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Research paper

Aldehyde dehydrogenase and HSP90 co-localize in human glioblastoma biopsy cells

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ABSTRACT

The concept of a stem cell subpopulation as understood from normal epithelial tissue or bone marrow function has been extended to our understanding of cancer tissue and is now the target of treatment efforts specifically directed to this subpopulation. In glioblastoma, as well as in other cancers, increased expression of aldehyde dehydrogenase (ALDH) has been found localized within a minority subpopulation of tumor cells which demonstrate stem cell properties. A separate body of research associated increased expression of heat-shock protein-90 (HSP90) with stem cell attributes. We present here results from our initial immunohistochemistry study of human glioblastoma biopsy tissue where both ALDH and HSP90 tended to be co-expressed in high amounts in the same minority of cells. Since 12% of all cells in the six biopsies studied were ALDH positive and 17% were HSP90 positive, by chance alone 2% would have been expected to be positive for both. In fact 7% of all cells simultaneously expressed both markers-a significant difference ($p = 0.037$). That two previously identified proteins associated with stem cell attributes tend to be co-expressed in the same individual glioblastoma cells might have clinical utility. Disulfiram, used to treat alcoholism for half-a century now, is a potent ALDH inhibitor and the old anti-viral drug ritonavir inhibits HSP90. These should be explored for the potential to retard aspects of glioblastoma stem cells' function subserved by ALDH and HSP90.

1. Preamble

> "Upon this, one has to remark that men ought either to be well-treated or crushed entirely. They can avenge themselves of lighter injuries. Of more serious ones they cannot..." otherwise known as "Never do an enemy a small injury." Machiavelli, ca. 1469-1527

2. Introduction

Glioblastoma remains one of the most aggressive and difficult to treat of all human cancers. Despite extensive surgical resection followed by radiotherapy and temozolomide (TMZ), an oral methylating chemotherapy agent, glioblastoma usually recurs within the original treatment area. Patients usually survive only 1–2 years after diagnosis. Local recurrence is seen even in those patients whose original tumor has been widely resected, with no visible tumor remaining on MRI scan. The development of more effective therapies for glioblastoma must incorporate strategies which address recurrence from residual single cells or microscopic cell clusters. Part of glioblastoma's resistance to chemotherapy and irradiation derives, as in the Preamble, from many compensatory growth enhancing changes that are triggered by current mainstay treatments [1–5].

Glioblastoma's repopulation of the surgical cavity even after intensive anti-tumor therapies involves the existence of a tumor stem cell sub-population that is a prime locus of the growth-enhancing compensatory changes triggered by treatment [6–9]. This stem cell hypothesis of glioblastoma (and cancer generally) states that there exists a minority of relatively quiescent and undifferentiated cells that, as with marrow haematopoetic stem cells, produce a variety of downstream daughter cell types as well as self-renew as other stem cells [6,8,9]. Non-stem cells constitute the bulk of a tumor. The attributes of stemness are thought to be relative quiescence, cytotoxic chemotherapy resistance, higher clonogenic and tumor initiation capacities than the non-stem population [6,8,9].

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Our team has been pursuing the potential of conceptually new avenues of approach. We, along with others, are exploring the potential of inherently non-cytotoxic, currently marketed drugs to inhibit growth promoting or apoptosis resistance pathways that have been identified as active in glioblastoma (recent examples: [10–15]). We envision these noncytotoxic interventions as adjuncts to improve the current standard Stupp Protocol [7] of maximal resection, TMZ, and irradiation.

Aldehyde dehydrogenase (ALDH) is expressed in higher amounts in a sub-set of cells within glioblastomas that show stem cell attributes like cytotoxic chemotherapy resistance and higher clonogenic capacity compared to the ALDH low expressing cells [10–12]. ALDH is considered a marker of stem cells in many other cancers [16–18].

We have also been interested in heat-shock protein-90 (HSP90), an ATP hydrolyzing, 90 kDa protein found as a homodimer, upregulated and playing a prominent role in cancer generally [19] and glioblastomas specifically [20–24]. Functioning to stabilize and correctly fold many client proteins, HSP90 is crucial to cell proliferation, cell survival, and most importantly here, for stem cell function. Core mediators of glioblastoma growth like endothelial nitric oxide synthase (eNOS) [25], epidermal growth factor receptor (EGFR) [26,27], and transforming growth factor-beta (TGF-beta) are HSP90 client proteins [28]. HSP90 is overexpressed specifically in the cancer stem cell sub-population [29,30].

