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Redox homeostasis of breast cancer lineages contributes to differential cell death response to exogenous hydrogen peroxide

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ABSTRACT

Aims: Cancer cells produce higher amounts of reactive oxygen species (ROS) than their normal counterparts. It has been suggested that a further increase in ROS concentration in these cells would lead to oxidative damage-driven death. Thus, we aimed to understand how the intra- and extracellular redox homeostasis differences set cell death response to ROS in breast cancer cell lines.
Main methods: Intra- and extracellular ROS generation was evaluated in tumoral (MCF-7 and MDA-MB-231) and non-tumoral (MCF10A) breast epithelial cells, as well as H_2O_2 concentration in the culture medium, glutathione peroxidase (GPx), total superoxide dismutase (SOD) and catalase activities, extracellular H_2O_2 scavenging capacity and total thiol content. Cell viability was determined after H_2O_2 exposure using the MTT assay.
Key findings: We have found an increased extracellular ROS production in tumor cells when compared to the non-tumoral lineage. MCF10A cells had higher H_2O_2 concentration in the extracellular medium. Moreover, extracellular H_2O_2-scavenging activity was higher in MDA-MB-231 when compared to MCF10A and MCF-7. Regarding intracellular antioxidant activity, a lower GPx activity in tumor cell lines and a higher catalase activity in MDA-MB-231 were observed. Thiol content was lower in MDA-MB-231. Additionally, tumor cell lines were more sensitive to H_2O_2 exposure than the non-tumoral cells.
Significance: The present report shows that the capability to generate and metabolize ROS differ greatly among the breast cancer cell lines, thus suggesting that redox balance is finely regulated during carcinogenesis. Therefore, our data suggest that therapeutic approaches targeting the redox status might be useful in the treatment of breast tumors.

1. Introduction

The maintenance of redox homeostasis is pivotal for normal cell physiology, and reactive oxygen species (ROS) are known to regulate several cellular events, including cell growth, differentiation, apoptosis, metabolism and others. Tumor cells usually produce larger amounts of ROS than its normal counterparts and this feature is believed to contribute to the tumor phenotype [1,2]. The greater amount of ROS is attributed to increased expression of enzymatic sources of ROS [3,4], increased production by non-enzymatic sources, *e.g.* abnormal mitochondrial respiration [5], impaired antioxidant activity or, more plausibly, a combination of these factors. *In vivo* studies have shown that mice lacking the intracellular antioxidant enzyme Cu-Zn Superoxide Dismutase (SOD1) develop hepatocarcinoma [6], while mice lacking Glutathione Peroxidase 1 (GPx1) and 2 (GPx2) develop intestine tumors [7], endorsing the concept of ROS as carcinogenic agents. Nevertheless, the mechanisms through which tumor cells take advantage of disruptions in redox homeostasis are yet to be clarified.

The role of ROS in producing DNA damage and genomic instability is extensively recognized and its relation with tumor initiation is well established [8]. However, ROS can directly or indirectly affect an astonishing number of molecular pathways related to cell proliferation, motility, angiogenesis and invasion. Small concentrations of ROS can stimulate proliferation, especially due to the oxidative inactivation of protein phosphatases, such as PTEN and PTP-1B and/or to direct

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activation of pathways such as $p38^{MAPK}$ [9], $p70^{S6K}$and $p90^{Rsk}$ [10], phospholipase D [11], JAK/STAT [12], JNK and ERK [13]. Regarding angiogenesis, both exogenous H_2O_2 and mitochondria-derived superoxide anion are able to stabilize HIF1-α, promoting the expression of pro-angiogenic downstream effectors [14,15]. Moreover, the epithelial-mesenchymal transition (EMT), involved in metastatic phenotype acquisition, is orchestrated by several pathways and molecules regulated at expression or activity level by ROS, such as TGFβ/Smad, Snail, E-Cadherin, β-catenin and matrix metalloproteinases (MMP) [2,16,17].

Redox homeostasis disturbances in tumor cells are associated not only with increased ROS production but also with aberrant antioxidant systems. The three known Superoxide Dismutase isoforms are reported to be modified in cancer and have been linked to carcinogenesis. Mn-Superoxide Dismutase (SOD2) is now considered a tumor-suppressor, found to be reduced or absent in several types of cancers. Some authors have suggested that SOD2 activity is also suppressed in human breast cancers, due to the loss of its regulator SIRT3 [18,19], while other groups have found an opposite result [20]. However, the experimental overexpression of SOD2 in breast tumor cell lines was shown to decrease cell proliferation and tumorigenicity, consistent with a tumor-suppressive role [21]. On the other hand, some reports have shown that SOD1 is overexpressed in human breast tumors. In fact, a SOD2-SOD1 switch has been proposed during oncogene-driven mammary cell transformation [22]. Extracellular SOD (SOD3) performs a unique role catalyzing superoxide dismutation to hydrogen peroxide in the extracellular space. Teoh-Fitzgerald and colleagues have recently shown that SOD3 protein and mRNA are suppressed in human breast tumors [23]. Importantly, new insights have placed ROS as a key-factor in BRCA1-related breast cancers etiology due to BRCA1-NRF2 interaction and influence on NRF2-dependent antioxidant ability [24].

