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

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-chloromercuryphenoylazo)-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...
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 cover-stained on ice for 5 min, followed by two rinses with 9:1 acetone:water. 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 bivariant 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 sulf hydrys 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 than 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 radiosensitivity 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.