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14. ABSTRACT
Background: Many cancers appear to arise from rare, primitive self-renewing cancer cells, that are biologically distinct from their more numerous differentiated progeny characterizing the malignancy. Although the clinical significance of these cancer stem cells (CSC) has been uncertain, our data suggest that they are responsible for many of the relapses that follow anticancer therapy. Objective/Hypothesis: We hypothesize that the dramatic initial response rates in ovarian carcinoma represent therapeutic effectiveness against the differentiated cancer cells making up the bulk of the tumor, while the high rate of relapses represent rare, resistant CSC. The overall objectives of these studies are to identify and characterize ovarian CSC, and to develop novel therapies that target these cells. Shared stem cell characteristics and pathways will be key elements of both the characterization and therapeutic investigations. Specific Aims: 1) Identify, isolate, and characterize ovarian CSC, and 2) Develop clinical approaches for eliminating ovarian CSC. Study Design:. Using a spontaneously developing rat ovarian carcinoma (FNAR) that mimics the human disease, strategies will be developed to isolate and characterize ovarian CSC. These results will be used to guide similar studies using human ovarian cell lines, and ultimately primary human ovarian carcinoma. Our preliminary data show that aldehyde dehydrogenase (ALDH) expression marks FNAR cells which initiate tumors in rats. These cells highly CD24, CD44 and CD47 when compared to the bulk tumor cells. Preliminary data demonstrate human ovarian cancer cell lines contain a parallel ALDH-expressing cell population. Potential therapeutic strategies will also be initially tested in the rat model and human cell lines; those with activity in these models will be evaluated against ovarian CSC from clinical specimens. Impact: The potential existence of ovarian CSC has enormous implications for the development of novel therapies for this disease. In particular, it would explain the transient remissions that characterize current treatments for ovarian carcinoma. Isolation and characterization of these cells will also facilitate the development of novel treatment approaches.

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Ovarian Carcinoma, Cancer Stem Cells, Aldehyde Dehydrogenase

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Introduction

Recent data suggest that many cancers arise from, and are maintained by, rare self-renewing cells that are biologically distinct from the more numerous differentiated cells that make up the bulk of the tumor. Although the clinical significance of these so-called cancer stem cells (CSCs) has been uncertain, our group and others have hypothesized that they are responsible for many of the relapses that follow anticancer therapy. Importantly, recent data in both hematologic malignancies and solid tumors from our group and others now for the first time strongly suggest that rare CSC populations are in fact relatively resistant when compared to the bulk tumor cells and responsible for relapse. Minimal residual disease (MRD), the cancer cells remaining during treatment-induced remissions, are enriched for cells with a CSC phenotype, and the presence or absence of these cells correlates with progression-free survival. Ovarian carcinoma is one of the most responsive solid tumors, with the majority of affected women now achieving complete remissions. Unfortunately, most affected women eventually relapse and die of the disease. We hypothesize that the initial clinical responses in ovarian carcinoma represent therapeutic effectiveness against differentiated cancer cells making up the bulk of the tumor, while the high rate of relapses represents rare, biologically distinct CSCs resistant to the therapies effective against the tumor bulk. The limited understanding about the phenotype of normal ovarian epithelial stem cells is an obstacle to identifying ovarian CSCs, if they exist. However, several characteristics that appear to be shared by normal stem cells from many tissues, such high expression of aldehyde dehydrogenase (ALDH) and the Hoechst side population (SP) phenotype, may serve as markers for CSCs from many malignancies. In particular, we have found that high expression of ALDH is highly expressed by, and can be used to isolate, normal stem cells and CSC from all the tissues that we have studied including, in preliminary data, ovarian CSCs. The overall objectives of these studies are to identify and characterize ovarian CSCs, and to develop novel therapies that target these cells. Shared stem cell characteristics and pathways will be key elements of both the characterization and therapeutic investigations. Combining stem cell biology expertise with expertise in gynecologic oncology/ovarian carcinoma and in animal models of cancer this proposal will: 1) Identify, isolate, and characterize ovarian CSCs*,* and 2) develop clinically applicable approaches for eliminating ovarian CSCs.

