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Determination of cellular glutathione:glutathione disulfide ratio in prostate cancer cells by high performance liquid chromatography with electrochemical detection

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ABSTRACT

A validated method has been developed for the simultaneous measurement of reduced and oxidized glutathione in de-proteinised cellular extracts. This has been used to compare models of malignant and non-malignant human prostate cell lines. Analysis of LNCaP and DU145 cells showed a glutathione to glutathione disulfide ratio of 8:1 and 32:1 respectively, whilst the control cell line, PZ-HPV7 displayed a ratio of 93:1. Results indicate that the more aggressive phenotype displays adaptation to increased oxidative stress via up regulation of glutathione turnover. It was also noted that in the LNCaP and DU145 cell line, glutathione was only responsible for ca. 60% and 79% respectively, of the total cellular reduced thiol; indicating the presence of other biological thiols.

1. Introduction

Prostate cancer (PCa) currently affects 1 in 9 men over the age of 65, and is the most prevalent cancer affecting men in 124 countries worldwide [1–4]. The incidence of PCa is strongly associated with ageing, and therefore progressive metabolic changes in cells may play an important role in PCa development [5]. Additional factors including genetic predisposition, androgen sensitivity, diet and lifestyle have also been shown to correlate with the development of the disease [5–7].

Increasing evidence from clinical and experimental studies has shown that oxidative stress is implicated in development and progression of PCa [8,9]. Often, changes in cellular redox state towards a position of increased oxidative stress are associated with initiation of carcinogenesis [10,11]. The nature of this association is noted to be complex, as transformed cells are in turn shown to generate increased levels of pro-oxidants and specifically reactive oxygen species (ROS), which at chronic levels are initiators of cell death [12,13].

Maintaining normal cellular redox state relies on a balance between pro-oxidant species and antioxidants. Glutathione is the major non-protein thiol found in nearly all mammalian tissues and is vital in maintaining redox balance by neutralising oxidising species [14–16]. Normal resting intracellular glutathione:glutathione disulfide ratios (GSH:GSSG), of approximately 100:1 have been reported, whereas this ratio in models of oxidative stress is reduced to 10:1 [14,17]. In cancer cell studies it is therefore evident that the measurement of GSH:GSSG, is important in determining the cellular redox status; a shift to increased GSSG being representative of an increased oxidative environment [11].

The desire to quantify GSH and GSSG has led to the development of a number of methods to analyse these compounds in plasma, urine, saliva and tissue [18–23]. Commonly, pre-column derivatisation is employed to improve both chromatographic separation and detection sensitivity. Additionally, many GSSG measurements are based upon the reduction of the disulfide followed by measurement of GSH [24]. In order to avoid sample pre-treatment some investigators have utilised electrochemical detection (ECD) to directly and simultaneously measure GSH and GSSG in plasma [25,26]. The sensitive nature of ECD lends itself to the analysis of GSSG which is often difficult to measure due to its low endogenous concentration. Recently, the development of a boron-doped diamond electrode (BDD) for use in ECD has been shown to overcome some of the

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classic problems associated with carbon electrodes, such as; instability of response when using high potentials necessary for disulfide analysis, high background noise from oxidation of aqueous mobile phase, and loss of sensitivity due to adsorption of material onto the electrode [27,28].

Direct measurement of the GSH:GSSG control and diseased cell extracts would provide a valuable insight into the redox state of tumour cells and are not reported for prostate cancer cell lines. Therefore we report the following sensitive, reliable and unambiguous method for the measurement of GSH:GSSG in cellular extracts.

2. Materials and methods

2.1. Chemicals

Analytical grade reduced glutathione, oxidized glutathione, cysteine, methionine, reduced cysteinylglycine, 5′5 dithiobis(2-nitrobenzoic acid), monosodium phosphate, sodium hydroxide, sodium 1-octanesulfonic acid, phosphoric acid and trichloroacetic acid were purchased from Sigma–Aldrich Co., (Poole, Dorset, UK). Electrochemical analysis grade acetonitrile was purchased from Fischer Scientific, (Loughborough, UK). Ultrapure, 0.2 μm filtered 18.2 mΩ-cm^2 water was obtained from a Diamond Lab Water System (Triple Red Laboratory Technology, Bucks, UK).

2.2. Cell lines

Cell lines were selected to represent prostate cancer models from healthy cells (PZ-HPV7) to malignant androgen sensitive (LNCaP) and androgen insensitive metastatic (DU145) cells. The PZ-HPV7Human Prostate, HPV-18-transfected control cell line (ATCC®-CRL 22211), LNCaP clone FGC, a human lymph node derived prostate carcinoma cell line (ATCC®-CRL-1740™), and DU145, derived from a brain metastatic site (ATCC®-HTB-81™), were obtained from LGC Standards (Middlesex, UK). Cells were received as frozen ampoules, and were revived according to the manufacturer's protocol.

