Int J Clin Exp Pathol 2016;9(3):3051-3056
www.ijcep.com /ISSN:1936-2625/IJCEP0020449

Original Article

Specific killing effect of cytotoxic T cells induced by OCT4 and Sox2 on lung adenocarcinoma stem cells

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Received November 24, 2015; Accepted January 23, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Objectives: The specific killing effect of cytotoxic T cells induced by OCT4 and Sox2 on lung cancer stem cells was investigated. Methods: CD40-activated B cells (CD40-B) were used as the antigen presenting cells (APCs) to induce the CSC-specific CTL with the peripheral lymphocytes of NSCLC patients, and the OCT4 and Sox2-specific T cell proliferation was observed to analyze the killing effect of the specific CTL on lung cancer CSCs. Results: The CD154 (CD40L) positive clone cell was successfully constructed via the lentiviral vector, and the peripheral blood mononuclear cells (PBMCs) were successfully extracted from lung adenocarcinoma patients. The PBMCs and the positive clone cells were co-cultured to prepare CD40-B cells, which were used as the APCs with the stimulation of OCT4 and Sox2 to construct the specific CTL. The killing effect of the CTL on the lung adenocarcinoma CSC, FC9 was tested, and the result verified the effective killing activity of the CTL on lung adenocarcinoma stem cells, with the optimum effect reached at the effector-target ratio of 40:1. Conclusions: The induced OCT4 and Sox2-specific CTL can effectively kill lung adenocarcinoma stem cells, providing new perspectives into the immunotherapy of lung cancer.

Keywords: OCT4, Sox2, cytotoxic T cells, 7410 lung adenocarcinoma stem cells

Introduction

Lung cancer is one of the malignancies seriously threatening human health. Surgery, radiotherapy, drug therapy, etc. are now employed clinically for the treatment of lung cancer. Drug therapy (including combined chemotherapy mainly with CDDP and EGFR target therapy) remains the primary choice because a majority of the confirmed patients have entered the mid-late stage [1, 2]. However, relapse and metastasis will occur in some patients due to drug resistance, and these metastatic lung cancer patients usually suffer poor prognosis. Therefore, effective killing of drug-resistance tumor cells remains crucial to control the disease progression and improve prognosis [3].

The drug-resistance mechanism of tumor cells is complex, which includes drug transport and metabolism, target gene mutation, anti-apoptosis gene expression, etc. [4, 5]. One of the primary causes leading to drug-resistance of tumor cells on the cellular level is a group of undifferentiated cancer cells in tumor tissues with the characteristics of stem cells, which are called the cancer stem cells (CSCs) [6]. Studies show that cytotoxic drug chemotherapy and target therapy can kill most tumor cells except CSCs [7]. CSCs are typically featured by self-renewal, entitling them the properties of high oncogenesis, metastasis, drug-resistance, etc. [8]. For lung cancer CSCs, the expression of pluripotent transcription factors, especially OCT4 and Sox2, is closely correlated to tumor relapse, metastasis and drug-resistance [9-11].

Given high resistance of CSCs to the existing cytotoxic drugs and target drugs, the specific cytotoxic T cells were used as an effective immunotherapy to kill this group of drug-resistance tumor cells. The tumor occurrence and development are a dynamic process concerning the interaction between tumor cells and the immune system. It remains unclear which lung cancer antigen is recognized by CTLs to display anti-tumor effects, but it is estimated based on the anti-tumor mechanism that the CSC-specific

Figure 1. Western blotting result of the expression product of the 293T cells transiently transfected with the CD154 lentiviral vector.

CTL therapy will be clinically effective by adoptive immunotherapy. In this study, the CSC-specific CTL targeting CT4 and Sox2 was constructed accordingly, and the killing effect of the CTL on lung adenocarcinoma cells was investigated, laying the foundation and providing new perspectives for the development of the immunotherapy based on targeting and killing drug-resistance CSCs.

Materials and methods

Construction of the CD154 lentiviral vector

CD40LG (CD154) was selected from the human placenta cDNA library and then digested by AgeI/NheI, which was then inserted into the GV341 lentiviral expression plasmid (Genechem, Shanghai, China). The constructed plasmid was then transiently transfected into 293T cells, and then the total protein was extracted for Western blotting. After package and concentration, 1 ml lentivirus was obtained (1×10^8 TU/ml).