Body

We have fully characterized the spontaneous rat ovarian carcinoma that is being used to model our characterization and treatment studies (Sharrow *et al J Ovarian Res.* 3:9, 2010,). The tumor, designated FNAR, could be serially transplanted into Lewis rats and propagated as a cell line *in vitro*, maintaining the properties of the original tumor. The FNAR cells displayed striking morphologic similarities to human ovarian carcinoma, resembling the endometrioid carcinoma subtype of surface epithelial neoplasms. The cells expressed estrogen receptor alpha, progesterone receptor, androgen receptor, her-2/neu, epithelial cell adhesion molecule, CA125, and nuclear beta-catenin. A gene expression profile showed upregulation of a number of genes that are also upregulated in human ovarian carcinoma. Thus, this reliable model of ovarian carcinoma should be helpful in better understanding the biology of the disease as well as the development of novel treatment strategies.

Studies into identifying and characterizing CSCs from hematologic malignancies have been greatly facilitated by a comprehensive understanding of cell surface antigen expression throughout lymphohematopoietic differentiation. In contrast, little is known about the cell surface phenotype associated with the growth and development of most non-lymphohematopoietic tissues. Thus, we have been studying the ability of the pan-stem cell marker ALDH1 to identify ovarian CSCs in this rat model, with the intent to use information in this model to interrogate

human ovarian cancer. Preliminary studies showed that 2-4% of the FNAR cells express high levels of ALDH, and these cells were responsible for the producing the tumor growth upon intraperitoneal (IP) injection. Conversely, the ALDH^{low} (>95% of the total cells) produced transient abdominal swelling and malignant ascites. However, after several passages of the line *in vitro*, the ALDH^{high} population disappeared (Figure 1A). After studying several culture

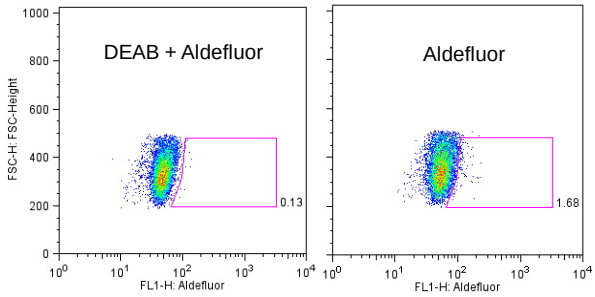

Figure 1A. The majority of FNAR cells cultured in standard medium do not express ALDH (DEAB is control for Aldefluor).

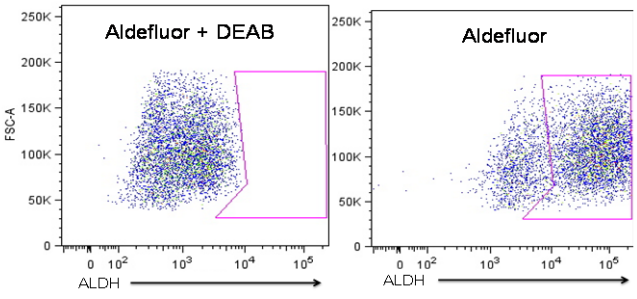

Figure 1B. The majority of FNAR cells cultured in stem cell KnockOut medium express high ALDH (DEAB is control for Aldefluor).

conditions, it became clear that serum led to terminal differentiation and loss of the ALDH^{high} subpopulation. After studying several different culture techniques, we found that upon transferring the cell line to a serum-free culture medium developed for growing undifferentiated embryonic stem cells (KnockOut SR, Invitrogen) the ALDH^{high} population not only reappeared, but after several passages represented the predominant population in the cell line (Figure 1B). Preliminary data shows that the ALDH^{high} cells maintained in KnockOut medium are phenotypically identical to the ALDH^{high} cells in the early FNAR passages, while the cells maintained in standard medium are phenotypically identical to the bulk FNAR cells in the early passages. That is, both populations express most ovarian cancer markers, including Her2/neu and EPCAM. However, in addition to ALDH, the FNAR ALDH^{high} cells (putative CSCs) expressed the stem cell markers CD24, CD44, and CD117 not expressed by the ALDH^{low} cells. Moreover, the ALDH^{high} FNAR cells do not appear to express any of the sex hormone receptors (ER, PR, or AR) expressed by the ALDH^{low} cells.