2.2.1. Cell culture

Cells were incubated in vented cap culture flasks under a humidified atmosphere of 5% CO_2 at 37 °C, and were sub-cultured 1:3 every 3–5 days when confluence had reached 80%. Spent media was discarded and the monolayer washed once with phosphate buffered saline. The cells were removed from the flask by addition of trypsin ethylenediaminetetraacetic acid (EDTA) solution (Sigma–Aldrich). An equal volume of trypsin soybean inhibitor (Sigma–Aldrich) was added to PZ-HPV7 cells. The cell pellet was collected by centrifuging at 430 × *g* for 5 min, and was re-suspended in sufficient fresh growth media to give a seed density of 2×10^6 for a T-75 flask. PZ-HPV7 cells were grown in keratinocyte serum-free media (K-SFM) containing 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml human recombinant epidermal growth factor (EGF), (Invitrogen GIBCO™, kit catalogue number 17005-042). DU145 cells were grown in Eagle's Minimum Essential Medium (EMEM), (ATCC® 30-2003™). LNCaP cells were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) growth media, (ATCC® 30-2001™.) Penicillin Streptomycin solution (10,000 units penicillin, 10 mg streptomycin/ml) was added to each growth media at a concentration of 2% (v/v). EMEM and RPMI were supplemented with 10% (v/v) heat inactivated fetal bovine serum (Biosera, Labtech International Ltd., East Sussex.)

2.2.2. Cell harvesting & quenching

Cells were harvested at 80% confluence with trypsin and re-suspended in a small volume of fresh growth media or trypsin soybean inhibitor. Cell counting was performed using a C-Chip® disposable haemocytometer (Labtech International Ltd., East Sussex). Cells were pelleted by centrifugation at 1000 rpm for 5 min. The pellets were immediately re-suspended in 1 ml of ice cold 0.9% (w/v) NaCl solution to quench the metabolism and wash the pellet. The cells were transferred to 1.5 ml micro-centrifuge tubes and centrifuged at 430 × *g* for 5 min. The supernatant was removed by gentle aspiration and the pellets were frozen at −80 °C until extraction.

2.2.3. Preparation of acid soluble fraction

Frozen cell pellets from each cell line were defrosted at +4 °C and re-suspended in 1 ml of 10% (w/v) trichloroacetic acid (TCA) in water. The cell suspensions were vortex mixed on full power for 10 s to ensure complete mixing. Cells were lysed on ice using a Soniprep™ (MSE UK Ltd., London) sonic probe set at 18 μm amplitude with a 10 s on pulse followed by a 10 s off period for 5 cycles. The tubes were vortex mixed for a further 30 s then left on ice for 30 min. The tubes were then centrifuged at 18,620 × *g* at +4 °C for 20 min. The supernatant was collected and stored on ice. The cell pellet was re-extracted with half the original volume of 10% (w/v) TCA as above, and the supernatant was combined with the first extract. The combined ASF was frozen at −80 °C until analysis.

2.3. Sample preparation

Individual stock standards were prepared in 10% (w/v) trichloroacetic acid (TCA) and stored at +4°C for a maximum of 1 week. Further dilutions were prepared in 10% (w/v) TCA. ASF was defrosted at +4 °C and vortexed for 1 min before transferring to an polypropylene sample vial with an integrated filter (0.2 μm, nylon) (Thomson Instrument Company, California, USA).

2.4. Total thiol determination

Total free thiol content was determined by colourimetry using Ellman's reagent [29]. Ellman's reagent was prepared by dissolving 5′5-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.4 M sodium phosphate buffer, pH 7.6, to a concentration of 100 μg/ml. Calibration standards were prepared by serial dilution of the GSH stock solution. A 100 μl aliquot of standard solution or ASF was added to 1900 μl of DTNB reagent and mixed on a vortex mixer for 30 s. Absorbance at 412 nm was read immediately in a 1 cm quartz cell using a double beam spectrophotometer (PerkinElmer, Seer Green, UK) against a blank of DTNB solution.

2.5. High performance liquid chromatography

Isocratic reversed phase HPLC was carried out on an integrated Agilent 1290 UHPLC system (Agilent Technologies, Delaware, USA), using a Gemini-NX C18 column (100 mm × 4.6 mm ID, 3 μm), (Phenomenex, Cheshire, UK). Column temperature was automatically regulated at 35 °C. Data was collected and analysed using ChemStation Software (Agilent Technologies, Delaware, USA). The mobile phase was 25 mM sodium phosphate in water (pH 2.65), and MeCN [96:4], containing 2 mM 1-octanesulfonic acid. The flow rate was maintained at 0.7 ml/min and the run time was 20 min, with a post time of 10 min to allow for electrode re-equilibration following the clean cell operation. Samples were analysed using 1 μl injections by means of an integrated Agilent autosampler, which incorporated a 10 s needle wash with mobile phase.

2.6. Electrochemical detection

Detection of disulphides and thiols was performed using an ESA 5040 analytical cell equipped with a BDD at anoperating voltage