Microregional Heterogeneity of Non-Protein Thiols in Cervical Carcinomas Assessed by Combined Use of HPLC and Fluorescence Image Analysis

Vojislav Vukovic, Trudey Nicklee, and David W. Hedley

Department of Medical Biophysics, Ontario Cancer Institute, Toronto, Ontario, M5G 2M9 Canada [V. V., D. W. H.], and Departments of Medical Oncology and Hematology [D. W. H.] and Oncologic Pathology [T. N., D. W. H.], Princess Margaret Hospital, Toronto, Ontario, M5G 2M9 Canada

ABSTRACT

Under low oxygen conditions, non-protein thiols (NPSHs, non-protein sulfhydryls) can effectively compete for DNA radicals sites and hence represent a potentially important cause of radiation resistance in the clinic. Intra- and intertumoral heterogeneity of glutathione (GSH) and cysteine were assessed in cryostat sections of multiple biopsies obtained from 10 cervical carcinomas by the combined use of a sensitive high-performance liquid chromatography (HPLC) method and a fluorescence image analysis technique to examine the spatial distribution of NPSHs in tumor tissue. Glutathione concentrations ranged from 1.98 to 4.42 mM; significant (≥1 mM) concentrations of cysteine, a more effective radioprotector than GSH, were found in some tumors. By HPLC, the intratumoral heterogeneity of NPSHs was relatively small compared with the intertumoral heterogeneity. The histochemical stain 1-(4-chloromercuryphenoylazo)-2-naphthol (mercury orange), which binds to GSH and cysteine, was used to determine the spatial distribution of NPSHs in tumor tissue. A comparison of NPSH levels in serial cryostat sections showed a close correlation between NPSH values determined by HPLC and mercury orange fluorescence quantification. Using fluorescence image analysis, an ~2-fold increase of NPSHs in tumor versus nonmalignant tissue was observed in the same section. Because some cervical carcinomas contain radiobiologically important levels of cysteine, agents that target the biochemical pathways maintaining tumor cysteine have therapeutic potential as adjuncts to radiotherapy in cervix cancer patients.

INTRODUCTION

GSH, a cysteine-containing tripeptide, is the most abundant NPSH in mammalian cells. It is involved in a wide range of biochemical processes (1), but it is particularly relevant to oncology because of its potential roles in resistance to chemotherapy and ionizing radiation (2–4). GSH is able to conjugate electrophilic drugs, such as alkylating agents and cisplatin, under the action of glutathione S-transferases. Recently, GSH has also been linked to the efflux of other classes of agents, such as anthracyclines, via the action of the multidrug resistance-associated protein, MRP. In addition to drug detoxification, GSH enhances cell survival by functioning in antioxidant pathways that reduce reactive oxygen species and maintain protein sulfhydryls in their reduced states (5–9). Glutathione and cysteine, the other radiobiologically important NPSHs, are able to effect the chemical repair of DNA radicals produced by ionizing radiation, in competition with oxygen, which stabilizes DNA radical sites. Cysteine concentrations are typically much lower than GSH when cells are grown in tissue culture, and the role of cysteine as an in vivo radioprotector is less well characterized. However, on a molar basis, cysteine protects DNA from the effects of ionizing radiation much more effectively than GSH (10–12). Furthermore, there is evidence that cysteine concentrations in tumor tissues can be significantly greater than those typically found in tissue culture (13, 14).

A number of studies have examined GSH levels in a variety of solid human tumors, often linking these to clinical outcome (14–26). Wide ranges of tumor GSH concentrations have been reported, and in general these have been greater (up to 10-fold) in tumors compared with adjacent normal tissues. Most authors have assessed the GSH content of bulk tumor tissue using enzymatic assays or GSH plus cysteine using HPLC. In general, only one tumor biopsy specimen was examined, and the problem of tumor heterogeneity was not addressed.

