Synergy of Dendritic Cell Vaccines and Avasimibe in Treatment of Head and Neck Cancer in Mice

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The main purpose of this study was to explore the antitumor effect and mechanisms of ACAT1 inhibitor combined with CSCs-DC vaccine.

Material/Methods: We isolated HNSCC CSCs and gained CSCs antigens, then used CSCs antigens to load dendritic cells (DC) and generated a CSCs-DC vaccine. We treated mice after surgical excision of established SCC7 tumors with CSCs-DC vaccine and/or ACAT1 inhibitor, and recorded local tumor relapse and host survival. T cells and B cells were harvested from mice treated with CSCs-DC vaccine and/or ACAT1 inhibitor. We tested antibody production and the death rate of CSCs killed by T cells.

Results: The tumors in the combined treatment group were smaller than in all other groups (P<0.01). The average survival time of the combined treatment group was 82 days and was the longest of all groups. Analysis of IgG levels secreted by B cell and CTL activity in spleens of mice found that results of the combined treatment group were the highest, and the results of the CSCs-DC group were lower than in the combined treatment group. The ACAT1 inhibitor group results were lower than in the CSCs-DC group and the combined treatment group results, but higher than in the PBS group, and the difference was statistically significant.

Conclusions: ACAT1 inhibitor enhanced the therapeutic effect of CSCs-DC vaccine in the treatment of the mouse HNSCC postoperative recurrence model. ACAT1 may play an important role in cancer immunotherapy.

MeSH Keywords: **Head and Neck Neoplasms • Neoplastic Stem Cells • Sterol O-Acyltransferase**

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Background

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant disease worldwide, with high morbidity and mortality [1]. Despite many advances in chemotherapy, radiotherapy, surgical techniques, and combination regimens, which have improved quality of life, the 5-year overall survival (OS) has still lingered at just 40–60% for the last 50 years [2].

With the developments of molecular biology, immunology, and genetic engineering, rapid progress has been made in the immunotherapy of HNSCC. PGE2 is a major product of cyclooxygenase 2 (COX-2) activity and has multiple mechanisms involved in malignant growth. PGE2 also inhibits T cell proliferation and NK cell activity, and it induces IL-10 production [3]. Klatka et al. found that COX-2 inhibition significantly enhanced proliferation of NKT cells stimulated with autologous DCs in patients with laryngeal cancer [4].

Cancer stem cells (CSCs) play an important role in the treatment failures, including local tumor recurrence, distant metastasis, and resistance to treatment. CSCs are a small subpopulation of cells within tumors; they can self-renew, differentiate, be tumorigenic, and contribute to tumor recurrence and metastasis. ALDEFLUOR/ALDH (aldehyde dehydrogenase) activity has been successfully used as a CSC marker in many kinds of cancers [5–8]. A recent study reported that ALDH^{high} murine squamous carcinoma SCC7 has tumor-initiating capacity [9]. Using CSCs antigens to load dendritic cell (DC) vaccine to treat syngeneic immunocompetent hosts (C3H mice) bearing SCC7, both local tumor relapse and pulmonary metastases were reduced and host survival was prolonged [10]. However, we also observed that the antitumor efficacy of DC vaccines is limited. Klatka et al. proved that there is a relation between the presence of laryngeal cancer and the expression of B7H1, B7H4, CD200, and CD200R regulatory molecules on the CD83+ Mo-DC pulsed with autologous cancer cell lysates. The expression of the aforementioned tolerogenic molecules on mature dendritic cells may be responsible for the failure of DC vaccines[11].

A recent study reported that Acyl-CoA: cholesterol acyltransferase 1 (ACAT1) inhibitor can promote the proliferation and function of CD8+T cells. To augment the antitumor efficacy of CSCs vaccines, we conducted combination therapy of ACAT1 inhibitors and CSCs vaccine in murine models of HNSCC, and explored the synergistic antitumor mechanisms of ACAT1 inhibitor combined with CSCs-DC vaccine.