Construction of the CD154 overexpression feeder cell

The CD154 virus was used to transfect the mouse embryonic fibroblast NIH3T3 (MOI = 5), and then 10 mg/ml puromycin was used to screen out the stably transfected cell clones two days after the transfection.

Extraction of the peripheral B cells and proliferation by co-cultural

Anticoagulant peripheral blood (10 ml) was collected from 10 lung adenocarcinoma patients, and then subjected to Ficoll gradient centrifugation to extract PBMCs. Then B cells were obtained via CD19 antibody magnetic separation. The CD40-B cells after two-week culture were subjected to immunofluorescence assay.

Ability of CD40-B cells to present antigens and induce CTLs

CD8+ T cells were extracted from PBMCs via magnetic separation and then mixed with CD40-B cells at the ratio of 5:1 as well as COT4 and Sox2 (each of 10 g/ml). Then incubation continued for two weeks.

Detection of the killing activity of CTL

The target cells were labeled with CFSE, and the controls were set: target cell blank control, target cell negative control and target cell positive control. Then mixed cultivation was performed: CD8+ T cells were collected and counted; 2×10^7 cells were centrifuged at 1500 rpm for 5 min, with the supernatant discarded; 1 ml 10% FCS-RPMI was added; CFSE-labeled PC9 cells were added into the FCM tubes respectively and then centrifuged at 1500 rpm for 2 min; incubation at 37°C continued for 24 h; PI staining and FCM detection were performed.

Computer operation, counting and calculation: % DT-e = experimental group DT; % DT-t = PC-t tube DT; % DT-s = NC-t tube DT. % DT = (CFSE+PI+ events/CFSE+PI+ events +CFSE+PI- events) × 100%. % specific cytotoxicity (% SC) = (% DT-e - % DT-c/% PC-t - % DT-c) × 100%.

Statistics

The statistical analysis was performed with SPSS 17.0. Qualitative variables were subjected to Chi-square test. Quantitative variables were subjected to analysis of variance. $P<0.05$ indicated statistical significance.

Results

Construction of the CD154 vector

The expression product of 31 kD was detected (**Figure 1**), which was consistent with the prediction, indicating successful construction.

CD154 overexpression feeder cell

The CD154 positive rate reached 96.9% by FCM (**Figure 2**).

Extraction of the peripheral B cells and proliferation by co-cultural

The result of separation showed an average amount of 1.78×10^6 B cells and an average

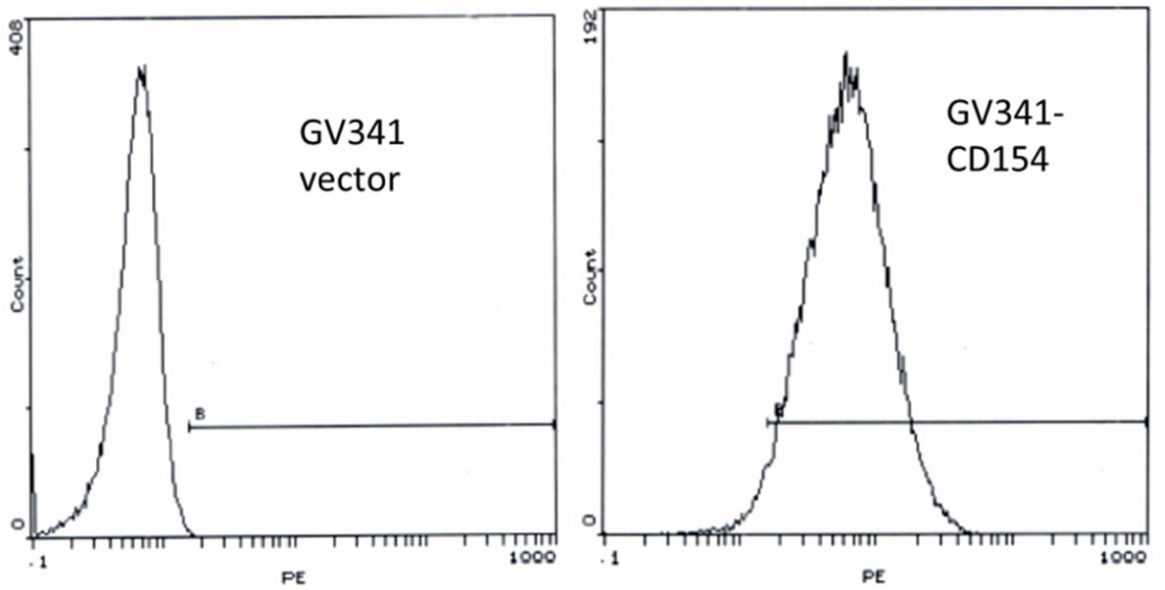

Figure 2. Analysis of the CD154 expression in the transfected NIH3T3 cells by FCM.

