Aldehyde dehydrogenase\textsuperscript{high} gastric cancer stem cells are resistant to chemotherapy

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Abstract. Cancer stem cells (CSCs) are known to influence chemoresistance, survival, relapse and metastasis. Aldehyde dehydrogenase (ALDH) functions as an epithelial CSC marker. In the present study, we investigated the involvement of ALDH in gastric CSC maintenance, chemoresistance and survival. Following screening for eight candidate markers (CD13, CD26, CD44, CD90, CD117, CD133, EpCAM and ALDH), five gastric cancer cell lines were found to contain small subpopulations of high ALDH activity (ALDH\textsuperscript{high} cells). We also examined the involvement of ALDH\textsuperscript{high} cell populations in human primary tumor samples. Immunodeficient NOD/SCID mice were inoculated with tumor tissues obtained from surgical specimens. ALDH\textsuperscript{high} cells were found to persist in the xenotransplanted primary tumor samples. In the immunodeficient mice, ALDH\textsuperscript{high} cells exhibited a greater sphere-forming ability \textit{in vitro} and tumorigenic potential \textit{in vivo}, compared with subpopulations of low ALDH activity (ALDH\textsuperscript{low} cells). Cell cultures treated with 5-fluorouracil and cisplatin exhibited higher numbers of ALDH\textsuperscript{high} cells. \textit{Notch1} and \textit{Sonic hedgehog} (\textit{Shh}) expression was also found to increase in ALDH\textsuperscript{high} cells compared with ALDH\textsuperscript{low} cells. Therefore, it can be concluded that ALDH generates chemoresistance in gastric cancer cells through \textit{Notch1} and \textit{Shh} signaling, suggesting novel treatment targets.

Introduction

The discovery of cancer stem cells (CSCs) in hematopoietic malignancies (1) has revealed that tumor tissues comprise a bulk of proliferating or differentiated tumor cells derived from small populations of self-renewing cells (2). Since their identification in leukemia, CSCs have been detected in solid tumors of the head and neck (3), gastrointestinal system (4), colon (5,6), breast (7) and brain (8,9). CSCs are tumorigenic, which is evident from xenotransplantation in immunodeficient mice, and are resistant to chemoradiation, whereas daughter cells are chemoradiation-sensitive (10,11). Recent studies have demonstrated that CSCs survive chemotherapeutic and radiation therapy in hypoxic regions of tumors (10,11). Studies of cell-autonomous mechanisms have revealed the involvement of anaerobic glycolysis in CSC maintenance and chemoradiation resistance (10,11). For example, CD13/aminopeptidase N, a liver CSC marker, regulates reactive oxygen species (ROS) through recycling reduced glutathione (GSH), thus contributing to intracellular ROS decrease following chemoradiation exposure (12). Similarly, intracellular ROS are suppressed after chemoradiation therapy through the activity of the hyaluronic acid receptor, CD44, an adhesion molecule expressed in cancer stem-like cells that directly interacts with pyruvate kinase M2, which is putatively involved in anaerobic glycolysis in CSCs (13). Furthermore, the CD44 variant (CD44v) has been shown to interact with xCT, a glutamate-cystine transporter, and to control intracellular GSH levels (14). CD44 abrogation has been shown to cause a loss of xCT from the cell surface, to suppress tumor growth in a transgenic gastric cancer (GC) mouse model and stimulate the p38 (mitogen-activated protein kinase) pathway (a downstream target of ROS) and the expression of the cell cycle inhibitor, p21(CIP1/WAF1), suggesting that CD44 plays a role in GSH synthesis and protection against ROS in gastrointestinal cancers (14). Taken together, these data indicate that cancer metabolism is critical for the initiation and progression of gastrointestinal CSCs.

In the present study, we investigated cell surface markers in gastric CSCs and after screening eight candidate markers...
(CD13, CD26, CD44, CD90, CD117, CD133, EpCAM and ALDH), we confirmed the involvement of aldehyde dehydrogenase (ALDH) in sphere formation, tumorigenicity and chemoresistance. Throughout the study of the ALDH pathway, a cancer metabolism regulator, we encountered stemness genes, suggesting novel molecular therapeutic targets.

Materials and methods

Cell lines and cell culture. The human GC cell lines, AGS, NUGC3, GSU, MKN1, MKN7, MKN28, MKN45 and MKN74, were cultured in RPMI-1640 medium (Sigma), supplemented with penicillin, streptomycin and 10% fetal bovine serum, in plastic culture dishes (Corning). Spheres were cultured in Gibco® Dulbecco’s modified Eagle’s medium with nutrient mixture F-12 (Invitrogen), supplemented with 20 ng/ml human recombinant epidermal growth factor (Promega), 20 ng/ml basic fibroblast growth factor (PeproTech Inc.), B-27® Supplement (Invitrogen) and N2 Supplement (Wako), in low-attachment dishes (Corning).