Material and Methods

Reagents

All standard chemicals were purchased from commercial sources and were all of analytic grade unless stated otherwise. ACAT1 inhibitor (avasimibe) was obtained from Selleck (Shanghai, China). The ALDEFLUOR™ Kit was purchased from StemCell Technologies (Cambridge, MA, USA). Purified anti-mouse CD3 Antibody, anti-mouse CD28 Antibody, anti-mouse CD40 Antibody, interleukin-2, and granulocyte-macrophage colony stimulating factor (GM-CSF) was purchased from Ebioscience (Wuhan, China). ELISA kits were purchased from Thermo Fisher Scientific (Wuhan, China). LDH release kits were purchased from Abcam (Cambridge, MA, USA). RPMI 1640 culture medium, fetal bovine serum (FBS), 0.25% trypsin digestion liquid, penicillin/streptomycin, and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Female C3H/He (C3H) mice were purchased from Wuhan University Research Center for Animal Experiments, China. Animals were housed 5 per cage and were kept under a controlled temperature of 22±1°C, humidity 55–60%, and 12-h light/12-h dark cycle throughout the experiment. Normal solid feed and water were available ad libitum. The feed was provided by the Wuhan University Research Center for Animal Experiments, China.

Culture of tumor cells

The squamous carcinoma cell line, SCC7, which is syngeneic to C3H mice, was obtained from ATCC (Manassas, VA, USA). The cell lines were cultured in complete medium consisting of RPMI1640 and supplements (10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin), and the cells were cultured at 37°C in a humidified CO_2 incubator.

ALDEFLUOR assay

The ALDEFLUOR Kit (StemCell Technologies) was used to isolate ALDH$^+$CSCs from the SCC7.

Preparation of DC vaccine

Bone marrow-derived murine cells were cultured in 10 ml complete medium (CM) supplemented with 20 ng/ml GM-CSF at a concentration of 0.2–0.4×10^6 cells/ml in non-tissue culture petri dishes (Corning) on day 0. Fresh medium supplemented with 20 ng/ml GM-CSF was added on day 3 (10 ml). On days 6 and 8, 10 ml of cultured cell suspension was taken from each dish, centrifuged, the was pellet resuspended in 10 ml of fresh

CM with 20 ng/ml of GM-CSF, and added back to each dish. On day 10, DCs were harvested. ALDH^{+} SCC7 cells were frozen and thawed 3 times to make cell lysate. Lysates of ALDH[+] SCC7 were added to DCs at a 1: 3 cell equivalent ratio. The DCs were then incubated at 37°C for 24 h with 5% CO_2. After incubation, the ALDH[+] lysate-pulsed DCs (CSCs-DC) were used as vaccine as specified in the subsequent experiments.

Tumor model and treatment protocols

C3H mice were inoculated subcutaneously with 0.5×10^6 SCC7 cells on day 0. On day 21, the mice were subjected to surgical tumor resection except for 1 group, which served as control. Animals with subcutaneous tumors removed were then divided into 4 groups (n=5 each) and were administered PBS, ALDH[+] SCC7 CSC-DC vaccine, ACAT1 inhibitor (avasimibe), and SCC7 CSC-DC vaccine combined with ACAT1 inhibitor at 24 h after tumor resection. The vaccination was repeated on days 29 and day 35. Each mouse was inoculated subcutaneously with 2×10^6 DCs per vaccine. An ACAT1 inhibitor was repeated on days 29, 31, 33, and 35. The abdominal cavity of each mouse was injected with ACAT1 inhibitor (avasimibe) at the dose of 15 mg/kg. Tumor volumes were measured 3 times per week. The volumes were calculated as: tumor volume=(width²×length)/2. Survival was monitored and recorded as the percentage of survivors after tumor inoculation. Mice with tumor size larger than 20 mm at the longest axis were euthanized for ethical reasons.

