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Combination Treatment with All-Trans Retinoic Acid Prevents Cisplatin-Induced Enrichment of CD133+ Tumor-Initiating Cells and Reveals Heterogeneity of Cancer Stem Cell Compartment in Lung Cancer

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Abstract: The existence of specific cellular subpopulations within primary tumors with increased tumorigenic potential and chemotherapy resistance (tumor-initiating cells, TICs) holds great therapeutic implications. Resistant cells can remain quiescent for long periods and be responsible for local relapses and metastasis. We and others have previously described in non–small-cell lung cancer the presence of cisplatin-resistant CD133+ cells with tumor-initiating potential and co-expression of CXCR4 as possible indicator of TICs with disseminating potential. In this study, we report, by in vitro cell fate tracing systems, heterogeneity within the TIC compartment with a highly quiescent pool and a slowly dividing subpopulation, both containing CD133+ cells but respectively enriched for CD133+/CXCR4- and CD133+/CXCR4+ cells. Pretreatment with differentiating agent all-trans retinoic acid counteracts cisplatin resistance specifically of the slowly dividing compartment indicating effect on CD133+/CXCR4+ cells. The same effects are appreciable also in vivo in patient-derived xenografts, where several cycles of all-trans retinoic acid and cisplatin treatment are able to stably reduce this fraction of TICs and tumor dissemination. Thus, partially affecting the heterogeneous TICs compartment, differentiating therapy has promising effects in counteracting cisplatin resistance of CD133+ cells, reducing both local tumor growth and dissemination. In addition, our approach discloses a further level of complexity of chemotherapy-resistant CD133+ TICs, revealing phenotypical and functional heterogeneity of the cancer stem cell compartment in lung cancer.

Key Words: Non–small-cell lung cancer, Retinoic acid, Cancer stem cells dynamics, Cisplatin resistance.

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Non–small-cell lung cancer (NSCLC), representing approximately 80% of all lung cancers, is the main cause of cancer-related deaths worldwide. Even though recently developed targeted therapies have been shown to provide some benefit in predefined subclasses of patients with tumors carrying specific mutations, platinum-based chemotherapy still represent the standard systemic treatment for NSCLC. However, 5-year survival rates have not raised substantially in the past decades, remaining as low as 20% for late stage disease (III–IV).[1] Cisplatin resistance is the consequence of a multifactorial event which involves a combination of features, such as drug inactivation by detoxifying factors, alterations in checkpoint and apoptotic proteins, and variation in intracellular drug accumulation.[2–4] However a rising number of observations also indicates that, in several tumor types, distinct cellular subpopulations persist after treatment and that these cells own intrinsic characteristics associated with a stem-like phenotype[5] and can be indicated as cancer stem cells or tumor-initiating cells (TICs). TICs characterized by increased tumorigenicity, self-renewal ability, and multipotency[6,7] have been described for several tumor types, such as myeloid leukemia,[8] glioblastoma,[9–11] melanoma,[12] and several epithelial cancers[13–18] including lung cancer.[19,20] Particularly, lung cancer TICs are detected by the expression of cell surface markers as CD133 or by the high activity of aldehyde dehydrogenase and finally by increased capacity to efflux the DNA-binding dye Hoechst 33342 that define the so-called side population cells.[21] Even if some controversies are still open about the choice of the most appropriate marker to enrich for TICs, almost all published studied demonstrating the drug resistance properties of lung

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MM, LR, UP, and GS designed the research; MM and GB performed the research; MM, LC, GB, and GS analyzed the data; MM, LR, and GB wrote the paper; GS and LR gave the study supervision. All authors participated in the critical revision of the report. Massimo Moro, Giulia Bertolini, Luca Roz, and Gabriella Sozzi contributed equally to this study.

Samples of primary NSCLC were obtained from patients undergoing surgical resection, who gave their informed consent after approval from the Internal Review and the Ethics Boards of the Fondazione IRCCS Istituto Nazionale Tumori. Animal studies were performed according to the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale Tumori, according to institutional guidelines.

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TICs reported the enrichment for a fraction of $CD133^+$ cells after chemotherapy, indicating the leading role of such cell subset in chemoresistance.[22]

Furthermore, the stem-like and tumorigenic phenotype has been associated to a slow proliferating fraction of cells within primary lines or cancer cells.[23,24] The peculiar characteristics of TICs, and in particular their capability to remain in a quiescent state,[25] are consistent with a lower susceptibility to replication-related drugs as previously described also in NSCLC, where $CD133^+$ TICs have been reported as being spared by Cisplatin treatment.[20] Moreover, in vitro CDDP treatment of NSCLC-derived spheres enriched for TICs induces a replication block (G2/M) and a more efficient DNA damage repair that can be prevented by treatment with inhibitor of checkpoint protein kinase (Chk1).[26] Recently, cancer stem cells (CSC) from NSCLC have also been shown to be susceptible to Bcl-XL inhibition indicating potential therapeutic strategies targeting TICs.[27]

We reasoned that an efficient strategy to target TICs could also rely on their mobilization from the quiescence state inducing a chemo-susceptible phenotype, as already proposed and substantiated for leukemic stem cells.[28,29] Altogether, this would deplete the tumor of its CSCs reservoir preventing relapse, metastasis formation and, eventually, increasing the efficacy of chemotherapy.

