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Research Paper

Influence of oxygen partial pressure on the characteristics of human hepatocarcinoma cells

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ABSTRACT

Most of the *in vitro* studies using liver cell lines have been performed under atmospheric oxygen partial pressure (21% O_2). However, the oxygen concentrations in the liver and cancer cells are far from this value. In the present study, we have evaluated the influence of oxygen on 1) the tumor cell lines features (growth, steady-state ROS levels, GSH content, activities of antioxidant enzymes, p66 Shc and SOD expressions, metalloproteinases secretion, migration, invasion, and adhesion) of human hepatocellular carcinoma cell lines, and b) the response of the cells to an oxidant stimulus (aqueous leaf extract of the *V. baccifera* plant species). For this purpose, three hepatocarcinoma cell lines with different p53 status, HepG2 (wild-type), Huh7 (mutated), and Hep3B (deleted), were cultured (6–30 days) under atmospheric (21%) and more physiological (8%) pO_2. Results showed that after long-term culturing at 8% *versus* 21% O_2, the cellular proliferation rate and the steady-state levels of mitochondrial O_2^- were unaffected. However, the intracellular basal ROS levels were higher independently of the characteristics of the cell line. Moreover, the lower pO_2 was associated with lower glutathione content, the induction of p66 Shc and Mn-SOD proteins, and increased SOD activity only in HepG2. This cell line also showed a higher migration rate, secretion of active metalloproteinases, and a faster invasion. HepG2 cells were more resistant to the oxidative stress induced by *V. baccifera*. Results suggest that the long-term culturing of human hepatoma cells at a low, more physiological pO_2 induces antioxidant adaptations that could be mediated by p53, and may alter the cellular response to a subsequent oxidant challenge. Data support the necessity of validating outcomes from studies performed with hepatoma cell cultures under ambient O_2.

1. Introduction

In physiological conditions, oxygen supply and diffusion into tissues are necessary for survival. The oxygen partial pressure results from the balance between oxygen delivery into an organ and its consumption. Although the pO_2 at ambient atmosphere is equivalent to 21%, tissue oxygenation progressively decreases as it reaches internal organs and tissues [1]. The level of O_2 and its distribution among the various tissues depends on the rate of capillary blood flow and the tissue metabolic activity. Consequently, in humans under physiological conditions, the pO_2 in well-irrigated organs such as lungs, liver and kidneys, ranges from 4% to 14% [2,3]. The oxygen concentration in tumor cells is heterogeneous and depends on the distance of the cell from the blood vasculature. Cells that reside far away from blood vessels can even become hypoxic, receiving inadequate amounts of oxygen [4]. Most of the *in vitro* experiments using cell cultures are typically performed in atmospheric O_2 levels (21%), thus, in a non-physiological environment. An inadequate (absent or in excess) oxygen tension in cell cultures can result in the production of reactive oxygen species (ROS) and the induction of oxidative stress [5–7], with consequences on the cellular behaviour leading to cell growth or death [8]. The change in the redox status of the cell may alter the expression of antioxidant enzymes, cell proliferation, migration and invasion [8,9]. Oxygen finely regulates cell activity from the gene level to the proteome expression [10]. It has been reported that the long-term culturing of transformed human and murine myeloid cell lines under atmospheric oxygen levels (21% O_2) or more physiological pO_2 (5% O_2) induced significant differential phenotype changes in free surface thiol expression, total GSH content, and sensitivity to hydrogen peroxide [11].

The p53 tumor suppressor protein plays key roles in regulating cell-cycle and apoptosis. The protein regulates the expression of various mitochondrial-targeted genes that affect pro-apoptotic proteins, leading to cell death [12]. p53 also possesses potent redox-regulating activity through modulating various ROS-generating and antioxidant

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enzymes, particularly p66 Shc and MnSOD [13,14]. p66 Shc has recently emerged as a redox sensor that transmits oxidative stress signals to DNA damage in hepatocytes [15]. Activated p66 Shc is localized in mitochondria, where the molecule generates hydrogen peroxide to initiate the apoptotic cascade [16,17].

In a previous work, we described that an aqueous leaf extract of the Amazonian *V. baccifera* plant species induced intracellular accumulation of ROS and toxicity to several human hepatocellular carcinoma cell lines cultured under atmospheric O_2. Results suggested that oxidative stress was involved in cell death [18]. In the present study, we have evaluated the influence of the oxygen partial pressure on 1) the tumor features (growth, steady-state ROS levels, GSH content, activities of antioxidant enzymes, p66 Shc and SOD expressions, migration, invasion, metalloproteinases secretion, and adhesion) of human hepatocellular carcinoma cell lines, and b) the response of the cells to an oxidant stimulus (*V. baccifera* leaf extract). For this purpose, three hepatocarcinoma cell lines with different p53 status, HepG2, Huh7, and Hep3B, were long-term (6–30 days) cultured under atmospheric (21%) and more physiological (8%) pO_2. HepG2 cells carry wild-type p53, in Hep3B the p53 gene is deleted [19], and p53 expressed in Huh7 conserves around 4% wild type transactivating activity [20]. Data suggest that the long-term culturing of human hepatoma cells under low pO_2 induces antioxidant adaptations that may modify the cellular response to a subsequent oxidant challenge, and support the necessity of using low, more physiological oxygen tensions in culturing tumor cell lines to draw conclusions applied to cancer biology from *in vitro* studies.