Purification and culture of host B cells and T cells

Spleens were harvested from animals subjected to various treatments at the end of the experiments. Splenic B cells were purified and activated in CM supplemented with lipopolysaccharide, anti-CD40, and IL2. The culture supernatants were collected and stored at −20°C for future experiments. Splenic T cells were purified and activated to generate CTLs, which were analyzed in LDH cytotoxicity assays.

CTL cytotoxicity

CTLs were generated from splenocytes harvested from animals by anti-CD3/CD28 activation and IL-2 expansion. CTL-mediated CSC cytotoxicity was tested using the lactate dehydrogenase (LDH) Release Assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) according to the manufacturer's protocol. The following formula was used to calculate cytotoxicity:

$$\% \text{ Cytotoxicity} = \frac{\text{Exprerimental} - \text{Effector spontaneous} - \text{Target spontaneous}}{\text{Target maximum} - \text{Target spontaneous}} \times 100$$

Antibody production

To test systematic immune responses conferred by different treatments, spleens were harvested at the end of experiments.
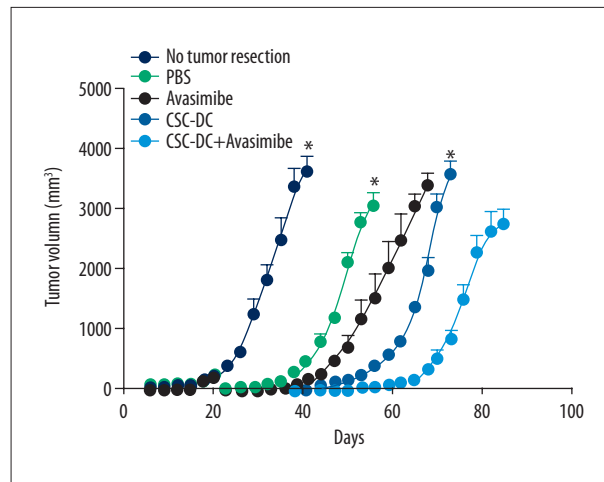

Figure 1. Tumor sizes of SCC7 tumor-bearing mice after surgical tumor resection and treated in different ways, as indicated, on days 29, 31, 33, and 35. Data are representative of 2 independent experiments. Error bars denote SEM * $P<0.01$.

After spleen B cells were activated, supernatants were collected and analyzed for IgG production.

Statistical analysis

All studies were done in triplicate. Survival analysis was determined by the log-rank test. Other data were evaluated by the unpaired t test (2 cohorts) or one-way ANOVA (>2 cohorts). Any P value <0.05 was considered statistically significant.

Results

CSC-DC vaccine combined with ACAT1 inhibitor significantly inhibited tumor growth and prolonged survival of head and neck SCC7 tumors-bearing mice after surgical resection.

We used SCC7-bearing mice to assess whether the addition of avasimibe could potentiate the antitumor activity of CSC-DC vaccine *in vivo*. To test the antitumor effect of CSC-DC vaccine and avasimibe, mice were treated with PBS as the control group. In experimental groups, mice were treated with CSC-DC vaccine, ACAT1 inhibitor, or CSC-DC vaccine combined with ACAT1 inhibitor. As shown in Figure 1, administration of either CSC-DC vaccine or ACAT1 inhibitor into the animal model significantly suppressed tumor recurrence compared to PBS-treated mice (P<0.01 at day 56). The CSC-DC vaccine combined with the ACAT1 inhibitor group significantly reduced tumor recurrence compared with the PBS control (P<0.01 at day 56), CSC-DC vaccine (P<0.01 at day 73), and ACAT1 inhibitor (P<0.01 at day 68).
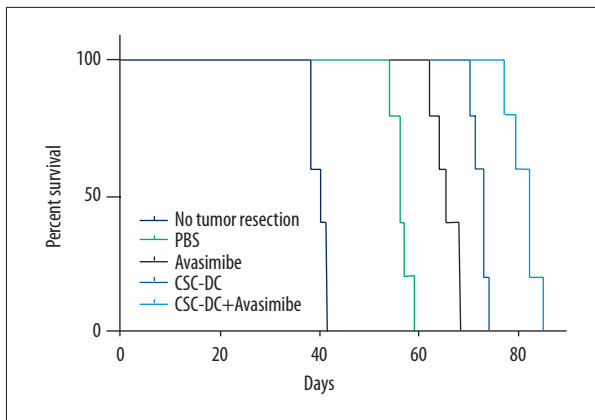
Figure 2. Survival of SCC7 tumor-bearing mice after surgical tumor resection and treated in different ways, as indicated, on days 29, 31, 33, and 35. Data are representative of 2 independent experiments. *P*<0.01.

