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ORIGINAL ARTICLE

An adoptive T cell immunotherapy targeting cancer stem cells in a colon cancer model

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Summary

Purpose: *Colon cancer is one of the most common malignancies worldwide. Cancer stem-like cells (CSCs) are a distinct subgroup of cancer cells that play a vital role in the development of cancer and also a role in the development of resistance against therapeutic agents. In this study we investigated the role of CSCs in colon cancer and evaluated the tumor-associated antigen CEP55 for targeting immunotherapeutically CSCs.*

Methods: *Side population (SP) cells from colon cancer cell line SW480, were isolated using DNA-binding dye Hoechst 33342. The cytotoxic activity of cytotoxic T lymphocytes (CTL) clone 41 for side population (SP) cells and main population (MP) cells was evaluated using ^{51}Cr release assay. The SP cells, MP cells and presorted cells from the colon cancer cell line were evaluated in NOD/SCID mice.*

Results: *The isolated SP cells showed resistance to the chemotherapeutic agents irinotecan and oxaliplatin, which suggests that targeting the CSCs can be a better strategy for the treatment of chemotherapy-resistant colon cancer. HLA class I and HLA-A24 in SP cells and MP cells were expressed at the same level. We used CTL clone 41 to corroborate the sensitivity of SP cells to cytotoxic T lymphocyte response. The cytotoxic responses of SP cells were to the same extent as they were in MP cells and pre-sorted cells. Adoptive transfer of CTL clone 41 in immunodeficient mice inhibited SW480-induced tumors, suggesting that this approach can be used for colon cancer immunotherapy.*

Conclusion: *Our novel findings suggest that colon cancer CSCs are sensitive to CTLs, and CEP55, a tumor-associated antigen, can be successfully used as active immunotherapy for targeting CSCs in colon cancer.*

Key words: *adoptive cell therapy, cancer stem cells, colon cancer, T cell therapy*

Introduction

Colon cancer is the second most frequently observed cancer in females and the third in males, globally [1]. Although the prevalence of colon cancer is less in Asian countries as compared to Western countries, in recent years there has been an increasing trend in Asian countries like China, Japan and Korea [2]. Cancer tissue contains heterogeneous population of cells. CSCs, also termed as 'tumor-initiating cells', are a distinct subgroup in the heterogeneous population that possesses the ability to regenerate cancer tissues, similar to a normal tissue regenerated through stem-like cells. It has also been observed that CSCs infiltrate tumors, self-renew and differentiate rapidly. Also, it has been reported that CSCs play an important role in tumor maintenance, recurrence, and metastasis. In recent years there have been several studies showing identification and isolation of CSCs from various types of cancers including colon cancer [3-7].

CSCs in immunotherapy models have attracted great attention over the past several years since the CSC theory was first suggested in 1875 by Cohnheim [8] and later re-identified in 1974

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by Pierce, who hypothesized that cancer develops from normal stem cells which undergo alterations in their pattern of proliferation and control [9,10]. In colon cancer, CSCs have been shown to reinitiate tumors which morphologically resemble primary colon cancer tissues when transplanted into immunodeficient mice [4]. It has also been observed that CSCs along with tumor infiltrating cells (TICs) have higher tumorigenic potential as compared to non-CSCs/TICs. Earlier studies have suggested that CSCs/TICs are resistant to chemotherapy and radiotherapy through various mechanisms, control over cell cycle quiescence, expression of drug transporters, DNA repair machinery, and resistance to apoptosis [11]. Studies from several investigators further support the hypothesis that solid and haematological malignacies comprise heterogeneous populations of cells organized in a hierarchical separation model [4-7]. These CSCs form the top tier of this hierarchy, therefore targeting CSCs is much desirable to achieve an effective therapeutic effect. In recent years, there has been increasing evidence to support that conventional treatment modalities in cancer are inefficient in eliminating the CICs, which will eventually lead to tumor relapse. Scientific evidence also suggests that a rare population of undifferentiated cells is responsible for tumor formation and maintenance [12,13], but this has not been explored in colorectal cancer.