2. Materials and methods

2.1. Reagents

Bis-(3-carboxy-4-nitrophenyl)-disulphide (DTNB), 3,4-dichloronitrobenzene (CDNB), glutathione, glutathione reductase, horseradish peroxidase (HRP), hydrogen peroxide, NADPH, nitro-blue tetrazolium (NBT), sulfosalicylic acid, trypsin, xanthine and xanthine oxidase (XOD) were all obtained from Sigma-Aldrich (St Louis, MO, USA). Anti-Cu,Zn-SOD antibody was purchased from Calbiochem (La Jolla, CA, USA), anti-Mn-SOD and anti-Shc antibodies from Millipore (Darmstadt, Germany), and Amersham ECL Western Blotting Detection Reagent from GE Healthcare (Chicago, Illinois, USA).

2.2. Culture and maintenance of cell lines

The human hepatoma cell lines HepG2, Huh7 and Hep3B were purchased from ATTC (American Type Culture Collection, Manassas, USA). These cells were maintained in Eagle's Minimum Essential Medium (EMEM) (ATCC) supplemented with 10% heat inactivated fetal bovine serum (FBS) (ATCC), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin (all from Sigma-Aldrich, St Louis, MO, USA). Shortly after establishment of consistent cell lines in 75 cm^2 flasks under 21% pO_2 at 37 °C in humidified atmosphere with 5% CO_2, each cell line was divided into two flasks and cultured under similar conditions except for the O_2 concentration (21% and 8% pO_2). Cells were cultured in a Thermo Fisher Scientific HERAcell incubator (Waltham, MA, USA) equipped with two gas monitoring systems, CO_2 and O_2/N_2 (nitrogen to reduce the oxygen levels). All media were preequilibrated to the O_2 conditions in the incubator before their use. All cell passages were performed quickly in the laminar flow cabinet when the cell monolayer reached around 75% of confluence. Cells were detached with a solution of 0.1% trypsin-0.04% EDTA and then harvested to perform subsequent experimentations. Cells adapted to the pO_2 regimen for a minimum of six days and a maximum of 30 days before the corresponding experiment.

The Ethical Committee for Researching with Biological Agents (CEIAB) from the University of the Basque Country, UPV/EHU, approved the protocol (M30_2015_2013_RUIZ SANZ).

2.3. Plant aqueous extract

The aqueous leaf extract of *V. baccifera* was prepared from infusions, as has been described in Lizcano et al. [21].

2.4. Cell proliferation assay

Cells cultured under both pO_2 conditions described in point 2.2 were seeded onto 96-well plates and cultured under both different oxygen conditions and at different cell densities (2,000, 2,500 and 3,000 cells per well). Their growth was registered every 24 h for 5 days, following the crystal violet stain method according to Gillies et al. [22]. This consisted in removing medium, washing the cells once with phosphate buffered saline (PBS) and fixing them for 15 min with a 3.7% formaldehyde solution. Then, the cells were washed twice with PBS and stained with a 0.25% crystal violet solution (Merck, Darmstadt, Germany) for 20 min in the dark. After this, plates were washed with running water and when they were dry, 150 µl of a 33% acetic acid solution was added in each well to dissolve crystal violet.

The absorbance was measured at 590 nm in a Synergy HT microplate reader (BioTek, Winooski, VT, USA). Considering that absorbance is proportional to the cell density, the obtained data were represented as exponential growth curves. Duplication times were derived from semi-logarithm representations of the absorbance *versus* the culture time, and were calculated using the following formula: $A=A_0\times 2^{t/DT}$; *DT* refers to the duplication time; *t* to the culture time and A_0 and A refer to absorbances at zero and at any time, respectively.

2.5. Intracellular ROS and mitochondrial O_2^- detection

Intracellular ROS levels were measured using the cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) probe (Molecular Probes, Eugene, OR, USA), which is deacetylated and oxidized inside the cell producing the 2′,7′-dichlorofluorescein (DCF) fluorescent compound. Cells cultured under both pO_2 conditions described in point 2.2 were seeded at a density of 2.5×10^5 cell per well onto 6-well plates and maintained under the two oxygen concentrations for additional 48 h, and before treatment (addition of *V. baccifera* extract). After that, cells were washed, resuspended in the corresponding medium (8% and 21% O_2) and incubated with H_2DCF-DA (20 µM) for 30 min at 37 °C in the dark. Then the probe solution was removed and, after washing with PBS, the cells were trypsinized and harvested to analyze the DCF fluorescence of the live cells by flow cytometry in a Beckman Coulter Gallios Flow Cytometer (λ_{exc}=485/20 and λ_{em}=528/20) in the General Research Services SGIker of the UPV/EHU (http://www.ikerkuntza.ehu.es/p273-sgikerhm/en/). At least 10,000 cells (events) were detected for each group. Data obtained from flow cytometry were analyzed using Summit 4.3 software (Dako, Hovedstaden, Denmark). Intracellular ROS levels were expressed as the mean fluorescence signal (arbitrary units) of the analyzed live cell population (10,000 events).

The mitochondrial superoxide anion levels were measured using the cell-permeant MitoSOX™ Red reagent (Molecular Probes, Eugene, OR, USA), which is selectively targeted to mitochondria and oxidized by superoxide. Cells were incubated in the corresponding medium (8% and 21% O_2) with MitoSOX (4 µM) for 30 min at 37 °C in the dark. The fluorescence intensity from live cells was analyzed by flow cytometry in a Beckman Coulter Gallios Flow Cytometer (λ_{exc}=485/20 and λ_{em}=620/20) in the General Research Services SGIker of the UPV/EHU. Results were expressed as the mean fluorescence signal (arbitrary units) of the analyzed live cell population (10,000 events).