The histochemical stain mercury orange (1-(4-chloromercurphenoylazo)-2-naphthol) has been shown previously to have high specificity for GSH in tissue sections, allowing semiquantitative assessments of intratumoral heterogeneity of NPSH. We recently refined the mercury orange histochemical technique by the use of digital image analysis to quantify labeling intensities in extensive areas of tumor tissue, acquired using fluorescence optics and a computer-controlled microscope stage (27). By the combined use of this technique and a sensitive HPLC method based on electrochemical detection, we have

Received 12/7/99; revised 2/10/00; accepted 2/17/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked
advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the National Cancer Institute of Canada, using funds raised by the Terry Fox Run.

2 To whom requests for reprints should be addressed, at Department of Medical Biophysics, Ontario Cancer Institute, 610 University Avenue, Toronto, Ontario, M5G 2M9 Canada. Phone: (416) 946-2262; Fax: (416) 946-2984; E-mail: david_hedley@pmh.toronto.on.ca.

3 The abbreviations used are: GSH, glutathione; NPSH, non-protein sulfhydryl; HPLC, high-performance liquid chromatography; IOD, integrated absorbance.

Downloaded from clinicancerres.aacrjournals.org on April 20, 2017. © 2000 American Association for Cancer Research.
shown that the intratumoral distribution of NPSHs is relatively homogeneous, whereas there is an ~2-fold range in values between individual tumors. Cysteine concentrations >1 mM were found in some samples, values that are potentially significant in terms of chemotherapy and radiation resistance.

**MATERIALS AND METHODS**

**Tumor Biopsies.** A total of 34 punch biopsies were taken from 10 cervix cancer patients who were entered into a clinical trial conducted at the Princess Margaret Hospital, Ontario Cancer Institute, studying the effects of oxygenation status on radiation treatment outcome (28). Nine of the tumors were squamous cell carcinomas, and one was an adenocarcinoma. Informed consent was obtained in accordance with institutional ethical guidelines. The biopsies (two to four per patient) were placed into cryovials containing Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA) and rapidly frozen in liquid nitrogen.

**Preparation of Tissue Sections.** Five-μm serial sections of tumor tissue were cut using a Tissue-Tek II Cryostat (Miles Laboratories, Naperville, IL). The first two sections were adhered to 3-aminopropyl triethoxysilane (Sigma Chemical Co., St. Louis, MO)-treated glass microscope slides. A third serial section was used for HPLC measurements of NPSHs. The first section was stained for NPSHs with the sulfhydryl-reactive dye mercury orange (Sigma). Mercury orange was first dissolved in acetone, and then distilled water was added to produce a final concentration of 75 μM in 9:1 (v/v) acetone:water. To minimize the loss of reduced thiols through oxidation, the sections were cut and rapidly placed in the mercury orange solution and stained on ice for 5 min, followed by two rinses with 9:1 acetone:water. After rinsing with PBS, the slides were covered with Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). The second section was fixed in 3.7% neutral buffered formalin for 10 min, rinsed, and stained with H&E.

**HPLC Measurement of NPSHs.** NPSHs were extracted using the method described by Koch and Evans (13). Briefly, the tissue section was rapidly placed into a vial with extraction buffer containing 50 mM sulfosalicylic acid and 50 μM of each of the iron and copper chelators EDTA, sodium diethyldithiocarbamate, and diethylenetriaminepentaacetic acid and kept at 4°C for 1 h. The samples were then centrifuged at 14,000 x g for 15 min, and the optical clear supernatant was aliquoted and stored at 4°C. Determinations of NPSHs were carried out by HPLC-based electrochemical measurement, usually within 24 h of extraction. The HPLC system consisted of a Waters 600E system controller, Waters Ultra WISP 715 sample processor, Waters 746 data module, and Waters 464 pulsed electrochemical detector, equipped with a mercury-coated dual gold electrode. The separation was carried out using a Supelco LC-18 reversible phase column (7.5 cm x 4.6 mm; bead size, 3 μm). To resolve GSH from cysteine, a low pH mobile phase (pH 2.0), consisting of 0.1 mM phosphoric acid with 3.3 mM heptanesulfonic acid in water and 10% methanol, was used, as described by Koch and Evans (13). Prior to separation, the mobile phase was purged with helium to displace dissolved O2 and reduce background current. GSH and cysteine concentrations were calculated by comparing the peak area of the samples with that of known standards. The consistency of measurement was maintained using external standards after every three samples. The volumes of the tissue sections used for HPLC measurements were obtained by the product of the section area, determined by digital microscopy of the parallel H&E section, and the section thickness.