Since we have separate bodies of evidence of both ALDH and HSP90 being specifically overexpressed in stem cell subpopulation(s), we set out to determine if one and the same stem cell expressed both or if ALDH and HSP90 demarked different stem cell sub-populations in glioblastoma. We indeed found that high expression of ALDH and high expression of HSP90 tended to co-occur in the same cells beyond what one would expect from chance alone in human glioblastoma biopsy tissues. Some potential treatment implications of these findings are given in the Discussion section below.

3. Methods

Formalin fixed, paraffin-embedded sections of glioblastoma biopsies were run for immunofluorescence, using primary antibodies anti-ALDH1A1 and HSP90a/β, using standard protocols. In brief, after deparaffination and rehydration, tissue sections were blocked with 3% BSA in PBS for 30 min and incubated with the first primary antibody overnight, one night for each antibody (goat polyclonal antibody ALDH1A1, dilution 1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, Cat. N. sc-22589; mouse monoclonal antibody HSP90a/β, dilution 1:100, Santa Cruz Biotechnology Inc., Cat. N. sc-13119). After washing with PBS, slides were further incubated with fluorescent secondary antibodies for 1 h at room temperature (Goat anti-mouse IgG TRITC-conjugated secondary antibody, dilution 1:50, Sigma–Aldrich, Saint Louis, MO, USA, Cat. N.T-5393; Donkey anti-goat IgG-FITC-conjugated secondary antibody, dilution 1:50, Santa Cruz Biotechnology Inc., Cat. N. sc-2024). Finally, nuclei were stained for 15 min with Hoechst 33342 (dilution 1:1000 in PBS, Sigma–Aldrich, Cat. N. B-2261). Further method details are available from the authors.

Slides were observed on a Leica CTR5000 fluorescent microscope. All observations were made at 400× magnification (HPF) by two independent observers (FR and FC) and the means of duplicate counts were used for statistical analyses.

Data obtained from immunofluorescence evaluations were plotted using Microsoft Excel® software (Microsoft Italia, Milan, Italy). Statistical analyses were performed using Statview® 5.0 (SAS Institute Inc., Cary, NC, USA). Mean and standard deviation (SD) of data were calculated. Moreover, correlation analyses were performed using the Spearman rank correlation method. Probability values of $p < 0.05$ were considered significant. Finally, to have an indication on the variability of immunoreactivity data obtained by the different observers, the coefficient of variation was calculated using both the interobserver and intraobserver kappa statistics.

4. Results

Table 1 shows the percentage of cells positive for ALDH1A1, for HSP90, and percentage of cells positive to both markers ("Merge"). In particular, ALDH1A1 was present in a percentage of cells between 6.6 and 15.6 (mean: 12%), while HSP90 was present in a percentage of cells between 11.2 and 20.2 (mean: 17%). Between 5.2 and 10.8% (mean: 7%) of glioblastoma cells were positive to both proteins. Fig. 1 shows an H & E stained glioblastoma biopsy from one of our six cases. Fig. 2 shows nearby slices stained for ALDH1A1, HSP90, and an enlarged merged micrograph where yellow or orange hues indicate staining for both ALDH and HSP90.

Statistical analyses (Table 1) showed the presence of a significant correlation between ALDHA1 and HSP90 ($r = +0.838$, $p = 0.0371$). Despite the small cohort of subjects, the "r" value is strong. Finally, inter-observer kappa statistics showed a value of 0.85 and intra-observer kappa statistics a value of 0.95.

5. Discussion

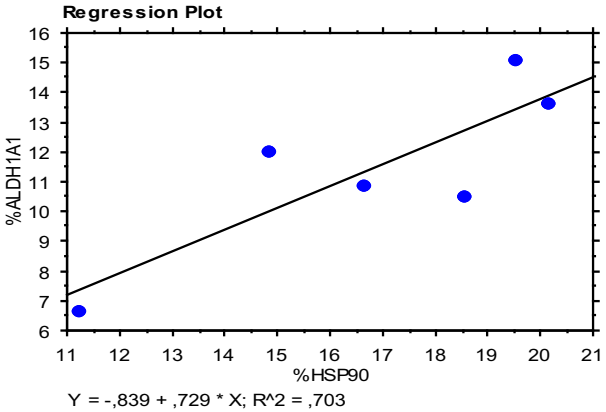
The concept of a stem cell subpopulation within a tumor is the subject of much effort to refine and define. Cancer stem cells, including those of glioblastoma are generally thought to be

Table 1
Percentage of cells positive for each marker (ALDH1A1, HSP90) and of double positive cells (merge). The percentage of positive cells was calculated on 10 HPF (400×) in each sample by two independent observers [FR & FC]; mean of observations is indicated in the table. The graph shows a significant correlation between ALDHA1 and HSP90 ($r = +0.838$, $p = 0.0371$) in examined samples.