In this study, to overcome CDDP resistance of $CD133^+$ NSCLC CSCs, we tested the capacity of all-trans retinoic acid (ATRA), in vitro and in vivo, to force the TICs fraction to differentiate to a more CDDP susceptible phenotype. ATRA has been reported to be effective in the cure and prevention of many types of cancer (reviewed in [30]), however, its differentiating role against solid tumors CSC's compartment is still poorly explored.[31–33] Using in vitro cell fate tracing systems and in vivo models of patient-derived xenografts (PDXs),[34] we show here that ATRA is able to interfere with the dynamics of the CSC compartment and prevents chemotherapy-related TICs increase.

MATERIALS AND METHODS

Cell Cultures

LT73 cells were derived in our laboratory from a primary lung tumor of a 68-year-old Caucasian male patient with lung adenocarcinoma. Cells were grown in RPMI-1640 (Lonza, Basel, Switzerland), supplemented with 10% fetal bovine serum (Lonza) and penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2.

PKH Labeling

LT73 cells were labeled with PKH67 Fluorescent cell linker (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instruction. In brief, cells were incubated 5 minutes at room temperature with PKH67 Dye Solution. Staining was stopped adding fetal bovine serum (Lonza) and labeled cells were analyzed by flow cytometry at different time points.

Culture of Cancer Tissue-Originated Spheroids

Cancer tissue-originated spheroids (CTOS) culture method was adapted from Kondo et al.[35]

In brief, tumor tissue was mechanically and then enzymatically digested in a solution of collagenase IV (5 mg/ml) and DNAse (100 U/ml; Sigma Aldrich, St. Louis, MO) in DMEM/F12 (Lonza, Verviers, Belgium) for 1 hour at 37°C and subsequently filtered through 100-μm and 40-μm cell strainers (Becton Dickinson, Franklin Lakes, NJ). The tumor tissue organoids retained in the strainer were washed in 30 ml of DMEM/F12 and centrifuged at 100*g* for 5 minutes. Tumor organoids were plated in stem cell medium SCM (described in [20]) and in 60 mm Petri dishes. Spheroids appeared in about 3 days. For culture expansion, spheroids were centrifuged at 100*g* for 5 minutes and incubated with a mild digestion solution of DMEM/F12 + collagenase IV 5 mg/ml at 37°C for 5 minutes.

Flow Cytometry Analysis

Single-cell suspensions were washed and incubated in staining solution containing 1% BSA and 2 mM ethylenediaminetetraacetic acid with specific antibodies at appropriate dilutions. For CD133 and CXCR4 staining, 10^6 cells were incubated with phycoerythrin-conjugated anti-CD133/1 (Miltenyi Biotec, Bergish Gladbach, Germany) and allophycocyanin-conjugated anti-CXCR4 (Becton Dickinson). Samples were acquired by FACS Calibur and analyzed with FlowJo_V10 software.

For lung dissemination analysis, a morphological gate allowing the identification of the highest percentage of human tumor cells in murine lungs was identified[36] and subsequent exclusion for $7\text{-}AAD^+$ dead cells and mouse $H2K^+$ cells was performed. This method was able to specifically detect as few as 10^3 single tumor cells in murine lungs.

Patient-Derived Xenograft Tumor Growth

All experiments were carried out with female SCID mice, 7–10 weeks old (Charles River Laboratories, Calco, Italy). Mice were maintained in laminar flow rooms, with constant temperature and humidity. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori, according to institutional guidelines. PDXs were established as described.[34] PDX111 (EGFRwt, KRASwt, LKB1wt, HER2wt, PIK3wt, BRAFwt) and PDX73 (EGFRwt, KRASwt, $LKB1^{K287X}$, HER2wt, PIK3wt, BRAFwt) were derived from a 77-year-old female and a 68-year-old Caucasian male patient, respectively, both with lung adenocarcinoma. For pharmacological experiments, mice were randomly distributed into equal groups (five mice per group, grafted in both flanks). Mice were treated with All-Trans Retinoic Acid (Sigma-Aldrich; 10 mg/kg gavage, qd × 5 × 3 weeks) and/or with Cisplatin (Teva, Petach Tikva, Israel; 5 mg/kg i.v. q7d × 3).

Immunofluorescence

10^4 LT73 cells were grown on Lab-Tek (ThermoFisher, Waltham, MA) slides and incubated with BSA 2% + NGS 5% blocking solution for 30 minutes, incubated with anti-human CD133/1 (Miltenyi; Biotec) for 1 hour at RT, then 30' at RT with AlexaFluor 488 goat anti human IgG (H+L) (Invitrogen) washed in tween 1× and mounted with the VECTASHIELD Mounting Medium, containing DAPI (Vector Laboratories, Burlingame, CA).