As shown in Figure 2, the mortality of the animals in the no surgery group started at day 38, and all of the animals eventually died during the following 3 days. The mortality of animals in the PBS group started at day 54, and all of the animals eventually died during the following 5 days. As compared to the PBS group, the survival was increased to 65 days by the treatment with the ACAT1 inhibitor (*vs.* PBS, P<0.01, log-rank test), and to 73 days by the CSC-DC vaccine (*vs.* PBS, P<0.01, log-rank test). The treatment with CSC-DC vaccine and ACAT1 inhibitor significantly increased animal survival compared with the other treatments or control mice to 82 days (P<0.01, log-rank test).

CSC-DC vaccination combined with ACAT1 inhibitor conferred host CSC-specific antibody responses

To test whether ACAT1 inhibitor improved specific anti-CSC immunity of CSC-DC vaccination, we removed the spleens after the total treatment, purified splenic B cells, and activated them. Then, we collected supernatants produced by B cells and tested the content of IgG. As shown in Figure 3, the results of ELISA assays indicated that the content of IgG produced in the CSC-DC vaccine and ACAT1 inhibitor group was highest compared with the other treatments or PBS (P<0.01 *vs.* all other groups). The content of IgG produced in the CSC-DC vaccine group and the ACAT1 inhibitor group were higher than in the PBS group (P<0.01 *vs.* PBS group).

CSC-DC vaccination conferred host CSC-specific CTL function

To examine the ability of CSC-DC vaccination combined with ACAT1 inhibitor to generate host CSC-specific CTL activity, we removed the spleens after the total treatment, purified splenic T cells, and activated them. Then, we measured the
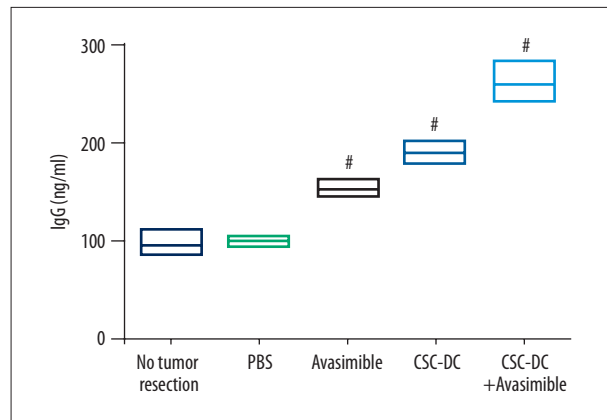

Figure 3. The content of the IgG measured by ELISA (n=3). IgG were collected as described in Methods from the spleens harvested from mice subjected to PBS, avasimibe, CSC-DC vaccine, or CSC-DC vaccine combined with avasimibe. Data were analyzed by *t* test. Error bars denote SEM, # *P*<0.01 compared with all other groups.
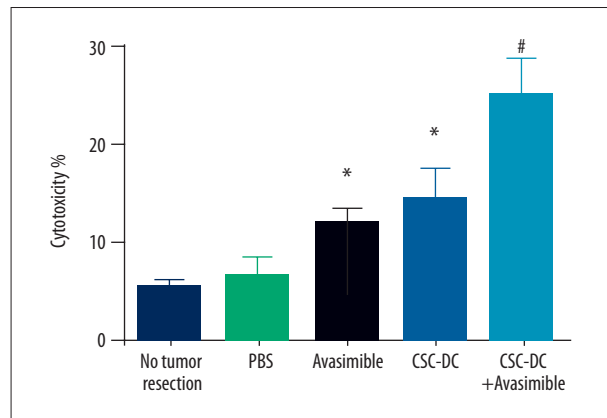