Similar studies have begun on human ovarian carcinoma cell lines. The well-characterized human ovarian carcinoma cell line SKOV3 contained essentially no ALDH^{high} cells after staining with Aldefluor even in KnockOut medium, similar to the FNAR cells in standard medium (Figure 2A). However, SKOV3 cells made resistant to taxol by our colleague Dr. Le-

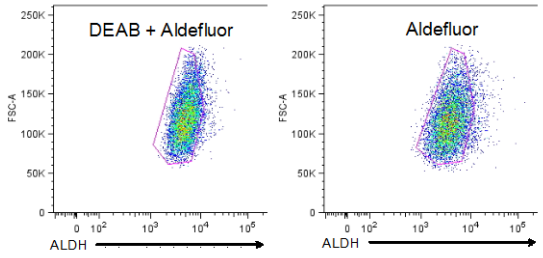

Figure 2A. SKOV-3 ovarian CA cells cultured in stem cell KO medium express no ALDH (DEAB is control for Aldefluor).

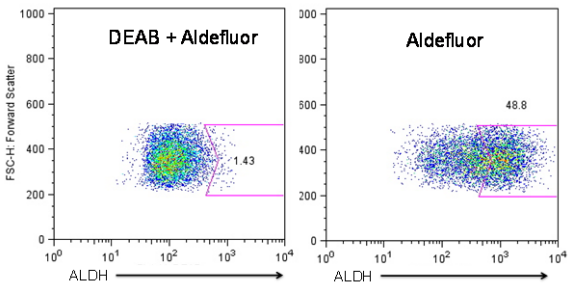

Figure 2B. Nearly half of taxol-resistant SKOV-3 ovarian CA cells cultured in stem cell KO medium express high ALDH (DEAB is control for Aldefluor).

Ming Shih in Johns Hopkins department of Pathology contained a very high number (nearly 50%) of ALDH^{high} cells, similar to the FNAR cells in KnockOut medium (Figure 2B).

Now that we have developed stable populations of the bulk tumor and ALDH^{high} subpopulations for both the rat FNAR and human SCOV3 cell lines, we have again begun completing the studies characterizing them. The preliminary cell surface antigen expression results described above suggest the ALDH^{high} cells from both the rat and human ovarian carcinoma cells have stem cell features phenotypically. The FNAR ALDH^{high} cells when sorted into 100 cell wells, completely recapitulate the heterogeneity of the cell line grown in knock-our medium (Figure 1B), while the ALDH^{low} cells only generate ALDH^{low} cells cells (Figure 1A). The ALDH^{low} cells grow adherently and have a doubling time of 18 hours, while the ALDH^{high} cells grow both attached and in suspension and have a doubling time of 72 hours. The taxol resistant ALDH^{high} SKOV3 cells similarly had a much longer doubling time that the ALDH^{low} SKOV3 cells.

It has been assumed that CSCs are relatively resistant to drugs, at least in part the result of stem cell characteristics shared with normal stem cells. We studied the drug sensitivity of the ALDH^{high} FNAR cells and the ALDH^{low} to three of the most commonly used drugs in ovarian cancer, all with different mechanisms of action and resistance: paclitaxel, carboplatin, and gemcitabine. The ALDH^{high} cells were relatively resistant to all 3 drugs (Figure 3A and B, data not shown for gemcitabine). Similarly, the ALDH^{high} SKOV3 were also relatively resistant to all three drugs (Figure 4). These data demonstrate that the putative CSCs from both the rat ovarian carcinoma and the human ovarian cancer line are resistant to drugs commonly used in the treatment of ovarian cancer.

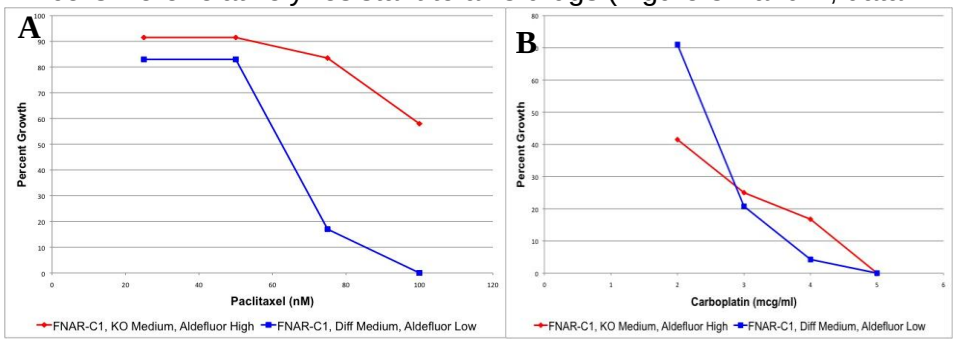

Fig 3. Relative drug sensitivity of ALDH^{high} (red line) and ALDH^{low} (blue line) FNAR cells. Cells were incubated with varying concentrations of taxol (A) and carboplatin (B) and LDA performed. Results are 3 separate experiments.