Patients	ALDH1A1	Hsp90	Merge	Expected if no association
121	10.45%	18.60%	5.46%	2%
131	6.60%	11.23%	5.18%	1%
141	15.60%	19.54%	10.78%	3%
202	10.80%	16.73%	7.66%	2%
252	13.58%	20.16%	7.26%	3%
273	12.00%	14.80%	6.16%	2%

Regression Coefficients
%ALDH1A1 vs. %HSP90

	Coefficient	Std. Error	Std. Coeff.	t-Value	P-Value
Intercept	-,839	4,054	-,839	-,207	,8462
%HSP90	,729	,237	,838	3,076	,0371



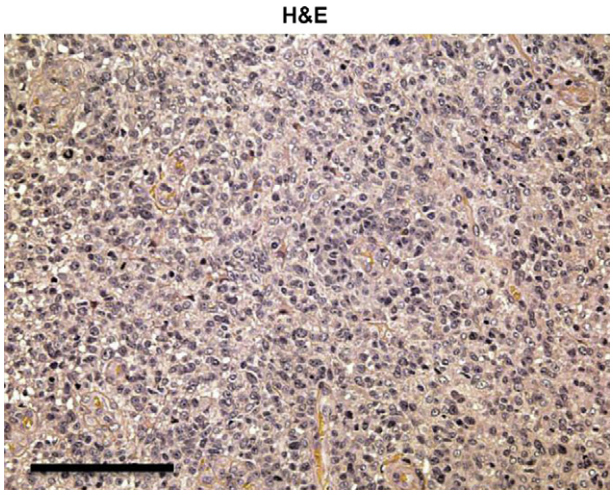


Fig. 1. H & E micrograph of same glioblastoma biopsy tissue as that immunostained in Fig. 2. Bar: 100 micron.

a CD133+ subpopulation within a tumor that is relatively quiescent and chemotherapy resistant, forming a reservoir of cells with high ALDH activity, with strong self-renewal capabilities, that is less differentiated than the majority population, that can generate semi-differentiated highly proliferative non-stem tumor cells that are CD133 negative and have lower expression of ALDH [16–18]. The tendency we documented here are suggestive of an association beyond chance alone for high HSP90 expression to co-localize within the same cells as does high ALDH expression, supporting previous contentions of others that HSP90 is also a stem cell marker in glioblastoma. Beyond being simply markers of stem cell

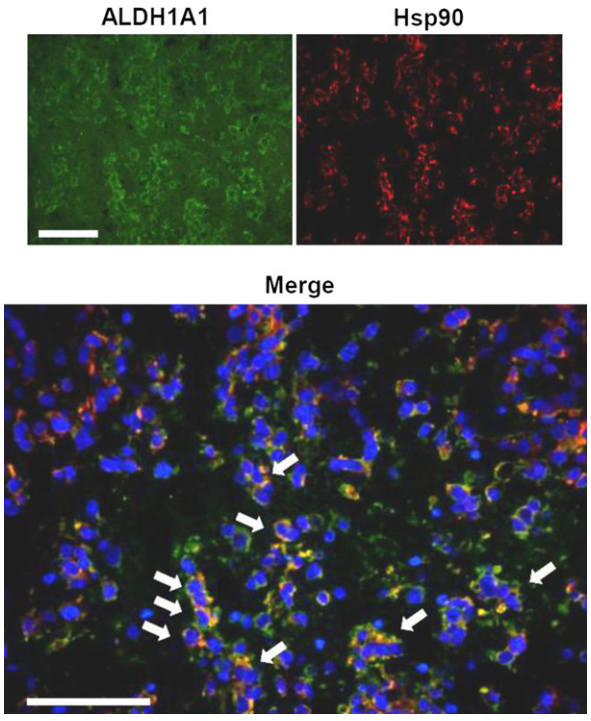


Fig. 2. Representative micrographs immunostained for ALDH1A1 (green) or Hsp90 (red) from same tissue block as in Fig. 1. Lower is a merging of both ALDH1A1 and Hsp90 micrographs where cells in hues of orange or yellow (arrows) are positive for both molecules. Bar: 100 micron. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

attributes, ALDH and HSP90 potentially function to mediate aspects of stem cell behavior.

