Novel Tumor Antigen-Specific Monoclonal Antibody-Based Immunotherapy to Eradicate Both Differentiated Cancer Cells and Cancer-Initiating Cells in Solid Tumors

Francesco Sabbatino,[a,] Yangyang Wang,[a,*] Xinhui Wang,[a] Joseph H. Schwab,[b] Soldano Ferrone,[a,b] and Cristina R. Ferrone[a]*

A growing body of experimental and clinical evidence strongly suggests that the resistance of cancer-initiating cells (CICs) to conventional therapies represents a major obstacle to the successful treatment of a malignant disease. To overcome this limitation a novel combinatorial tumor antigen (TA)-specific monoclonal antibody (mAb) strategy has been developed. In this strategy TA-specific mAbs are combined with chemotherapeutic agents and/or small molecules that inhibit aberrantly activated signaling pathways in cancer cells and especially in CICs. The in vitro results we have obtained indicate that this strategy is very effective in eradicating both differentiated cancer cells and CICs in several types of malignant disease. If the in vitro results have in vivo relevance, the strategy we have designed may have an impact on the treatment of malignant diseases.
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A growing body of experimental and clinical evidence indicates that most, if not all types of malignant tumors contains a variable, although low number of malignant cells with stemness characteristics.[1,2] These cells, which are termed cancer stem cells (CSCs) or cancer-initiating cells (CICs), have attracted much attention since they are believed to play a major role in the resistance of malignant diseases to conventional chemotherapy and/or radiotherapy and to be the cause of metastatic spread and disease recurrence.[1,3,4] The latter two are the major causes of morbidity and mortality in cancer patients.[5] These clinical findings have prompted a number of investigations to characterize this subpopulation of cancer cells and to develop therapeutic strategies to eradicate CICs. Here, we describe the markers that have been used to identify and isolate CICs from cell lines and from surgically removed tumors in solid malignancies and the characteristics of this subpopulation of cancer cells. Next, we discuss why we have selected tumor antigen (TA)-specific monoclonal antibody (mAb)-based immunotherapy instead of T-cell–based immunotherapy to develop a combinatorial strategy to eradicate CICs. Then, we describe the TA systems we have used to test the validity of our hypothesis. We also present some in vitro results that prove the efficacy of the strategy we have designed. Lastly, we discuss the significance and potential clinical relevance of the results we have obtained.

IDENTIFICATION AND ISOLATION OF CICs

Due to the close relationship with tumor initiation, progression, metastasis, and drug resistance, identification and isolation of CICs from the total cancer cell population is essential to assess the ability of therapeutic strategies used for the treatment of malignancies to inhibit and eradicate CICs. The latter are relatively rare and lack a unique morphology that distinguishes them from their differentiated progeny *in vitro* and *in vivo*.[6] Therefore,

[a]Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA.
[b]Department Orthopaedic Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA.
* Francesco Sabbatino and Yangyang Wang contributed equally to this work.
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Address correspondence to Cristina R. Ferrone, MD, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, 55 Fruit St, Boston, MA 02114. E-mail: cferrone@mgh.harvard.edu
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identification of CICs mainly has relied on their ability to grow indefinitely as tumor spheres in vitro and to induce tumors when injected in low number in immunodeficient mice.[7]

Several markers and methods have been used to identify and isolate CICs in cell lines and surgically removed tumors in many types of human malignancies. The methods can be essentially divided into two groups. One relies on the use of mAbs recognizing molecules that have been shown or postulated to be selectively, if not specifically, expressed by CICs. The cell surface markers used include CD133 in brain cancer,[8] SSEA-1 and CD44 in head and neck cancer,[9] epithelial specific antigen (ESA), CD44, and CD24 in breast cancer,[10] CD133 in lung carcinoma,[11] CD133, CD44, epithelial cell adhesion molecule (EpCAM) or CD24 in pancreatic ductal adenocarcinoma,[12,13] CD133, CD44, CD166, EpCAM, or CD24 in colon cancer,[14–16] CD44, CD133, or high expression of α2β1 in prostate cancer,[17] CD133 in osteosarcoma,[18] and CD133 or CD15 in chordoma.[19] The other method used to identify CICs relies on the measurement of metabolic activities that are increased in CICs. High activity of the adenosine triphosphate (ATP)-binding cassette (ABC) transporters has been used to identify and isolate CICs in brain cancer, lung cancer, and melanoma.[8,20,21] ABC proteins are membrane transporters that can pump various distinct and structurally unrelated small molecules (ie, cytotoxic drugs and dyes) out of cells at the expense of ATP hydrolysis. Normal stem cells and CICs appear to express high levels of ABC transporters.[22–24] This phenomenon could contribute to the multidrug resistance of CICs because many anti-tumor drugs can be pumped out, thereby resulting in their low intracellular concentration.[20,25] The ABC superfamily includes the multidrug resistance proteins (MRPs/ABCC), as well as the breast cancer resistance proteins (BCRP/ABCG2) and P-glycoprotein (P-gp/ABCB1). Expression levels of these ABC transporters in malignant stem cells can be determined by protein quantification or by treatment of cells with Hoechst 33342 dye.[25] In the latter case, CICs have been identified in vitro as cells that exclude the intracellular dye.

