Determination of Cellular Glutathione: Glutathione Disulphide 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 GSH:GGSG 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 GSH 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.

Keywords

High Performance Liquid Chromatography; Electrochemical Detection; Prostate Cancer; Oxidative Stress; Glutathione; Disulphide

Abbreviations

LMWT	low molecular weight thiol
PCa	prostate cancer
ROS	reactive oxygen species
GSH:GSSG	glutathione: glutathione disulphide
BDD	boron doped diamond electrode
ASF	acid soluble fraction

Introduction

Prostate cancer (PCa) currently affects 1 in 9 men over the age of 65, and is the most prevalent cancer affecting men [1-4]. The incidence of PCa is strongly associated with aging, 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].

There is increasing evidence from the literature suggesting oxidative stress is implicated in development and progression of the disease [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 in turn are shown to generate increased levels of pro-oxidants, 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 disulphide 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 produced numerous methods to analyse the levels in plasma, urine, saliva and tissues [18-23]. Commonly, pre-column derivatisation is employed in order to improve chromatography and sensitivity. Additionally, many GSSG measurements are based upon the reduction of the disulphide 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 [25, 26]. The sensitive nature of ECD lends itself to the analysis of GSSG which can be difficult to measure due to its low endogenous concentration. Recently, the development of a boron-doped diamond electrode (BDD) has been shown to overcome some of the classic problems associated with carbon electrodes used in ECD, such as; instability of response when using high potentials necessary for disulphide 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 disease state cell extracts would provide a valuable insight into the redox state of tumour cells. To our knowledge, these measurements are not reported for prostate cancer cell lines. Therefore we report the following sensitive and unambiguous method for the measurement of GSH:GSSG in cellular extracts.

Materials and Methods

Chemicals

Analytical grade glutathione reduced, glutathione oxidized, cysteine, methionine, cysteinylglycine reduced, 5'5 dithiobis-(-2-nitrobenzoic acid), monosodium phosphate , sodium hydroxide pellets, sodium 1-octanesulfonic acid, phosphoric acid solution 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).

Cell lines

Cell lines were selected to represent prostate cancer models from healthy (PZ-HPV-7) to malignant androgen sensitive (LNCaP) and androgen insensitive metastatic (DU145). The PZ-HPV7 Human Prostate, HPV-18-transfected control cell line (ATCC®-CRL 2221 1), 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.

1.1.1 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 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 1000 rpm for 5 minutes, and was re-suspended in sufficient fresh growth media to give a seed density of $2x10^6$ for a T-75 set-up. 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 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 foetal bovine serum (Biosera, Labtech International Ltd, East Sussex.)

1.1.1 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 minutes. 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 cell were transferred to 1.5 ml micro-centrifuge tubes and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed by gentle aspiration and the pellets were frozen at -80 °C until extraction

1.1.2 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 vortexed on full power for 10 seconds to ensure complete mixing. Cells were lysed on ice using a Soniprep™ (MSE UK Ltd, London) sonic probe set at 18 micron amplitude with a 10 second on pulse followed by a 10 second off period for 5 cycles. The tubes were vortexed for a further 30 seconds then left on ice for 30 minutes. The tubes were then centrifuged at 15,000 rpm at 4 °C for 20 minutes. 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.

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 ^{o}C and vortexed for 1 minute before transferring to an injection vial. All standards and samples were filtered prior to injection using nylon filter vials (Thomson Instrument Company, California, USA).

Total Thiol Determination

Total free thiol content was determined based upon reaction with DTNB (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. 100 µL of standard solution or ASF was added to 1900 µL of DTNB reagent and mixed on a vortex mixer for 30 seconds. Absorbance at 412 nm was read immediately in a 1 cm quartz cell using a double beam spectrophotometer (Perkin-Elmer, Seer Green, UK) against a blank of DTNB solution.

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 x 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 mins, with a post time of 10 mins to allow for electrode re-equilibration following the clean cell operation. 1 µL injections were performed by the integrated Agilent autosampler, which incorporated a 10 second needle wash with mobile phase.

Electrochemical Detection

Detection was performed using an ESA 5040 analytical cell equipped with a BDD electrode operating at +1400 mV (vs. Pd reference), and programmed with a pre injection clean cell operation at +1900 mV for 50 second. An ESA 5020 guard cell was operated at +900 mV between the pump and autosampler to pre-oxidise the mobile phase. An ESA CoulArray® system was used to organise signals, and CoulArray Data Station Software (Thermo-Scientific, Waltham, MA, USA) was used to acquire data and manage the electrodes.

Results and Discussion

Validation

Method Validation was performed in accordance with the Federal Drug Administration (FDA) guidelines for Bioanalytical Method Validation [30]. This was chosen as it is more specific for bioanalytical methods compared to the ICH guidelines for Validation of Analytical Procedures [31].

Lower Limits of Detection

The lower limits of detection (LLOD) were established for GSH and GSSG by sequentially reducing the concentration of a standard mix until a signal to noise ratio of 3:1 was achieved for each analyte using triplicate injections. LLOD for GSH and GSSG were determined at 1.2 and 1.1 nM respectively.