Adoptive cell therapy (ACT) based on autologous T cells is an upcoming field of personalised cancer therapy. Various strategies like tumor infiltrating lymphocytes (TILs), chimeric T cells (CART cell), γδ T cells and ex vivo expansion of autologous T cells with tumor associated cells (TAA) are currently being explored to target cancer cells. In the present study we have tried to isolate colon CSCs as SP cells and to study their contribution to the development of chemotherapy resistance in the colon cancer cell line SW480. We also aimed at evaluating whether the CSCs were sensitive to CTL response and efficiency of adoptive T cell immunotherapy targeting colon CSCs, using the centrosomal protein CEP55 as antigen.

Methods

Cell line

Colon adenocarcinoma cell line SW480 (HLA-A*0201/2402) was purchased from the American Type Culture Collection (ATCC, Manassas, USA). The cell line was cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA) [14].

Side population analysis

Side population analysis was performed as described previously by Goodell et al., with minor modifications [15]. Trypsinized cultured cells were washed with phosphate buffer saline (PBS) and resuspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum at 37°C. After preincubating the cells for 10 min, the cultured cells were labeled with DNA-binding dye Hoechst 33342 dye (Lonza, Walkersville, MD) at a concentration of 5 µg/mL for 90 min, with or without an ABC transporter inhibitor, verapamil (Sigma-Aldrich, St. Louis, USA) at 50 µmol/L concentration. To label dead cells counterstaining with 1 µg/mL propidium iodide was performed. Finally, 1×10^6 viable cells were analyzed and sorted using a BD FACSCalibur II fluorescence-activated cell sorting system (BD Biosciences, NJ, USA). After excitation of Hoechst dye at 355 nm, the fluorescence was measured at two wavelengths using optical filters 405 DF20 (450/20 nm band-pass filter O [Hoechst Blue] and 635LP (635 nm long-pass edge filter [Hoechst Red]). Propidium iodide labeling was measured through a 630/BP30 filter for discrimination of dead cells.

Animal model

Four to eight week-old female nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were purchased from the Chinese Academy of Medical Sciences, and all the experimental procedures involving animals were conducted under a protocol reviewed and approved by the Ethics Committee of The Central Hospital of Taian, Taian, China. All efforts were made to minimize the number of animals used and to ameliorate their suffering.

The SP cells, MP cells, and presorted cells from the colon cancer cell line were mixed 1:1 by volume with Matrigel (BD Biosciences, San Jose, USA) and were injected subcutaneously (s.c.) into the dorsal side of NOD/SCID mice. The size of the tumor in mm^3 was assessed with Vernier calipers every 7 days and was calculated as Tumor Size = (Longest diameter × Shortest diameter^2)/2.

Flow cytometric analysis and monoclonal antibodies

Since recognition of HLA class I presented TAA is necessary to evoke tumor specific immune response, we tested the expression of HLA class I molecules and TAA. Cells were incubated with mouse specific monoclonal antibodies (Mabs) anti-pan HLA class I (WG/32) and anti-HLA A24 (C7709A2.6 hybridoma) at saturation concentration for 30 min, washed with PBS, and stained with secondary antibody (polyclonal goat anti-mouse antibody) coupled with fluorescein isothiocy-