Cell staining and flow cytometry. Cultured cells were harvested and stained using an Aldefluor® stem cell detection kit (StemCell Technologies) for 45 min at 37°C. To stain cell surface markers, cells were incubated on ice with antibodies against CD44, CD26, CD117, CD90 (all from BD Biosciences), EpCAM (BioLegend) and CD133 (Miltenyi). Isotype antibodies were used as the negative controls. Discrimination between live and dead cells was carried out using the Live/Dead® Fixable Yellow Dead Cell Stain kit (Invitrogen). Mouse cells were identified by anti-H2kd (eBioscience) and anti-mouse CD45 (eBioscience) antibodies.

Primary surgical specimens and xenografts. Tumor tissues were digested into single cells with collagenase (Roche) and DNase (Worthington) at 37°C for 1 h. Staining for fluorescence-activated cell sorting (FACS) analysis was performed, as described above. For xenografting, cells were injected subcutaneously with Matrigel® into NOD-SCID mice. All the animal experiments were performed with approval of Animal Experiments Committee of Osaka University.

RNA extraction, cDNA synthesis and quantitative PCR. Total RNA was extracted using TRIzol® reagent. cDNA was synthesized using SuperScript® (Invitrogen). Quantitative PCR was performed using LightCycler® 480 Real-Time PCR system. All procedures were performed according to the manufacturer’s instructions.

Statistical analysis. Statistical significance was determined using the Student’s t-test. Analyses were performed using JMP software.

Results

Screening of CSC markers in GC cell lines. We examined novel markers in gastric CSCs, if: i) they had been previously reported in other tumor types and for which useful antibodies were available for FACS analysis; ii) they were expressed in small populations (<50%) in the cell lines; and iii) these observations were evident in more than half the cell lines.

We investigated the functions of cell surface markers and intracellular molecules (ALDH) to establish a functional detection system. We screened six GC cell lines (AGS, NUGC3, GSU, MKN7, MKN1 and MKN45) for gastric CSC markers using eight candidate markers expressed in other CSCs (CD13, CD26, CD44, CD90, CD117, CD133, EpCAM and ALDH) (11). As shown in Table I, FACS analysis revealed a high expression of EpCAM in all the cell lines (almost 100% positive cells), whereas CD90, CD117 and CD133 expression was uniformly undetectable or negative. CD26 expression was positive (>50%) in four of the cell lines, but undetectable or negative in the other two, suggesting that CD26 expression depends on individual cell lines rather than the heterogeneous conditions of cell line subpopulations. CD13 expression was detected in only one cell line, GSU. Conversely, the investigation of ALDH indicated that the proportion of cells highly expressing ALDH (ALDHhigh cells) was relatively small (6.2-45.5%) compared with the other markers (CD13, CD26, CD90, CD117, CD133 and EpCAM; Table I and Fig. 1A). Moreover, the ALDHhigh cell populations reproducibly disappeared upon the addition of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB), indicating the specificity of detection in ALDHhigh cell populations. Reportedly, ALDH1A1, a substrate for DEAB inhibition, has been shown to be responsible for ALDH activity in CSCs (15). Thus, we focused on ALDH activity.

Cells expressing high levels of ALDH also express CD44. We examined CD44 expression, reportedly a CSC marker in breast, colon, esophageal and gastric cancers (11,13,14). Two-dimensional analysis data indicated that ALDHhigh cell populations represented only a small subpopulation of CD44-positive cells, suggesting that ALDH is a good candidate as a CSC marker in GC (Table I and Fig. 1B).

Cells expressing high ALDH exist in xenografts in immunodeficient mice. We then examined the involvement of ALDHhigh cell populations in human primary tumor samples. Primary tumor tissues from surgical specimens were obtained with written informed consent and inoculated into immunodeficient NOD/SCID mice. Approximately 30% of inoculated primary samples formed tumors in the mice after several weeks. The probability of tumor formation is likely influenced by tumor tissue viability (nutrients, necrosis and therapy-related damage), vasculogenesis in the mice (dependent on local conditions) and CSC conditions within primary samples. The tumor sample from a patient was subjected to FACS analysis. The data indicated that 57% of the human living tumor cells (separated by FACS using the Live/Dead Fixable Yellow Dead Cell Stain system and distinguished from mouse cells using anti-H2kd and CD45 antibodies) expressed active ALDH (Fig. 1C), indicating that ALDHhigh cells are present in primary human tumor sample.

Sphere formation and tumorigenicity of populations expressing high levels of ALDH. We then examined stemness in ALDHhigh cells. A culture of FACS-sorted ALDHhigh cells in serum-free medium resulted in the frequent formation of large spheres compared with cells expressing low ALDH (ALDHlow cells; Fig. 2A and B), suggesting that ALDHhigh cells possess a greater self-renewal ability, a critical charac-
We examined tumorigenicity in vivo by inoculating FACS-sorted ALDH\textsuperscript{high} and ALDH\textsuperscript{low} MKN45 cells subcutaneously into NOD/SCID mice. We performed a limiting dilution experiment by reducing the number of inoculating cells. The inoculation of 500 ALDH\textsuperscript{high}, but not ALDH\textsuperscript{low} cells resulted in tumor formation in three out of four mice (Fig. 2C). The inoculation of 5,000 cells resulted in tumors being formed from the ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells (Fig. 2C). Taken together, these observations indicate that, although multiple factors may be involved, ALDH function is closely associated with the initiation, maintenance and progression of CSCs in vitro and in vivo.