Figure 4. CTL cytotoxicity measured by the LDH assay (n=3). CTLs were generated as described in Methods from the spleens harvested from mice subjected to PBS, avasimibe, CSC-DC vaccine, or CSC-DC vaccine combined with avasimibe. Target cells were ALDH+CSC SCC7. Data were analyzed by *t* test. Error bars denote SEM; * *P*<0.05 compared with PBS group and no tumor resection group; # *P*<0.05 compared with all other groups.

CTL activity of these T cells on CSCs using the LDH assay. As shown in Figure 4, CTLs generated from the combined group mediated significantly greater cytotoxicity in CSCs compared with the CTLs generated from PBS, CSCs-DC vaccine, or ACAT1 inhibitor (P<0.05; Figure 4), but there was not significant difference between the CSC-DC vaccine group and the ACAT1 inhibitor group.

Discussion

ACATs play key regulatory roles in cholesterol metabolism and they catalyze the synthesis of cholesterol esters from free cholesterol and fatty acyl-Co A [12]. Two isoforms of ACAT have been reported to date (ACAT-1 and ACAT-2). ACAT-1 is widely expressed in tissues, but ACAT-2 is expressed only in the liver and small intestine. ACAT is an enzyme resident in the endoplasmic reticulum; it prevents conversion of cholesterol into cholesterol ester by accumulation of cholesterol [13]. Numerous ACAT inhibitors have been developed to treat or prevent atherosclerosis [14]. These inhibitors can be divided into several classes, including fatty acyl amides (e.g., SaH 58-035, CI-976), urea-based compounds (e.g., CL 277,082, and PD132301-2), and compounds with increased water solubility (e.g., PD 138142-15, CI-1011, or avasimibe). Recent studies have focused on ACAT1 and its effects on cancer cell growth. ACAT1 inhibitor shows anti-neoplastic activity in a variety of experimental models *in vivo* and *in vitro*. Matsumoto et al. [15] identified strong expression of ACAT-1 in clear cell type renal cell carcinoma, and upregulation of ACAT-1 leads to high ACAT enzymatic activity, which accelerates the accumulation of cholesterol ester and is associated with tumor grade. Lee et al. [16] reported that ACAT-1 inhibitor significantly reduced cholesteryl ester storage in lipid droplets and elevated free cholesterol levels, which led to suppression of proliferation and apoptosis of colon cancer cell lines. However, Uda [17] found that proliferation of CEM-CCRF cells was slightly affected by ACAT inhibitor, CE content in lipid droplets was significantly higher than those in control cells, and the enzyme activity was continuously inhibited. Antalis et al. [18] found that estrogen receptor-negative (ER(–)) breast cancer cells had higher expression of ACAT1 as compared to ER(+) breast cancer cells, and proliferation of ER(–) breast cancer cells was reduced by inhibition of ACAT. Bemlih et al. [19] showed that avasimibe, a specific inhibitor of ACAT, inhibited the growth of the cells in glioma cell lines (U87, A172, and GL261). Paillasse et al. [20] found that inhibition of cholesteryl ester formation and ACAT activity by Sah58-035 decreased cell growth by 34% and invasion by 73% (CCK2R-E151A). In mice, treatment with ACAT-1 inhibitor notably suppressed tumor growth and extended survival time [16]. De Medina et al. [13] reported that auraptene inhibits ACAT and binds to ERs, which correlates well with the control of growth and invasiveness of tumor cells in intact cancer cells of murine and human origins. ACAT/cholesterol esterification is a novel pathway that contributes to tumor cell proliferation and invasion. On the other hand, data show that overexpression of ACAT1/2 in human breast cancer cells using a lentiviral approach directly promotes tumor growth and lung metastasis [21]. Targeting ACAT1 is a promising therapeutic strategy in the treatment of human cancer.