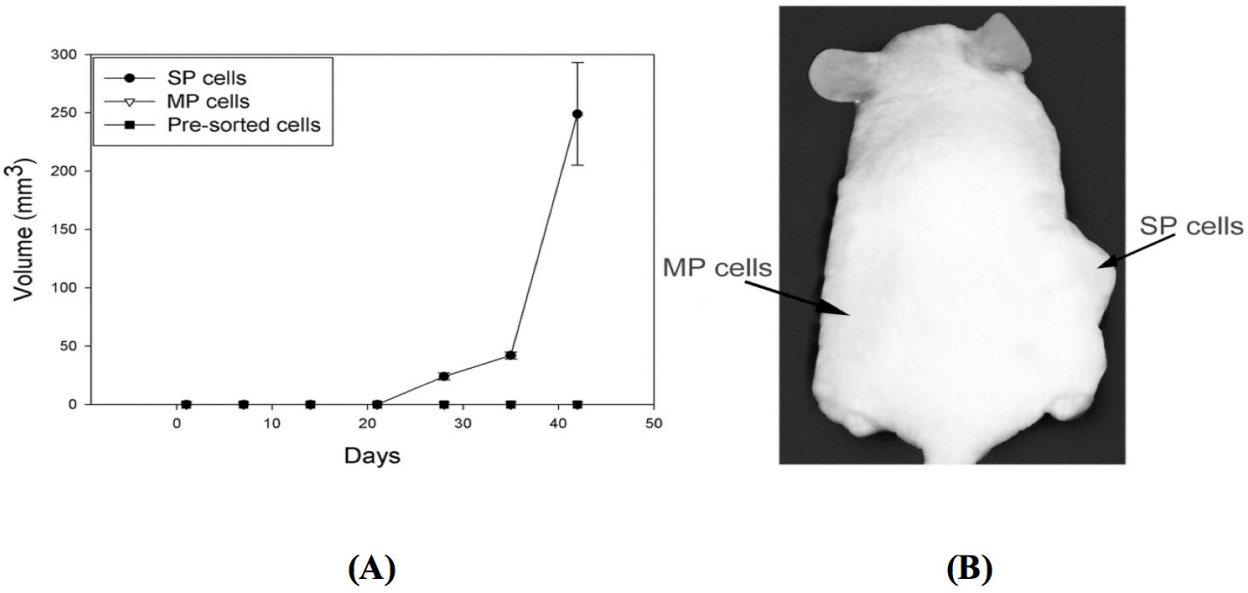

Figure 1. A: SP cells, MP cells, and presorted cells from colon cancer cell line SW480. Data are means±SD. P=0.04 indicates differences between cell types according to Mann-Whitney U-test. **B:** Arrows showing tumor growth in a NOD/SCID mouse at the SP cells and MP cells injection site; cells were inoculated subcutaneously into the left and right side of the back.

anate for 30 min. Samples were analyzed using a BD FACSCalibur flow cytometry system (Becton Dickinson, San Jose, CA). Anti-pan HLA class I (W6/32) and anti-HLA-A24 monoclonal antibodies (C7709A2.6 hybridoma) were purchased from ABcam (Cambridge, USA).

Survival studies for oxaliplatin and irinotecan

SP and MP cells of the SW480 cell line were isolated as per the protocol described in a previous study [15] and seeded on 96-well culture plates at a concentration of 1×10^4 cells per well for each population of cells. The cells in both populations were treated with oxaliplatin (1 and 5 μg/mL) or irinotecan (40 and 400 μg/mL). The viability of the cells was determined after 72 hrs of exposure to the chemotherapeutic agents using the SOD assay kit, which was performed as per the manufacturer's instructions (Sigma-Aldrich, St. Louis, USA).

Cytotoxicity assay

The cytotoxic activity of CTL clone 41 for SP cells, MP cells, and pre-sorted cells was evaluated using 51Cr release assay as described by Inoda et al. [16]. Briefly, SP cells, MP cells and pre-sorted cells were labelled with 100 μCi of ^{51}Cr for 1 h at 37°C, washed 4 times with PBS, and resuspended in AIM-V medium (Invitrogen, Grand Island, USA). Using 96-well culture plates the ^{51}Cr-labeled target cells (2000 cells/well) were then incubated with varying concentrations of effector cells for 6 hrs at 37°C. Using a gamma counter the radioactivity of the culture supernatant was determined. The percentage of cytotoxicity was calculated using the formula: % Specific Lysis = (Experimental Release – Spontaneous Release) × 100/(Maximum Release – Spontaneous Release). Target cells were treated with 100 units/mL interferon-γ for 48 hrs before the assay.