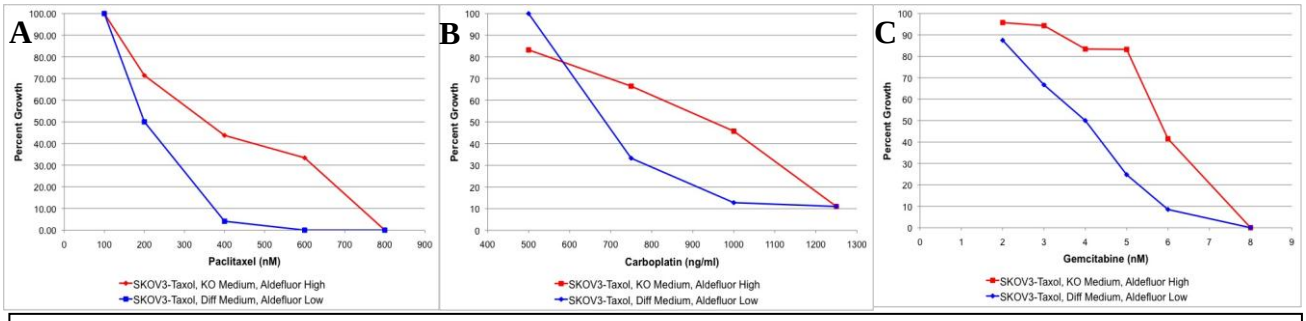

Fig 4. Relative drug sensitivity of ALDH^{high} (red line) and ALDH^{low} (blue line) FNAR cells. Cells were incubated with varying concentrations of (A) paclitaxel, (B) carboplatin, and (C) gemcitabine and LDA performed. Results are 3 separate experiments.

The two subpopulations from the FNAR lines have been transplanted intraperitoneally into female Lewis rats in three separate experiments. The results from the first experiment with the FNAR cells are available; the others are yet too early. All four rats transplanted with 50K unseparated FNAR cells died of overwhelming ovarian CA at a median of 177 days. Only one of

four rats receiving 50K ALDH^{low} cells from the KnockOut medium died, at 100 days. All rats transplanted with 50K ALDH^{high} cells grown in KnockOut medium died, at a medium of 200 days. These data are consistent with early, transient engraftment from the ALDH^{low} cells, with later, but persistant and fatal engraftment from the ALDH^{high} cells. We are currently performing similar experiments with the Taxol resistant SCOV3 cells grown in standard and knock-out medium, and the preliminary results parallel those with the FNAR cells. The ALDH^{high} cells recapitulate the heterogeneity of the cell line grown in knock-our medium (Figure 2B), have a longer doubling time and grow in suspension, while while the ALDH^{low} cells only generate ALDH^{low} cells cells (Figure 2A). Similar experiments transplant experiment using NOD/SCID-IL2Rγ^{null} (NSG) mice have also been performed with the Taxol-resistant SCOV3 cells and the results pending.

We have also performed expression arrays on the two subpopulations from both cells lines. Table 1 shows a genes of interest in a comparison of the FNAR ALDH^{high} and ALDH^{low} cells Not surprisingly, ALDH1 is markedly upregulated in the ALDH^{high} cells. In addition, the stem cell markers WT1 and CD47 are also upregulated. CD24 has also been reported to be downregulated in ovarian (Meng *et al Clin Exp Metastasis,* Epub ahead of print May 18, 2002) and breast cancer (Al-Hajj et al Proc Natl Acad Sci USA 100:3983–3988, 2003) and we have previously showed that GPI-anchored proteins, like CD59, are down-regulated in cancer stem cells. Interestingly, CD44, which has been reported to be upregulated in ovarian and breast cancer, is significantly down-regulated in the FNAR ALDH^{high}. The Wnt and Hedgehog (Hh) signaling pathways are also upregulated in the ALDH^{high} cells. The Taxol-resistant SCOV3 ALDH^{high} showed a similar expression pattern when compared to the SCOV3 ALDH^{low} cells (Table 2).

Gene	Fold upregulated	p value
ALDH1A2	+39-fold	0.02
WT1	+4.5-fold	0.02
CD47	+2.1-fold	0.001
CD44	-16.7-fold	0.001
CD59	-3-fold	0.03
CD24	-1.8-fold	0.005

Table 1. Expression differences between the ALDH^{high} FNAR cells, as compared to ADLH^{low}.

