Bromodeoxyuridine amplifies free-radical-mediated DNA damage

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Elevated oxygen concentrations and paraquat, a superoxide-generating compound, induce an arrest of cells in the G2 phase of the cell cycle, which can be enhanced by adding bromodeoxyuridine (BrdU) to the culture medium. Experiments with the lipophilic peroxide cumene hydroperoxide and the free-radical scavenger vitamin E demonstrate that the BrdU-dependent G2 arrest is not mediated by lipid peroxidation. The BrdU-dependency of arrest in the G2 phase can be used as a sensitive cell biological assay to detect DNA damage elicited by oxygen free radicals.

INTRODUCTION

Complex and cumbersome methods to detect modified base residues and DNA strand breaks are required to assess the mechanisms by which oxygen free radicals

damage DNA [1]. By example, quantification of thio-barbituric acid-reactive material, resulting from degradation of deoxyribose after attack by hydroxyl radicals [2–4], can be proposed as a method of detecting the deleterious action of oxygen free radicals. As this putative

Fig. 1. Bivariate cytograms of human diploid fibroblasts cultured for 72 h at 5 or 35% oxygen

Panels on the left represent cultures exposed to 100 μ M-BrdU. The abscissa shows BrdU-quenched Hoechst 33258 fluorescence, and the ordinate displays unquenched ethidium bromide fluorescence. The first cycle (G0/G1, S, G2/M) is represented by the rightmost series of signal clusters, whereas the signals of the second and third cycle are progressively shifted leftwards due to the BrdU quenching of Hoechst 33258 fluorescence. The arrows indicate the cluster representing cells in the G2 compartment of the first cycle.

Abbreviations used: BrdU, bromodeoxyuridine; G2, G2 phase of the cell cycle; FBS, foetal-bovine serum; Chp, cumene hydroperoxide.

method may detect only a single type of DNA damage, it will be limited in its analytical power regarding the destructive effects of oxygen free radicals. A more global approach takes advantage of the observation that oxygen at concentrations higher than 5% (v/v) provokes a permanent arrest of cells in the G2 phase of the cell cycle [5,6]. The extent of this G2 arrest can be modulated by exposing cells to the radiosensitizer bromodeoxyuridine (BrdU) [6]. Here we demonstrate a putative synergism between oxygen free radicals and BrdU by using paraquat, a superoxide-generating compound [7,8]. Additional experiments with the lipophilic peroxide cumene peroxide and the free-radical scavenger vitamin E were performed to decide whether the BrdU-dependent G2 arrest is mediated by lipid peroxidation. The results of these experiments suggest that flow-cytometric measurement of BrdU-enhanced arrest in the G2 phase provides a sensitive assay for the biological action of oxygen free radicals at the level of a cell population.

MATERIALS AND METHODS

Confluent cultures of human diploid fibroblasts were rendered quiescent by exposure to 0.1% foetal-bovine serum (FBS) (Flow Laboratories) for 48 h. Subsequently, cells were trypsin-treated, plated at a density of 2500 cells/cm² and cultured for 72 h in Minimal Essential Medium (GIBCO), supplemented with 10% FBS and a

concentration series of BrdU and deoxycytidine (Sigma). Cell culture was performed at 37 °C in a humidified incubator in an atmosphere containing 5% (v/v) CO₂ and a regulated concentration of O₂. The desired oxygen concentration was obtained by mixing oxygen or nitrogen with the air in the incubator and continuous monitoring with a Clark-type oxygen electrode (Heraeus, Hanau, Germany). All culture flasks were carefully wrapped in aluminium foil to avoid exposure to light of short wavelengths. Cells were harvested by trypsin treatment under illumination with red light only. Subsequently, cellular DNA was stained with Hoechst 33258 and ethidium bromide, and cells were analysed by bivariate flow cytometry as described previously [9,10].