**Transmitted Light Microscopy.** The H&E sections were imaged using a MicroComputer Image Device (Imaging Research, Inc., St. Catharine’s, Ontario, Canada) linked to a Sony DXC-970 MD, 3CCD color video camera mounted on a Zeiss Axioskop microscope fitted with a Ludl Biopoint motorized stage. Using 10 x 0.25 N.A. objective lens and an automated mini program, a microscopic field-by-field digitized tiled image of the entire tumor section was obtained. These images, showing the cellular morphology of the biopsies, were used as a guide for subsequent mercury orange fluorescence measurements.

**Fluorescence Microscopy and Image Acquisition.** A second MicroComputer Image Device image analysis system was used to tile the entire tumor section stained with mercury orange. This system has similar computer hardware and software to that used for transmitted light microscopy but is linked to a Xillix MicroImager (Xillix, Vancouver, British Columbia, Canada) mounted on an Olympus BX50 reflected fluorescence microscope fitted with a Ludl Biopoint motorized stage. Using the 10 x 0.3 N.A. objective lens, tiled field images were obtained using an excitation filter centered at 540 nm, which optimally excites mercury orange. Fluorescence emission was collected using a 585/40 nm band pass filter.

**Image Processing and Analysis.** Digitized mercury orange fluorescence and H&E images were saved as 24-bit TIFF files. Subsequently, the images were converted to 8-bit grayscale images using Adobe PhotoShop 5.0 software. Further image processing was performed using an application written in the Interactive Data Language (IDL 5.1; Research Systems, Inc., Boulder, CO). The H&E images were inverted and subsequently thresholded at a brightness level that corresponded to three times the lowest brightness value in the background. The total number of positive pixels was used as reference to find the appropriate threshold value for the mercury orange fluorescence image. After thresholding, the positive pixel addresses from the mercury orange binary image were used on the original grayscale image to collect the brightness values from corresponding pixels. The mean IOD was calculated by dividing the total sum of positive pixel brightness values by pixel number. Background was defined as the average pixel value in three randomly chosen image regions outside the tissue section and subtracted from the positive pixel brightness values.

To assess the mercury orange fluorescence levels in tumor cells and nonmalignant cells within a tumor, areas predominantly populated with tumor cells were chosen on the H&E image, and the corresponding areas of the mercury orange fluorescence image were manually outlined using the Adobe PhotoShop software. The mean brightness from 5 to 10 randomly chosen areas within one section was averaged and compared with areas containing predominantly nonmalignant cells.
RESULTS

NPSH Measurements using HPLC. Peaks representing GSH and cysteine were readily resolved in all of the samples, with no additional peaks to indicate the presence of GSH fragments, such as the dipeptides Cys-Gly or γ-Glu-Cys, in detectable amounts. The mean values for GSH and cysteine concentrations of all of the samples analyzed (n = 34) were 2.86 mM (SE, 0.15) and 0.75 mM (SE, 0.06), respectively. Fig. 1 shows the mean values and SDs for the multiple biopsies obtained from individual patients. As seen, the intertumoral variability of GSH ranged from 1.98 to 4.42 mM. The intertumoral heterogeneity of the GSH tumor levels was significantly greater than the intratumoral heterogeneity, as determined with one-way ANOVA (P < 0.01). The intertumoral variability of mean cysteine concentrations ranged from 0.34 to 1.40 mM. As with GSH, intertumoral heterogeneity of the cysteine tumor levels was significantly greater than the intratumoral heterogeneity (one-way ANOVA, P < 0.01).