Table I. Screening for common CSC markers using six GC cell lines.

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-<1%. +, <50%. ++, 50-100%.

Figure 1. Aldehyde dehydrogenase (ALDH) and the other marker expression in gastric cancer cell lines. (A) ALDH in five gastric cancer (GC) cell lines. Diethylaminobenzaldehyde (DEAB) was used to inhibit ALDH activity, to show the specificity of detection. (B) Double detection of CD44 and ALDH in GC cells. ALDH\textsuperscript{high} cells are a small fraction of CD44\textsuperscript{+} cells. (C) Study of human primary GCs. NOD/SCID mouse xenografted tumor contains ALDH\textsuperscript{high} cells.
Chemoresistance in cells highly expressing ALDH and the underlying mechanisms. Our aim was the identification of novel molecular therapeutic targets. Reportedly, CSCs can survive toxic injuries and chemoradiation therapy (2,10,11). To combat this, we explored the effect of chemotherapeutic agents commonly used to treat GC. The exposure of the cell cultures to cisplatin and 5-fluorouracil (5-FU) increased the number of ALDH<sup>high</sup> cells (3.1-4.4% with cisplatin and 31.0% with 5-FU treatment; Fig. 3A), indicating that exposure to these chemotherapeutic agents causes an accumulation of surviving ALDH<sup>high</sup> CSCs. To elucidate the molecular mechanisms underlying this chemoresistance, we examined gene expression changes in candidate pathways (11). We found that Notch1 and Sonic hedgehog (Shh) expression was increased in ALDH<sup>high</sup>, compared to ALDH<sup>low</sup> cells, suggesting that the survival of ALDH<sup>high</sup> cells following chemotherapy is associated with increased Notch1 and Shh signaling.
Discussion

The demonstration of an association between ALDH and tumors was first shown in breast cancer (16) and subsequently in pancreatic (17), liver (18), colorectal (19), head and neck (19), thyroid (20) and lung (21) cancers. In lung cancer, ALDH activity has been shown to be selective for adenocarcinoma stem cells, depending on Notch signaling (21), in agreement with our observations of gastric CSCs. We demonstrated that in GC, Notch and Shh signaling may be important for both CSC maintenance and the generation of chemoresistance, providing the rationale for further study of therapy-resistant ALDHth CSCs. ALDH is widely used as a marker to identify and isolate various types of normal stem cells and CSCs (22). In GC, several markers reportedly characterize CSCs: CD133 (23), CD44 (23-26), side-populations identified by FACS (27), CD44 and EpCAM (25), CD54 (26) and CD90 (28). Of these markers, CD44 and ALDH are involved in aerobic glycolysis during cancer metabolism. Although an association between ALDH and the clinicopathological features of GC has been reported (29), the relevance of ALDH to chemoresistance has yet to be fully investigated; another study detected no association between immunohistochemical staining for ALDH and prognosis in GC patients (23). In this study, we examined for the first time the involvement of ALDH in chemoresistance and identified a candidate underlying molecular mechanism for this resistance.

GC is the second major cause of cancer-related mortality worldwide and is prevalent across Asia. Helicobacter pylori (H. pylori) infection was identified in 1982 by Marshall and Warren in patients with chronic gastritis and gastric ulcers (30). H. pylori-associated GC has been investigated in order to elucidate the mechanisms underlying gastric tissue damage. In general, the two mechanisms by which H. pylori promotes cancer are as follows: i) enhanced production of free radicals proximal to the H. pylori infection site, increasing the host cell mutation rate; and ii) pregenetic factors that transform host cell phenotypes by altering adhesion proteins or inflammation-related cytokines/chemokines, such as tumor necrosis factor-α or interleukin-6. Thus, H. pylori infection causes enhanced migration or invasion of damaged epithelial cells, without additional tumor suppressor gene mutations (31). Those non-cell autonomous mechanisms are likely facilitated by the hypoxic microenvironment of tumors, since recent studies have implicated hypoxia in inflammatory reactions provoked by H. pylori infection (32). Indeed, hypoxia-inducible factor-1α is mediated by the induction of a ROS-inducible protein (apurinic/apyrimidinic endonuclease 1) and its enhanced interaction with the transcriptional coactivator, p300, leads to transformed phenotypes in H. pylori-infected gastric epithelia (33). Although H. pylori infection and related atrophic gastritis are closely associated with GC, hypoxia and its related metabolism play a critical role in tumor initiation and progression in the stomach and likely in other organs (34). Further studies are warranted to elucidate the association between H. pylori infection and ALDH-positive CSCs in hypoxic areas and to evaluate the eradication of H. pylori infection and GC treatment by surgery, chemotherapy and molecular targeting of therapy-resistant CSC functions.

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