Gene	Fold upregulated	p value
ALDH1A1	+33.7-fold	<0.001
Wt1	Not on array	NA
CD47	+1.83-fold	0.01
CD24	-11.5-fold	<0.001
AR	-2.6-fold	<0.001
MUC1	-2.8-fold	<0.001

Table 2. Expression differences between the ALDH^{high} Taxol-resistant SCOV3 cells, as compared to ADLH^{low} cells.

To date, there are limited reports of growing primary ovarian cancer in immunodeficient mice. However, many ovarian cancer cell lines grow IP in immunodeficient mice, and the pattern of growth of other human cancers in immunodeficient mice tends to parallel that seen clinically. As detailed in the proposal, we have attempted to develop an immunodeficient mouse model for primary ovarian carcinoma. CD45^{+} cells represent a high percentage of the cells present in the ascites in many if not most women, and there is some data these inflammatory cells might inhibit engraftment. Thus, we isolated the EPCAM^{+}/CD45^{-} cells from the malignant ascites of a woman with ovarian carcinoma, and injected 1 x 10^5 cells IP into 3 NOD/SCID mice irradiated with 200 cGy. All 3 mice developed malignant ascites and peritoneal studding with human ovarian cancer. These data form the basis of subsequent studies looking at engraftment with subsets of fresh, explanted tumor isolated by CSC phenotype

In summary, it appears that ALDH expression appears to isolate a population of cells from both the rat ovarian carcinoma model and a human ovarian cancer cell line that have characteristics of CSCs as others have recently suggested (Kryczek *et al Int J Cancer*. 130:29-39, 2012). These cells grow more slowly and in suspension, recapitulate the heterogeneity of the line, are relatively drug-resistant *in vitro,* and express stem cell markers. The second specific aim is to develop treatments that target ovarian CSCs, with the ultimate intent of moving

promising approaches into the clinic. This aim has just begun until the second year of the grant, and was delayed with the loss of the $ALDH^{high}$ subpopulation. With characterization of the $ALDH^{high}$ and $ALDH^{low}$ subpopulations, these experiments are about to begin. Our preliminary data in hematologic malignancies suggest that inhibitors or telomerase and Hedgehog signaling are prime candidate drug for targeting the ovarian CSCs.

Key Research Accomplishments

Initial characterization of putative ovarian CSCs in FNAR model and human ovarian cell lines.

Using specialized culture medium, $ALDH^{high}$ sublines for both the FNAR rat model and the SCOV-3 human ovarian CA have been developed and are facilitating our characterization of the putative ovarian CSCs.

Preliminary data that the $ALDH^{high}$ sublines are phenotypically and functionally CSCs.

Reportable Outcomes

Sharrow AC, Ronnett BM, Thoburn CT, Barber JB, Giuntoli RL, Armstrong DK, Jones RJ, Hess AD. Identification and characterization of a spontaneous ovarian carcinoma in Lewis rats. J Ovarian Res. 3:9, 2010

Sharrow A, Ghiaur G, Gerber, Jones RJ. Cancer Stem Cell Principles. In, Translational Cancer Research: Molecular Therapeutics (Eds, Anderson, K and Kurzrock) Wiley, Hoboken, NJ, 2013 In Press.

Personnel

Jones, Richard, PI (1.2 calendar months)

Hess, Allan, Co-Investigator (.60 calendar months)

Giuntoli, Robert, Co-Investigator (.60 calendar months)

Sharrow, Allison, Graduate Student (12 calendar months)

Rogers, Ophelia, Research Specialist (1.8 calendar months)

Conclusion

We have developed culture conditions – primarily with the use of the serum-free KnockOut medium developed for growing undifferentiated ES cells – that permit large scale propagation of the putative ovarian CSCs in both rat and human cell lines. These putative CSCs phenotypically and biologically function as ovarian CSCs. The potential existence of ovarian CSCs has enormous clinical implications. Not only it would explain the transient remissions that characterize current treatments for ovarian carcinoma, but isolation and characterization of these cells will also facilitate the development of novel treatment approaches. Preliminary experiments suggest that $ALDH^{high}$ cells from fresh explanted ovarian cancer from ascites also appear to have CSC characteristics and may grow in NSG transplants are. Investigations into clinically-translatable treatments that target ovarian CSCs are underway.