NPSH Measurements Using Image Analysis. To compare NPSH levels in tumor cells and nonmalignant tissues and to assess the intracellular NPSH distribution, adjacent tumor tissue sections were stained with mercury orange and H&E. Representative tiled images covering the entire sample area, composed from more than 30 × 10 objective viewing fields, are shown in Fig. 2. The mean mercury orange fluorescence values, integrated across the entire tissue area, are shown in Fig. 3. As with the HPLC measurements, the intertumoral heterogeneity of mercury orange fluorescence intensities was significantly larger than the intratumoral heterogeneity (P < 0.01, one-way ANOVA). In Fig. 4, these values are shown as bivariate plots versus HPLC-determined concentrations of GSH (top panel) and NPSH (GSH + cysteine; bottom panel). A close correlation between these two methods was obtained for GSH (r² = 0.92) and for NPSH (r² = 0.76).

Measurement of NPSH levels within individual sections was done by manually outlining areas containing predominantly tumor cells and areas of nonmalignant tissue, using the H&E image as a guide. As can be seen in Fig. 2B, within areas that consisted substantially of viable tumor tissue, the labeling intensity with mercury orange was relatively homogeneous, similar to our recent findings using human cervical cancer xenografts (27). The mercury orange fluorescence levels were higher by a factor of approximately two in areas containing predominantly tumor cells compared with areas of nonmalignant cells (Table 1). As shown in Fig. 5, within individual tumors the NPSH contents of tumor and nontumor tissue were closely correlated (r² = 0.82).

DISCUSSION

In this report, we have examined the heterogeneity of NPSH levels in multiple biopsies obtained from patients with cervical carcinomas who were entered into a study investigating the effects of hypoxia on the response to radical radiotherapy (28). The major findings are that the intertumoral heterogeneity of the concentrations of GSH and cysteine exceeds the intratumoral heterogeneity, and that cysteine concentrations ≥1 mM were found in some samples, confirming an earlier report by Guichard et al. (14).

There is an extensive literature showing that elevated cellular GSH levels can produce drug resistance in experimental models because of drug detoxification or the antioxidant effects of GSH. In addition, radiation-induced DNA radicals can be repaired nonenzymatically by GSH and cysteine, indicating a potential role for NPSH in radiation resistance. Cysteine is the more effective radioprotector but is usually present in lower concentrations than GSH. Whereas under fully aerobic conditions this radioprotective effect appears to be relatively minor, NPSHs compete more effectively with oxygen for DNA radicals under the hypoxic conditions that exist in some solid tumors and hence might play a significant role in radiation resistance in the clinic.

Radiotherapy is a major treatment modality for cervical carcinomas. Randomized clinical trials show that patient outcome is significantly improved when this is combined with cisplatin-based chemotherapy, and combined modality therapy is now used widely. It is important to establish the clinical relevance of GSH and cysteine to drug and radiation resistance because of the potential to modulate these using agents such as buthionine sulfoximine, an irreversible inhibitor of γ-glutamylcysteine synthetase that can produce profound depletion of GSH in tumor and normal tissues (29, 30). However, before the effects of such treatments can be fully evaluated, it is necessary to develop methods for accurate NPSH measurements in solid tumors.

Bulk NPSH levels have been determined in a wide range of tumor types using enzymatic assays that measure GSH and/or GSSG and less frequently using HPLC methods that can also measure cysteine. Typically, these studies have reported elevated tumor GSH relative to adjacent normal tissue and intertumoral heterogeneity in GSH content. These findings are consistent with the idea that GSH could play a clinically significant role in drug resistance, although it should be noted that relatively few studies have the sample size and follow-up duration necessary to detect a significant relation between tumor GSH
Fig. 2  Tiled H&E and mercury orange fluorescence images. Tiled images of parallel tumor biopsy sections stained with H&E and mercury orange (A and B, respectively), composed of more than 30 individual ×10 objective viewing fields.
content and response to chemotherapy; hence, there are no consistent clinical data to support this idea.