Winn assay

The SP cells from SW480 cells were co-cultured with CTL, clone 41, at a ratio of 1 SP cell to 10 CTL cells. The resulting suspension (200 μL with 1×10^6 CTL clone 41 and 1×10^5 SP cells) was injected s.c. into the dorsal side of NOD/SCID mice. A control group consisting of mice was injected with SP cells alone. The tumor size was assessed every week.

Adoptive transfer of CTL

NOD/SCID mice were injected s.c. on the dorsal side with SW480 SP cells 1×10^3 cells in 1ml). After 3 weeks, on development of palpable tumor, 5×10^4 Cep55/c10orf3_193 (10)-specific CTL clone cells or PBS were injected intravenously (i.v.). The procedure was repeated at week 4. The same procedure was repeated 4 weeks after inoculation with SP cells. The tumor size was assessed weekly.

Statistics

For the survival studies, cytotoxicity assay, xenograft model, adoptive transfer model and Winn assay, the data were analyzed using the Mann-Whitney *U* test. Results were expressed as means ± standard deviation

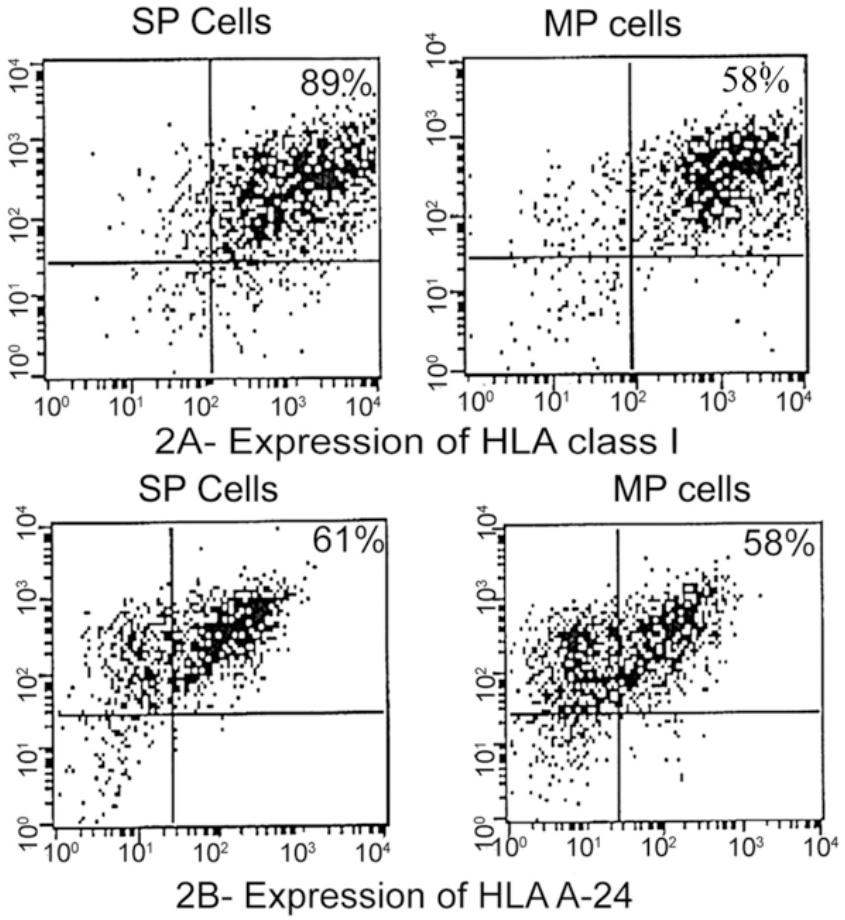

Figure 2. A: Expression of HLA class I molecules. **B:** Expression of HLA-A24 molecules.

and a p value <0.05 was considered as statistically significant difference. For statistical analyses SPSS 22 software (IBM, Armonk, NY) was used.