Lower Limits of Quantitation & Precision

The lower limits of quantitation (LLOQ) and the intraday precision were assessed simultaneously by injection of a mixed standard of each thiol at twice the LLOD concentration. The retention time and analyte peak area response for each thiol were measured over six consecutive injections and coefficient of variation was calculated. Variation values of < 1 % were achieved, demonstrating high precision. Interday precision was assessed by six injections of a standard solution containing 0.05 mM GSH and GSSG over six consecutive days, and calculating the coefficient of variation of the signal peak area response for each LMWT. Between injections the sample was capped and stored at +4 °C. RSD values of < 20 % were obtained, complying with biological method validation (Table 1).

Table 1: LLOQ and Precision Data

Linearity

Linearity was assessed by triplicate 1 µL injections of mixed LMWT standards over a range of concentrations from the LLOQ for each LMWT up to a concentration at which the response is no longer deemed to be linear. This was indicated by a decrease in the R^2 value of the trend line, using a minimum of 6 data points. Investigations of the plots confirm linearity over the stated concentration range, as shown by minimum R^2 values of 0.99 and 0.98 for GSH and GSSG respectively.

Table 2: Linearity Validation Data.

HPLC-ECD Analysis of ASF

Analysis of the ASF samples confirmed the presence of reduced and oxidised glutathione in both all cell lines. The method provides resolution of these analytes from the complex matrix in less than 20 minutes (Figure 1).

FIGURE 1

Calibration plots were used to determine the concentration of the analytes in the ASF. The levels of GSH and GSSG were determined to be 84.1 nM and 0.9 nM respectively in PZ-HPV7 ASF, 44.5 nM and 5.9 nM in LNCaP ASF and 240.4 nM and 7.6 nM in DU145 ASF. These concentrations were then translated using cell counts to reflect the cellular content of GSH and GSSG in each cell line (Table 3).

Table 3: Quantitation results as determined by external standard calibration and Ellman Assay

The data demonstrate that GSH contributes to nearly 99 % of the total free thiol present in PZ-HPV7 cellular extract, with a GSH:GSSG ratio of 89:1, which is near to the expected of ratio of 100:1 observed in resting cells [17]. In contrast LNCaP cells exhibited a diminished GSH:GGSG ratio of 8:1, which is indicative of increased oxidative stress as would be expected when cell growth is upregulated in cancer cell lines [13, 32]. However, DU145 exhibited a higher GSH:GSSG ratio of 32:1 compared to LnCap despite an increased malignant phenotype, associated with a significant increase in intracellular GSH concentration (47.45 femtomole/cell), 6.7 fold and 5.5 fold higher than that measured in LNCap and the control cell lines respectively.

A hallmark of cancer cell energy metabolism is the shift towards a high rate of glycolysis and a defective Krebs cycle, known as the Warburg effect [33], combined with a flux of glucose towards

the pentose phosphate pathway (PPP), a major source of NADPH required for glutathione reductase activity [34-36] (Figure 2).

FIGURE 2

Further more, whilst GSH accounts for nearly 100% of free thiol in the control cell line, its contribution reduces to 59.02 % in LNCap and 78.82 % in DU145, indicating that additional LMWT are present. Due to the up-regulation of glutathione synthesis and antioxidant activity it is likely that the turnover of precursor thiol metabolites might be increased in LNCaP and DU145 cells. Cysteine and cysteinylglycine are free thiols found in the glutathione cycle with the ability to take part in redox reactions and react with Ellman's reagent. Therefore it is possible that these thiol metabolites contribute to the remaining free thiol in LNCaP cells. Other reporters have also indicated that glutathione increase in response to increased ROS in metastatic cell lines [38, 39], and that it contributes to a reduced percentage of the total free thiol in LNCaP cells [40].

Taken together, our data reflect an adaptation of malignant cells to resist against oxidative stress owing to stimulation of GSH synthesis and increased regeneration of GSH after oxidation which would otherwise deplete GSH level and lead to cell death [41].

Further work is currently underway to identify and quantify the other contributing thiols as part of a wider study to investigate the potential roles of these thiols in PCa development.

Conclusion

A validated ECD method has been used for the simple, direct and simultaneous determination of GSH: GSSG in the complex matrixes of cellular extracts without the need for sample pre-treatment. Analysis of representative models of prostate cancer malignancy was performed to better understand the role of oxidative stress and glutathione activity. Comparison of the immortalized normal cell line, PZ-HPV7 and the non-malignant/malignant cell lines, LNCaP and DU145, has confirmed important metabolic differences with respect to GSH and GSSG content, as would be

expected when proliferation is increased. LNCaP displays evidence of increased oxidative stress as shown by the increased GSH: GSSG ratio compared to the levels shown in PZ-HPV7, whilst DU145 exhibited increased antioxidant capabilities, indicating that the more aggressive phenotype displays adaptation to increases oxidative stress.

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Figure Headings:

Figure 1: BDD chromatograms showing representative injections of PZ-HPV7, LNCaP and DU145 ASF. Gemini-NX C18 column (100 mm x 4.6 mm ID, 3 µm). Column temperature: 35 ˚C. Mobile phase: 25 mM sodium phosphate in water (pH 2.65), and MeCN [96:4], containing 2 mM 1-octanesulfonic acid. Flow rate: 0.7 ml/min. Detection: BDD +1400 mV.

Figure 2: Neutralisation of peroxide by GSH and subsequent regeneration utilising NADPH [37].