Relatively few studies have reported on cysteine levels in human cancers. However, an earlier HPLC-based study of cervical carcinomas by Guichard et al. (14) reported cysteine concentrations $>$1 mM in a significant number of cases. Compared with the present study, considerably greater intertumoral heterogeneity in cysteine and GSH were found by Guichard et al. (14), possibly because of differences in analytical technique, but the overall findings are similar. The fact that the variability in cysteine levels is greater than that for GSH suggests that these two thiols are regulated differently in tumors. Because cysteine is a more efficient DNA radioprotector on a molar basis than GSH (11), the finding of high cysteine levels possibly explains the failure of GSH depletion to result in significant radiosensitization of tumors in vivo. In addition to its ability to repair radiation-induced DNA radicals, cysteine has the potential to detoxify cisplatin, a cytotoxic agent now routinely combined with radiotherapy to treat locally advanced cervical carcinomas.

A major advantage of enzymatic and HPLC assays is that they are quantitative; however, they do not provide information on the intratumoral variability of NPSHs. In addition, biopsies from solid tumors can contain variable proportions of tumor and nonmalignant cells, or viable and necrotic tissue, making the interpretation of such measurements even more complex. Alternatively, sulfhydryl-reactive staining procedures with subsequent detection by flow cytometry or fluorescence microscopy can be used to assess cellular heterogeneity of NPSHs. Because of the potential for background labeling of protein sulfhydryls and practical difficulties establishing and maintaining an accurate calibration, these methods are generally considered to be semiquantitative.

In the present study, a strong correlation was found between the HPLC-determined GSH concentration and mercury orange fluorescence when these methods were applied to serial cryostat sections. This indicates that under carefully standardized conditions, the mercury orange technique is able to give a quantitative estimate of tissue GSH levels. The correlation was less strong with respect to NPSH (GSH + cysteine) determinations, possibly because of greater solubility of the reaction product between mercury orange and cysteine. Because of this and the relatively greater concentrations of GSH, we consider it unlikely that the mercury orange technique gives meaningful information about the intratumoral heterogeneity of cysteine.

By comparing areas populated predominantly with tumor cells and areas of nonmalignant cells within the same tumor section, we have found that mercury orange fluorescence intensity in tumor cells is approximately two times higher that in nonmalignant cells. This is in agreement with our previous work in cervical tumor xenografts (27) and the observation that GSH levels in many tumor types are increased 2-fold above levels found in normal tissues (17–20, 23–25, 31–33). The close correlation seen between the NPSH contents of malignant and nonmalignant tissues within the same tumor is relevant to the question of whether the heterogeneity of NPSHs seen in human cancers is attributable to autonomous tumor factors, such as...
expression of GSH-synthesizing enzymes, relative to systemic factors that determine the availability of GSH precursors.

By dual fluorescence staining for mercury orange and the hypoxia marker EF5, we have shown recently that NPSH levels are ~50% greater in hypoxic regions of ME180 and SiHa human cervical cancer xenografts, relative to better oxygenated tumor tissue (27). This finding suggests that tumor cells actively regulate NPSH levels in response to the tumor microenvironment. Because of the greater protection afforded by NPSH under hypoxic conditions, this response is likely to enhance the overall radioresistance of the tumors. In future experiments, we plan to develop the wide field fluorescence image analysis technique to address the underlying mechanisms of tumor GSH and cysteine regulation by examining the intratumoral relationships between hypoxia, mercury orange labeling intensities, and the expression of NPSH-regulating enzymes such as γ-glutamylcysteine synthetase and γ-glutamyltranspeptidase. These experiments will be done using xenograft models and biopsies obtained from cervix cancer patients being treated with EF5 as part of our research program investigating the mechanisms of hypoxia in human cancers.

REFERENCES


Microregional Heterogeneity of Non-Protein Thiols in Cervical Carcinomas Assessed by Combined Use of HPLC and Fluorescence Image Analysis

Vojislav Vukovic, Trudey Nicklee and David W. Hedley


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/6/5/1826

Cited articles
This article cites 31 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/6/5/1826.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/6/5/1826.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.