Results

Isolation of colon cancer stem cells

Using SP cell analysis for isolating colon CSPs, the SP cells were 3.0%. These cells were found to be explicitly inhibited by verapamil, indicating that they are specific for ABC transporter expression and showed tumor initiating activity compared with MP cells (Figure 1).

Expression of HLA and cancer-associated antigens in SP cells

HLA class I and HLA-A24 expression in SW480 SP cells was found to be at same level as in MP cells (Figure 2, A and B). These observations suggest that SP cells carry the same sensitivity to CEP55-derived antigenic peptide as MP cells and can be used as a means for comparing the immunogenic susceptibility of MP cells and SP cells with CTL response (Figure 2).

Resistance to chemotherapeutic agents

SW480 cells were found to be resistant to both irinotecan and oxaliplatin compared with MP cells (Figures 3 and 4).

Immunological susceptibility of CSCs to CTL response

Both SW480 derived SP cells and MP cells showed similar susceptibility to CTL clone 41 and the pre-sorted cell lines (Figure 2). This validates further that the colon CSCs are capable of being recognised by CTLs. To further examine the cytotoxic activity of the CTL clone 41 in vivo, Winn assay was performed. SW480 SP cells with or without CTL clone 41 were injected s.c. on the dorsal side of NOD/SCID mice. CTL clone 41 was found to significantly inhibit the tumorigenicity of SW480 SP cells (Figure 5). These findings con-

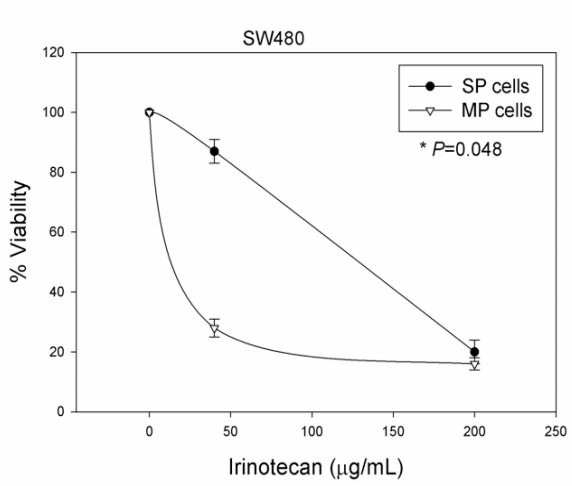

Figure 3. Sensitivity to the chemotherapeutic agent irinotecan; data are mean±SD (p=0.048).

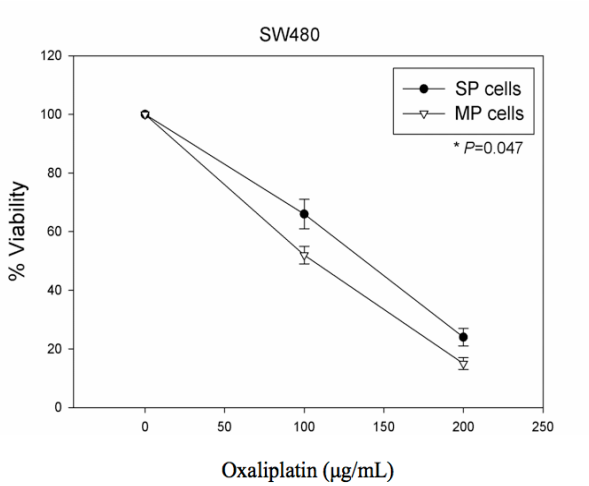

Figure 4. Sensitivity to the chemotherapeutic agent oxaliplatin; data are mean±SD (p=0.047).

firm that CTLs could identify CSCs both *in vitro* and *in vivo* (Figure 5).

