10⁻²</td>
<td>(-99.0)</td>
<td>BH2-OSP1</td>
<td>HMF</td>
</tr>
<tr>
<td>DAPI</td>
<td>8.803 × 10⁻⁴*</td>
<td>7.799 × 10⁻⁴*</td>
<td>(-11.4)</td>
<td>VANOX-T AH-2</td>
<td>Liver (Rat 3w)</td>
</tr>
<tr>
<td>PI</td>
<td>1.060 × 10⁻⁴</td>
<td>2.335 × 10⁻⁵</td>
<td>(-78.0)</td>
<td>BH2-OSP1</td>
<td>Liver (Rat 3w)</td>
</tr>
<tr>
<td>Feulgen</td>
<td>1.065 × 10⁻³</td>
<td>—</td>
<td>(-70.8)</td>
<td>BH2-OSP2</td>
<td>Liver (Rat 3w)</td>
</tr>
<tr>
<td>Feulgen</td>
<td>3.111 × 10⁻⁴</td>
<td>—</td>
<td></td>
<td>BH2-QRFL</td>
<td>—</td>
</tr>
</tbody>
</table>

*2-mercaptoethylamine hydrochloride

The slope (sec⁻¹) of photofading for several probe dyes with and without 1 mM NaN₃ is listed in this table. Restraint (Inhibiting) percentage (%) by the NaN₃ for the slope without NaN₃, the model of microscope, and the name of the cell sample are also listed for several probe dyes in this table.
405 nm. The red-fluorescence (F650) intensity decreased more compared to the green (F530) one. When 1 mM NaN₃ was added to the sample, the decreases was inhibited 70% at 650 nm and 54% at 530 nm as shown in Table 1. The PI- and Feulgen-fluorescence intensity were also quenched 78% and 71% by adding 1 mM-NaN₃, respectively. In the case of DAPI, it was quenched less about 11%. It was concluded that the bleaching phenomena at AO-red, PI and Feulgen fluorescence were caused by singlet oxygen predominantly from the data of the NaN₃ quenching rates.

Detection of the free radicals produced in the photosensitization by ESR

The ESR signal intensities of the TMPO-NO produced from TMPD by singlet oxygen and the DMPO-OH produced by OH radicals were estimated from the data of hyper fine splitting constant (hfsc) in the previous papers [7,8,10] and were compared for each photosensitizations as shown in Fig. 2. These intensities were normalized with a standard as shown in the Materials and Methods section in this text. It was found that the new ESR spectrum (DMPO-DAPI radical) presented in DAPI reaction. The ESR signal intensity belonged to the radical group (Type I). It was found that the relative yields of radicals (Type I: DMPO-OH or DMPO-DAPI) and singlet oxygen (Type II: TMPD-NO) dependent on the fluorescence probes as shown in Fig. 2.

In conclusion, it needs to add the best scavengers depending on the photosensitizing reaction types [5,11] (Type I= free radicals; Type II=singlet oxygen) to prevent the photobleaching on the microfluorometry, for examples, NaN₃ for the predominant Type II reaction.

IV. Acknowledgments

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V. References