Another metabolic activity that has been used widely as a marker for the identification and isolation of CICs in many types of malignancies is represented by the aldehyde dehydrogenase (ALDH) activity. In agreement with the literature, we have found that cells which display a high level of ALDH activity, referred to as ALDH^{bright} cells, are present in many types of tumors. They include head and neck, triple-negative breast, lung, pancreatic, colon, prostate, and ovarian cancers (Figure 1).[7,26–33] ALDH is a family of enzymes that are involved in the maintenance of cellular homeostasis by metabolizing both endogenous and exogenous reactive compounds.[34] ALDH enzymes are known to modulate several cell functions, including proliferation, differentiation, and survival, as well as the cellular response to oxidative stress.[35] Therefore, these enzymes may be considered as a marker for these cells and also may play a functional role in terms of self-protection, differentiation, and/or expansion of stem cell populations. High activity of different ALDH isoforms has been identified in normal stem cells and in CICs. ALDHA1, along with the ALDH3A1 isoform, has been the most extensively used to identify and isolate CICs. Levels of ALDH activity are measured using an ALDEFLUOR kit (Stem Cell Technologies, Vancouver, BC, Canada). In combination with diethylaminobenzaldehyde (DEAB), an inhibitor of the ALDH1A1, -A2, -A3 and ALDH3A1 isoforms, the flow reagent ALDEFLUOR identifies cell subpopulations with different ALDH activity levels.[36] This information is not provided by testing cells with ALDH-specific mAbs since the level of the enzyme expression does not correlate with its functional activity.

Viable cells are required to measure ABCB5 (P-gp/ABCB5) function and ALDH activity. This requirement represents a major limitation to the analysis of surgically

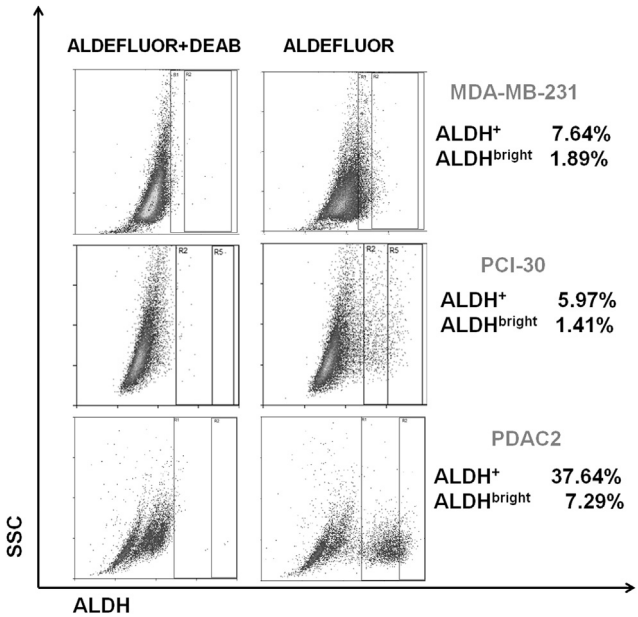

Figure 1. ALDH activity expression as a marker of CICs in triple-negative breast cancer (TNBC), head and neck squamous cell carcinoma, and pancreatic adenocarcinoma human cell lines. TNBC MDA-MB-231, head and neck squamous cell carcinoma PCI-30 and pancreatic adenocarcinoma PDAC2 cells were incubated with ALDEFLUOR with or without the DEAB inhibitor to detect ALDH activity. ALDH^{bright} cells were identified as those ALDH^{+} cells with twice the mean fluorescence intensity of the ALDH^{+} cell population. Both percentages of ALDH^{+} and ALDH^{bright} cells are shown for each cell line.