Discussion

In this study, we provided evidence to support the hypothesis that CSCs play a vital role in the development of colon cancer and that they also play a role in the development of resistance to chemotherapeutic agents. We have also shown that CSCs can be successfully isolated using SP cell analysis in an established colon cancer cell line (SW480). To date, the identification of CSCs has been limited to leukemia [17], and solid tumors like breast cancer [18] and glioma [19]. We have also shown successfully that targeting these CSCs could possibly lead to immunotherapy for colon cancer.

In this study, SP cells from the colon cancer cell line SW480 were isolated using DNA-binding dye Hoechst 33342 and were found to exhibit higher tumorigenicity than MP cells. This supports the hypothesis that SW480 cells were enriched with CSCs. We used flow cytometry and the DNA-binding dye Hoechst 33342 to isolate SP cells from for SW480 colon cancer cell line. Furthermore, the isolated SP cells also showed resistance to therapeutic agents used in chemotherapy (irinotecan and oxaliplatin), which suggests that targeting CSCs can be a better strategy for the treatment of chemotherapy-resistant colon cancer. CSCs from SP cells were first reported by Goodell et al. [15], and have also been isolated from several types of cancer in earlier studies [20-28]. The immunogenicity of SP cells was also evaluated through expression of HLA class I and tumor rejection antigen CEP55; these findings are in line with earlier studies [13,22].

The expression of HLA class I and HLA-A24 in SP cells and MP cells was at the same level. We used CTL clone 41 to corroborate the sensitivity of SP cells to cytotoxic T lymphocyte response [22]. The cytotoxic responses of SP cells were of the same extent as in MP cells and pre-sorted cells. Adoptive transfer of CTL clone 41 in immunodeficient mice inhibited SW480-induced tumors, suggesting that this approach can be used for colon cancer immunotherapy.

This study is the first of its kind, providing evidence that CSCs can be a valid target for colon cancer immunotherapy and can very well be extended to human studies. In an earlier study, SP cells isolated from HT29, HGT101, Caco2, and HRA19a1 gastrointestinal cancer cell lines have been found to lack CSC population [16]. In the present study, we have successfully demonstrated that CSCs in SW480 colon cancer cell line showed higher tumorigenicity than MP cells. Furthermore, the isolated CSCs were also found to express stem cell markers, including LGR5 and SOX2, at higher levels than the MP cells. The colon cancer CSCs were also found to express HLA class I molecule and the tumor associated antigen CEP55, and, interestingly, CSCs were sensitive to CTL response both in vitro and in vivo. CSCs have also been shown to be sensitive to γδT cells [29]. It has also been shown that both CTLs and γδT cells immunologically target cancer cells in a study by Todaro et al. PROM1-positive (CD133+) colon can-