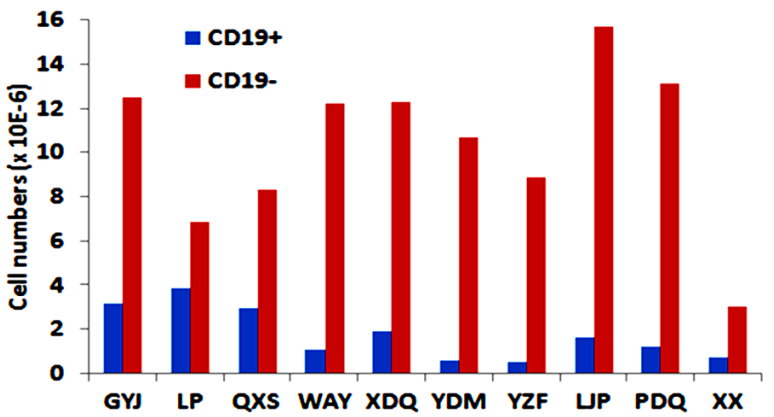

Figure 3. Amounts of B cells obtained from the 10 blood samples by CD19 antibody magnetic separation.

yield of 15.27% with the 10 blood samples (**Figure 3**).

The B cells from the HLA-A2+ blood samples and the tCD154 feeder cells were selected for in vitro incubation and proliferation. The amount of B cells was found to increase from 2 × 10^6 to at least 5 × 10^6 after one-week co-culture. The CD40-B cells after two-week culture were selected for immunofluorescence assay, and the result showed the positive expression of CD19 antigen (**Figure 4B**). The FCM result revealed a positive rate of 91% for the co-stimulatory molecules HLA-ABC and CD86 in the amplified CD40-B cells (**Figure 4D**, **4E**), which indicated a potential antigen-presenting ability of these B cells.

Ability of CD40-B cells to present antigens and induce CTL

The amount of the CD8+ T cells was increased by five times after counting. The FCM result showed that OCT4 and Sox2 could effectively stimulate the proliferation of CD3+CD8+ T cells with the positive rates reaching 87% (P1) and 94% (P2) respectively. CD40-B cells could induce the proliferation of CD8+ T cells when loaded with OCT4/Sox2 antigen peptides (**Figure 5**).

Detection of the killing activity of CTLs

The test standard was set with the unlabeled FC9 target cells as the baseline. After four-hour incubation, CFSE+PI- and CFSE+PI+ cells reached 1833 and 25341 respectively, and % DT = 6.75% (**Figure 6**). % DT of each group was calculated and then % Specific cytotoxicity (% SC) was obtained: effector-target ratio = 10:1, % DT = 21.04, % SC = 15.32; effector-target ratio = 20:1, % DT = 44.80, % SC = 40.80; effector-target ratio = 40:1, % DT = 70.49, % SC