Already in 1990 both human glioblastoma growing in nude mice and human glioblastoma biopsy tissue were shown to have greatly increased ALDH activity [31]. Then in 2010 ALDH was shown to facilitate clonal sphere formation in established glioblastoma cell lines [16]. High levels of ALDH functioned to keep these cells in an undifferentiated stem cell-like state [16].

Disulfiram is an ALDH inhibitor marketed and in clinical use to treat alcoholism since the 1950's [18]. Previous in vitro research has empirically documented augmented in vitro cytotoxicity when disulfiram was added to standard cancer chemotherapy agents: cisplatin [32], TMZ [14], paclitaxel [33], gemcitabine [34,35], docetaxel [36], cyclophosphamide [37], 5-fluorouracil [38], and doxorubicin [39].

The only clinical study we are aware of looking at disulfiram during human cancer treatment used grotesquely high disulfiram levels, over 3000 mg/day [40], while the usual dose when treating alcoholism is 250 mg by mouth once or twice daily. This study found no statistical benefit when disulfiram was added to cisplatin [40] but the dropout rate was so high in both cisplatin alone and the cisplatin plus disulfiram groups that data evaluation was difficult. Although not statistically significant, the only patient to achieve a complete remission was in the combination group [40]. A single case study showed complete and sustained remission of a stage IV metastatic melanoma to zinc supplemented disulfiram [41].

Glioblastoma biopsies show immunohistochemical over-expression of several HSP's, among them HSP90 [42]. One of the first anti-HIV drugs to come to market was the now-generic protease inhibitor ritonavir. Ritonavir binds Hsp90 with a Kd of 7.8 μmol/L and partially inhibits HSP90's chaperone function [43–46].

Although this is a weak inhibitor, it is the one we have available currently marketed. Ritonavir has limited ability to penetrate the blood–brain barrier, but the common anti-fungal drug ketoconazole at 200 mg by mouth daily increased CSF ritonavir by 178% (95% CI, 59%–385%), from 2.4 to 6.6 ng/mL [47]. Haupt et al. just published a list of 64 signaling kinases that are HSP90 client proteins, many of which have been shown to be important in giving rise to stem cell attributes and glioblastoma growth and apoptosis resistance-perhaps most important of them those of TGF-beta pathway [46].

In discussing the HER group of tyrosine kinases Citri et al. said "the dependence of ErbB-2 upon Hsp90 reveals an Achilles heel, which opens a window of opportunity for combating cancers driven by the ErbB/HER signaling network." [21]. Although we don't agree with applicability of the term Achilles heel (glioblastoma, like any strong foe, has too many overlapping, cross-covering growth stimulating pathways to have any single Achilles heel) we do understand and agree with the utility of inhibiting HSP90's role in assisting EGFR and other HER members' signaling. Experimental, geldanamycin-related HSP90 inhibitors have shown anti-glioblastoma activity in animal models [48,49] suggesting that ritonavir might do so as well.

Our results confirm in and extend to human glioblastoma biopsy tissue previous findings of Lee et al. in xenografted cell lines from human breast cancer [30] where ALDH and HSP90 tended to co-localize in the stem cell sub-population.

6. Conclusions

At a high normal clinical dose of 500 mg once daily no added burden to patients would be expected from disulfiram use as adjunct to the Stupp Protocol in glioblastoma treatment other than the necessity for abstinence from alcohol. Ritonavir at 400 mg orally twice daily did not separate from placebo side effect-wise.

Given the deadly nature of glioblastoma and the meagerness of progress we've seen over the last decades it is time to try new conceptual approaches. One such is to target growth facilitating pathways with these two non-cytotoxic, currently marketed drugs The minority stem cell population is more resistant to cytotoxic drugs and irradiation than are the majority non-stem population. We have shown that glioblastoma putative stem cells co-express higher amounts of ALDH and HSP90 than do the non-stem population. As in The Preamble, on our way to crush the enemy entirely, by depriving glioblastoma's stem cells of those protective attributes mediated by ALDH and HSP90 we might make our current cytotoxic chemotherapies less slight injuries.

7. Postscript

> "Drug discovery for complex and heterogeneous tumors now aims at dismantling global networks of disease maintenance..." [Siegelin et al. in ref. 49, referring to glioblastoma]. Adding disulfiram and ketoconazole-assisted ritonavir to current treatment of glioblastoma might prove to be a step in such project of comprehensive dismantling.

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