A large body of evidence obtained both *in vivo* and *in vitro* associating ROS with cancer has fostered the assumption that antioxidant therapy would be beneficial for cancer patients. Estimates point out that 40–85% of breast cancer patients use antioxidant supplementation during cancer treatment [25]. This concept is widespread both in medical community and in general population, even though epidemiological data do not strongly support the existence of benefits. As a matter of fact, huge epidemiological studies analyzing the effect of dietary intake of antioxidants on cancer prevalence and outcomes show very little, if any, effect in overall cancer risk in most populations studied [26–28]. Additionally, recent *in vivo* studies revealed that antioxidants may actually stimulate lung cancer in mice [29] and stimulate distant metastasis of melanoma [30,31], warning scientific community that ROS influence on cancer might be more complex than previously predicted and that this subject requires much more attention than it has been given before submitting patients to these therapeutic approaches. Therefore, a better comprehension about the redox status in cancer is imperative for development of new therapeutic strategies.

Studying how tumor cells manage to maintain ROS levels within an increased but viable range and how they are able to cope with excessive and deleterious amounts of ROS is an interesting field. However, very little is known about the molecular mechanisms regulating redox homeostasis in breast tumor cells, rendering any attempt to use redox systems-targeted therapies to be premature for this cancer type. Thus, in the present study we aimed to characterize the redox homeostasis of three of the most used breast epithelial cell lines, in order to better understand their redox physiology, which might be helpful to find new approaches in breast cancer therapy.

2. Material and methods

2.1. Chemicals, reagents and cells

All chemicals and reagents were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), unless otherwise specified. Non-tumoral human epithelial breast cell lineage MCF10A, ER-positive breast tumor cell MCF7 and triple-negative breast tumor cell MDAMB231 (donated by Dr. Franklin David Rumjanek - Federal University of Rio de Janeiro, Rio de Janeiro, Brazil) were maintained in phenol red-free DMEM medium containing 10% fetal bovine serum (Gibco®/Life Technologies, Carlsbad, CA, USA), penicillin and streptomycin (2%) and amphotericin B (1 mg/mL), whereas MCF10A's medium was further supplemented with cholera toxin (100 ng/mL), EGF (20 ng/mL), insulin (10 μg/mL) and hydrocortisone (500 ng/mL). All cells were maintained at 37 °C in an atmosphere of 5% CO_2/95% air.

2.2. Intracellular ROS levels

Cells were dissociated and incubated with 10 μM H_2DCF-DA (Invitrogen®/Life Technologies) for 30 min at 37 °C. Mean fluorescence intensity was detected by flow cytometry using a BD FACSAria™ III instrument (Becton Dickinson, East Rutherford, NJ, USA). Excitation and emission settings were 495 nm and 520 nm, respectively.

2.3. Extracellular H_2O_2 generation

Extracellular H_2O_2 production was quantified by the Amplex Red/HRP assay, which detects the accumulation of a fluorescent oxidized product. 1×10^5 cells in phenol red-free Balanced Salt Solution (BSS) were incubated with D-glucose (1 mg/mL), SOD (100 U/mL; Sigma), HRP (0.5 U/mL; Roche) and Amplex Red (50 μM; Molecular Probes) and the fluorescence was measured in a microplate reader (Victor X4; PerkinElmer) for 40 min in the wavelength of 530 nm excitation and 595 nm emission. H_2O_2 generation (nmol $H_2O_2 \times h^{-1} \times 10^5$ cells) was calculated using standard calibration curves.

2.4. Antioxidant enzymes activity

1×10^6 cells of each lineage were seeded in 10 cm Petri dishes. Twenty-four hours before protein extraction, culture medium was replaced by fresh medium and immediately before protein extraction these media were aspirated, centrifuged at 3000 ×*g* to remove any detached cells and stored at −70 °C until extracellular antioxidant assay.

Attached cells were washed 2× with BSS and harvested in 100 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 5 mM DTT. Homogenates were centrifuged at 3000 ×*g* for 15 min at 4 °C. Supernatants were sonicated at 20 Hz for 10 s and stored at −70 °C until activity assays. Protein concentrations were determined by Pierce's BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA). Catalase activity was assayed following the method of Aebi [32] and expressed as units per milligram of protein (U/mg). GPx activity was assayed by following NADPH oxidation at 340 nm in the presence of an excess of glutathione reductase, reduced glutathione and *tert*-butyl hydroperoxide as substrates, and expressed as nmol of oxidized NADPH per milligram of protein (nmol/mg). The total activity of SOD was determined according to the method described by Crapo et al. [33].

2.5. Measurement of thiol groups

Total thiols were determined in a spectrophotometer (Hitachi U-3300) using 5,5-dithionitrobenzoic acid (DTNB). Thiols react with DTNB, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB^-), which ionizes to the NTB^{2-} di-anion in water at neutral and alkaline pH. The NTB^{2-} was quantified in a spectrophotometer by measuring the absorbance of visible light at 412 nm.

2.6. Real-time PCR

Total RNA was extracted using RNeasy Mini kit (Qiagen, Venlo, Netherlands) and 2 μg were used for reverse transcription with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real-time PCR was performed with SYBR Green Master