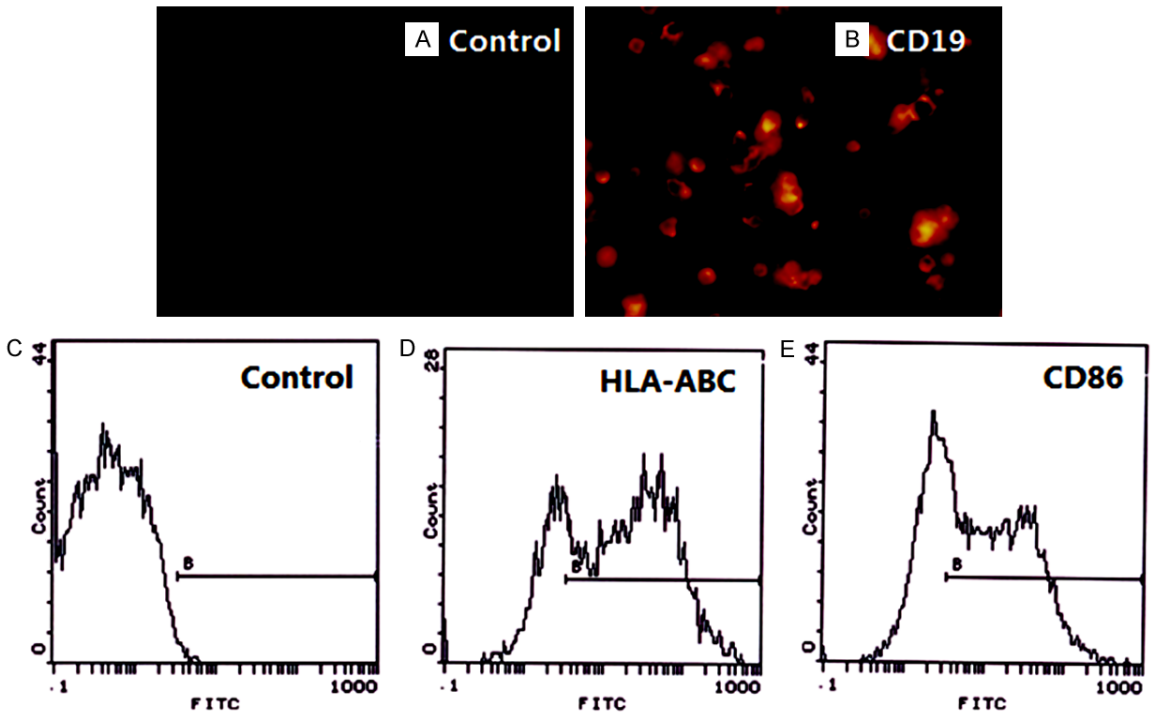

Figure 4. Amplification of the peripheral B cells co-cultured with the NIH3T3 feeder cells.

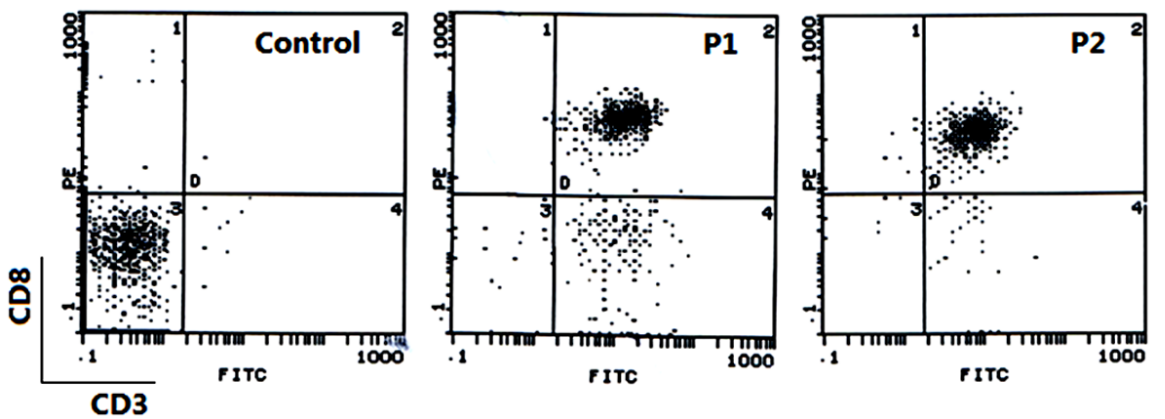

Figure 5. Proliferation of OCT4/Sox2-specific CD8+ T cells stimulated by CD40-B APCs.

= 68.35. The intergroup differences were statistically significant ($P<0.05$). The highest % DT and % SC appeared at the effector-target ratio of 40:1, indicating effective killing activity of the specific CTL on the FC9 cell line.

Discussion

The expression of the pluripotent transcription factors OCT4 and Sox2 is closely correlated to tumor relapse, metastasis and drug-resistance [9]. Recently, the relevant study shows significantly up-regulated OCT4, Sox2 and Nanog in the drug-resistance lung cancer cells in vitro induced by CDDP [12]. Via the transfection assay, the introduced heterologous OCT4 gene can promote the reverse differentiation of lung cancer cells to CSC-like cells with high drug-resistance, while the introduced OCT4-specific siRNA can silence the OCT4 expression to remarkably recover the sensitivity of tumor cells to chemotherapeutics, indicating that OCT4 and OCT4-positive cells play an pivotal role in chemotherapy resistance of lung cancer