RESULTS AND DISCUSSION

Fig. 1 shows that the cellular Hoechst 33258 fluorescence intensity (on the abscissa) is quenched when BrdU is incorporated during the S phase of the cell cycle, whereas the fluorescence of ethidium bromide (on the ordinate) is unaffected by the incorporation of BrdU [9,10]. Thus resting cells and those that have traversed up to three sequential cell cycles can be distinguished [6,10]. The major difference between the cytograms from cultures grown at 5 and 35% oxygen is the relative number of cells in the G2 compartment of the first cycle (arrows). By selecting the appropriate region of the cytogram,

Fig. 2. BrdU-dependency of G2 arrest of cells cultured for 72 h at 5, 20 or 35% oxygen (O₂), at 5% oxygen with 5, 10 or 20 μM-paraquat (Pqt), at 5% oxygen with 10, 20 or 30 μM-Chp (c), or cells cultured with 1 i.u. of vitamin (Vit. E)/ml of culture medium in the absence or presence of 10 μM-paraquat (d)

Linear-regression lines were obtained from the data by a least-squares approximation, and slopes representing increase in the percentage of cells arrested in G2 with increasing BrdU concentration were calculated. Slopes are 0.007 and 0.008 for cells cultured at 5 and 20% oxygen respectively, and 0.026 for cells at 35% oxygen. Paraquat gave slopes of 0.016, 0.021 and 0.025 at 5, 10 and 20 μM-paraquat respectively. Further abbreviation: Cont., control.

reducing the data to a one-dimensional histogram, and cell cycle fitting with a conventional algorithm, the fraction of cells arrested within any particular cell cycle compartment can be computed [9]. In the experiment shown in Fig. 1 the arrest in the G2 phase at 5% oxygen amounts to 3.4%, but is increased to 8.2% at 35% oxygen.

Fig. 2(a) displays the BrdU-dependency of G2 arrest in cells cultured at 5, 20 and 35% oxygen. With increasing BrdU concentrations, cells maintained at 5 and 20% oxygen exhibit a slight linear increase in G2 arrest, whereas a greater increase occurred at 35% oxygen. To assess the hypothesis that oxygen provokes this BrdU-dependent G2 arrest through the generation of oxygen free radicals, cells were cultured with a concentration series of paraquat, which intracellularly generates superoxide radicals [7,8]. The increase in G2 arrest of cells maintained at 5% oxygen with 5, 10 and 20 μ M-paraquat is shown in Fig. 2(b). Paraquat induces a dose-dependent rise in G2 arrest with increasing concentrations of BrdU, akin to the effect of 35% oxygen. Also, bleomycin, provoking a free-radical attack on the deoxyribose moiety in DNA [11], elicited a BrdU-dependent G2 arrest (results not shown).

The observed BrdU-dependent arrest in G2 might be induced by a direct attack of oxygen free radicals upon DNA or might result from the induction of lipid peroxidation. The latter process can be investigated by using lipophilic organic peroxides, such as cumene hydroperoxide (Chp) [12,13], and vitamin E, a lipophilic free-radical scavenger. Fig. 2(c) shows the BrdU-dependent G2 arrest elicited by 10–30 μ M-Chp. The G2 arrest of cells cultured with Chp is slightly greater than that of the control culture at 5% oxygen, but does not significantly increase with BrdU concentration. Thus Chp fails to mimic the BrdU-dependent G2 arrest of elevated oxygen or paraquat. This result, however, does not rule out the possibility that elevated oxygen concentrations or paraquat, both inducing lipid peroxidation akin to diquat [14], may elicit a BrdU-dependent G2 arrest via this mechanism. Another test for lipid peroxidation as a putative mechanism of the BrdU-dependent arrest in G2 is whether the effect of paraquat is diminished by incubation with vitamin E. As shown in Fig. 2(d), vitamin E does not interfere with the BrdU-dependent G2 arrest induced by 10 μ M-paraquat. Therefore the BrdU-dependent arrest in G2 is not mediated by lipid peroxidation.

The enhancement of G2 arrest by BrdU is reminiscent of the increased vulnerability of BrdU-substituted DNA to irradiation [15], which has been related to elevated generation of thymidyl-like radicals resulting from free-radical attack upon DNA [16]. Our results suggest that cell culture in the presence of BrdU with subsequent bivariate Hoechst 33258/ethidium bromide flow cytometry constitutes a rapid and sensitive method to detect effects of oxygen free radicals in a biological assay system.

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