An increasing number of studies have shown that cancer stem cells (CSCs) play a crucial role in tumor recurrence, metastases,

and therapeutic resistance [22]. Tumor cell populations have been proposed to be composed of 2 parts: CSCs and the differentiated cancer cells. CSCs are a subpopulation of cells from the original tumor that are molecularly and phenotypically distinct, and they are able to drive tumor initiation, proliferation, and spread. Most importantly, CSCs are resistant to radiotherapy and chemotherapy, which often allows them to survive after traditional therapy and lead to tumor recurrence and metastases [23,24]. Many recent studies have explored strategies to eliminate CSCs. To eliminate CSCs, the primary task is to identify and isolate them. Recent research indicates that there are various cancer stem cells markers. CD133 could be a marker useful in characterizing laryngeal cancer stem cells [25]. ALDH1 is related with cervical cancer stem cells [26]. ALDH is a tumor antigen used to identify and isolate CSC from tumors. ALDEFLUOR/ALDH has been successfully used as a marker to isolate CSCs in many kinds of human cancers, including thyroid carcinoma [27], retinoblastoma, prostate cancer [28], breast cancer [29], ovarian carcinoma [30], and HNSCC [31]. In recent studies, novel therapies were developed to target CSCs. The administration of dendritic cells loaded with CSCs induced a tumor-specific immune response and delayed tumor growth [32]. Visus et al. generated CSC-specific CD8 T effector cells *in vitro* and tested their ability to eliminate CSCs *in vitro* and *in vivo*. Results showed that CSC-specific CD8(+) T cells eliminated CSCs, inhibited tumor growth and metastases, and prolonged survival of xenograft-bearing immunodeficient mice. Freshly purified allogeneic NK cells can recognize and kill colorectal carcinoma-derived CICs rather than the non-CIC counterpart of the tumors (differentiated tumor cells) [33]. Furthermore, the growth of CSCs was inhibited by antibodies [34]. The results strongly support the potential of CSC-based immunotherapy to selectively target CSCs [35]. Several studies have previously reported that CSC-DC vaccine significantly inhibited tumor recurrence and prolonged animal survival compared with non-CSC-DC vaccinations [36,37]. CD8+T cells have a central role in antitumor immunity, but their activity is suppressed in the tumor microenvironment [38]. Recently, Wei et al. reported that inhibiting cholesterol esterification in T cells by genetic ablation or pharmacological inhibition of ACAT1 led to a potentiated effect or function and enhanced proliferation of CD8+ but not CD4+ T cells [39]. In the present study, we assessed the effect of CSC-DC vaccines combined with ACAT1 inhibitor in controlling tumor recurrence. Our data showed that mice in the combined group had smaller tumors and longer survival. Then, we examined the ability of CSC-DC vaccines combined with ACAT1 inhibitor to elicit CSC-specific humoral and cellular immune responses. We collected splenocytes from the treated mice and generated CTL and B cells. The results showed that combined treatment-primed CTLs significantly killed the scc7 ALDH+-CSCs compared with the CTLs generated from PBS, CSCs-DC, or ACAT1 inhibitor. In addition, the combined treatment-primed host B cells

produced antibody that was at a significantly higher level than the antibodies produced from PBS, CSCs-DC, or ACAT1 inhibitor-primed B cells. Our results suggest that the use of ACAT1 inhibitor combined with CSC-DC vaccination can offer greater benefit in treating cancer, and can enhance T and B cell activation as a method to improve CSCs-DC vaccine-induced anti-CSC immunity. Further characterization of ACAT1 will provide better understanding in more effective therapeutic targeting.

Conclusions

ACAT1 inhibitor promotes the antitumor effect of CSCs-DC vaccine. This effect is caused by ACAT1 inhibitor-enhanced humoral and cellular immune functions of the host.

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