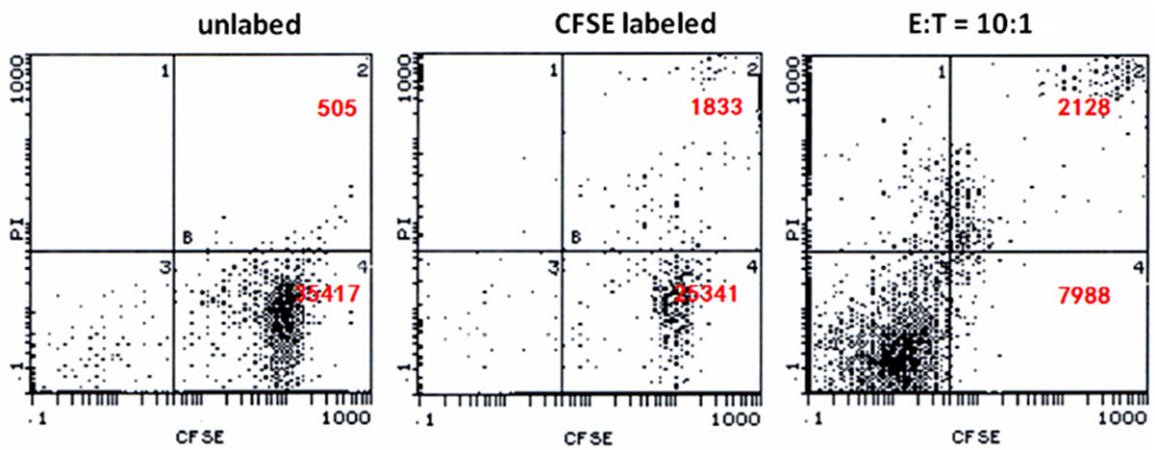

Figure 6. Killing effect of the specific CTL on lung cancer CSCs (FC9).

[9, 13]. Meanwhile, our previous studies [14] suggest that OCT4 and OCT4 are independent prognosis factors of lung cancer. Therefore, OCT4 and OCT4 were used as the specific antigens to induce CTLs and the killing effect of the specific CTL on the drug-resistance lung cancer CSC cell line was investigated in this study. The culture and amplification of the CTL specific to pluripotent factors have rarely been reported abroad. Therefore, our study is progressive in a certain sense.

Peripheral B cells can be used as the primary APCs in place of DCs to activate CTLs due to their antigen presenting capacity and easy culture and amplification in vitro [15]. Recently, the CD40-B-induced CTL has been an advanced technology [16, 17]. The lung cancer stem cell-specific CTL was activated and amplified in vitro with both CD40-B APC and acDC technologies which were easy and stable with high reproducibility. CD40-B APCs have been successfully prepared through this method in our previous work and used to stimulate the peripheral T lymphocytes of lung cancer patients. CD8+ T lymphocytes were successfully cultured and amplified in vitro, laying the foundation for this study [18]. Meanwhile, we have been dedicated to the study of the lung cancer CSC for years. In cooperation with the lung cancer stem cell study group of Shanghai Cancer Institute, we separated a group of lung adenocarcinoma CSCs with the characteristics of the bronchioalveolar stem cell (BASC) from the human lung adenocarcinoma cell line. This group of CD24/IGF-1R positive lung adenocarcinoma CSCs, which had the CD24+/IGF-1R+ phenotype, was manifested by high expression of the marker proteins of stem cells, such as OCT4, Sox2 and Nanog. The functional experiment also corroborated high invasiveness and oncogenicity of these CSCs. Oncogenesis could be triggered in NOD/SCID mice with only 100 transplanted cells, which had oncogenicity 1000 times that of the CD24/IGF-1R negative cells. Furthermore, the in vitro culture system of the lung cancer CSC has been successfully established, with which continuous culture of lung cancer CSCs can be performed without the loss of undifferentiation status [19]. Recently, induced by CDDP and gefitinib, the double-drug-resistance cell line was established with PC9 cells, and high expression of OCT4 and Sox2 was also observed in this cell line. Therefore, CD40-activated B cells (CD40-B) were used in this study as APCs to induce the CSC-specific CTL with the peripheral lymphocytes of NSCLC patients, and OCT4 and Sox2-specific T cell proliferation was observed [20]. The results showed that the induced OCT4 and Sox2-specific CTL could effectively kill lung cancer CSCs, laying the foundation for the clinical use of this technology in tumor treatment.

Disclosure of conflict of interest

None.

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