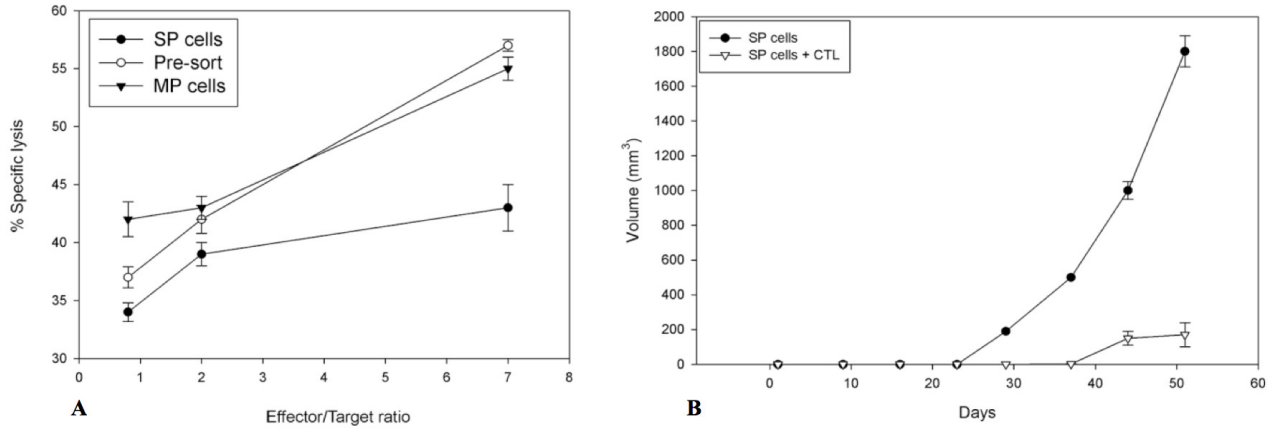

Figure 5. A: Cytotoxicity of CTL clone 41. **B:** 51Cr release assay. Data are mean±SD (p=0.049).

cer CSCs were found to upregulate CFLAR (c-FLIP) and BCL2L1 through secretion of IL-4, thereby inducing resistance to chemotherapy [29]. Another study by Saigusa et al. reported that relapse of rectal cancer after first or second line therapy was related to the overexpression of CSC markers such as Oct3/4, CD133 and SOX2 [30], indicating that colon CSCs are resistant to apoptotic cell death. Since lymphocytes can cause apoptosis of the target cells, sensitivity of these cells to immunotherapy raises concern.

The current study is in line with the findings of Todaro et al. [29] that colon CSCs are sensitive to T cell induced apoptosis, suggesting that both CTLs and γδT cells can be used as immunotherapy for colon cancer. Ogino et al. [31] showed that CTL response to cancer was due to long survival of patients with colon cancer and there was no specific antigen recognition by γδT cells. The study did not evaluate the TILs; however, the study showed that immunological response can be engineered to control cancer. In another study, Wei et al. [32] reported that CSCs derived from glioma suppressed the proliferation of T cells and induced apoptosis of T cells. They also induced apoptosis of T cells through activation of CD274 (B7-H1) soluble LGALS3 (galectin-3) and induced regulatory T cells.

Therefore we hypothesize that suppression and inhibition of immune response may be due to CSCs and for a successful immunotherapy, immunosuppression due to CSCs needs to be addressed as well CSCs. CSCs derived from other organs have been reported to be resistant to both irinotecan and oxaliplatin [20,21]. However, our study also suggests that CTLs can effectively target the CSCs. Keeping in mind the suppression of CTL through secretion of IL-10, we suggest that using anti-IL-10 monoclonal antibody along with adoptive transfer of the T-cell-receptor-induced T cells and CSC-specific CTL clones seems reasonable. Since colon CSCs and non-CSCs population were sensitive to CEP55-specific CTL and a number of TAA indentified by others [33,34], an adoptive T cell immunotherapy using these peptides may be a rationale approach. The present study has shown 10 to 100-fold higher tumorigenicity in colon CSCs than in non-CSCs. Given the fact that 1 L of peripheral blood contains $3x10^9$ cytotoxic T cells (CD8), and effector/target ratio = 0.3, about $3x10^6$ total peptide-specific CTLs for a 1 cm tumor diameter may be required in the whole blood to actively target the CSC population. Some studies have also shown that for active elimination of colon cancer and leukemia CSC Mab for δ-like 4 ligand (DLL4), insulin-like growth factor-1 receptor (IGF-1R), and CD47 may be useful [35-38].

Conclusion

In contrast to the use of Mabs, adoptive transfer of tumor specific CTLs provides memory cells that can offer active immunity for several years. This can be very much desirable for preventing relapse and recurrence of cancer after first line therapy. Our novel findings suggest that colon CSCs are sensitive to CTLs and CEP55, a tumor-associated antigen can be successfully used as active immunotherapy for targeting CSCs in colon cancer.

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