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RESEARCH ARTICLE

Renal cell carcinoma derived exosome as natural nanoparticles inhibits T cell proliferation and induces their apoptosis

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ABSTRACT

Objective(s): To evaluate the anti-tumor effect of the renal cell carcinoma ACHN cell-exosome on human T cell biological parameters.

Methods: Human T cell was isolated from healthy donors. ACHN-cell derived exosome was procured and expression of the CD9 and CD63 was assessed using western blotting. The effects of the ACHN- exosome on T cell proliferation was measured using MTT assay within 24, 48, 72 and 96 hours of treatment with 500-2000 ng/ml exosome. Also, the apoptotic cell percentages in treated cells were assessed by Annexin/PI staining and flowcytometry upon exposure with 500 and 2000 ng/ml exosome within 96 hours of treatment.

Results: According to results, exosome therapy inhibited T cell proliferation more evidently at higher concentration. Also, treatment with exosome caused an improvement in the apoptosis of the T cell within 96 hours of treatment.

Conclusions: Concerning the results, ACHN- exosome has negative effect on T cell viability and proliferation, facilitating immune evasion.

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INTRODUCTION

Renal cell carcinomas (RCC), tumors initiating in the renal epithelium, make up about 90% of kidney cancers [1]. The entity is composed of more than ten histological and molecular subgroups, with clear cell RCC (ccRCC) being the most prevalent and contributing to most deaths caused by tumors [2]. Localized RCC may be successfully treated with surgery, but metastatic RCC showed strong resistance to conventional chemotherapy [3, 4]. However, the treatment of metastatic RCC has advanced significantly thanks to specific medications like axitinib, sunitinib, sorafenib, bevacizumab, everolimus, temsirolimus, and pazopanib. They impede the mechanistic target of rapamycin (mTOR) complex, which inhibits angiogenesis, from recognizing the vascular endothelial growth factor (VEGF) and binding to its receptor (VEGFR) [5-7].

Exosomes are tiny, bilayered extracellular vesicles that can range in size from 30 to 150 nm.

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Many proteins, signaling pathways, and organelles are involved in the production and release of exosomes [8-10]. Hepatocellular carcinoma was shown to have increased serum extracellular vesicle (EV) concentrations, but there was no evidence that this was the case in ccRCC [11, 12]. Tumor-derived exosomes (TEXs), according to a significant number of studies, may enable tumor cells to evade immune surveillance and ultimately immune system-mediated death. TEXs can affect the innate immune system by disrupting the IFN signaling pathway. For instance, the negative effects of exosomes from tumor cells render programmed cell death protein 1 ineffective [9, 10, 13].

According to earlier findings, the mTOR signaling route, ERK signaling pathway, or STAT signaling pathway are all more activated in ccRCC [14]. However, there is presently no proof that TEXs could promote ccRCC treatment resistance by enhancing these signal pathways [15].

T cell malfunction poses a significant obstacle to the effectiveness of immunotherapy for cancer. T cell is essential and participates in every step of the antitumor response [16]. Extracellular vesicles (EVs) released by tumors are one of the primary causes that can cause T cell malfunction, according to emerging research [17]. Patients with cancer have high levels of tumor-derived EVs in their serum, tissues, and tumor microenvironment, which act as crucial cancer cell messengers [13]. Additionally, EVs produced from tumors can transport a variety of immune suppressive signals driving T cell dysfunction for tumor immunity [18].

Herein, we investigate the effect of the RCC-derived exosome on the biological function of human T cells.

MATERIAL AND METHODS

Isolation of Human T Lymphocytes

A healthy donor's blood was obtained, and the tubes were centrifuged at 500 x g for 45 minutes at room temperature. Peripheral blood mononuclear cells (PBMC) have now been centrifuged into the top cell layer, separating from other blood components. The first hazy band is the PBMC layer as seen from the top down. The blood's upper, transparent, yellowish phase was carefully removed. The PBMC layer was then transferred to a 15 mL or 50 mL conical tube using a P1000 micropipette. The PBMC were centrifuged at 500 x g for 5 minutes after being washed twice with PBS.

Culture of Human T Lymphocytes

PBMC were pipetted into a culture flask containing RPMI 1640 (Gibco, Thermo Fisher Scientific, US), 10% FBS (Gibco, Thermo Fisher Scientific, US), 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, US), and 1 g/mL phytohemagglutinin of RPMI 1640 medium (PHA) (Gibco, Thermo Fisher Scientific, US). The medium was carefully removed after one hour of incubation, put to a conical tube, and centrifuged at 500 x g for five minutes. A flask was used to hold the suspended cell pellet while it was incubated at 37°C.

Exosome isolation

We collected ACHN cell culture supernatant in a 50mL falcon tube at 72h after seeding and centrifuged at room temperature (RT) for 10min at 2000 x g (removal of cell debris and larger apoptotic bodies). Supernatant then transferred to a new falcon tube and spin at 4°C for 30min at 10,000 x g (removal of large microvesicles and smaller apoptotic bodies). Finally, the supernatant was filtrated using a 50mL syringe and a 0.22μm bacterial filter (removal of smaller microvesicles in the size of 200-500nm).

Western blotting

We used RIPA lysis buffer (Thermo Fisher Scientific Inc.) and followed the manufacturer's instructions in brief. The process involved complete lysis of all isolated proteins and then semi-dry blotting to transfer an equivalent amount (about 50 g) of the entire extracted protein to PVDF membranes after being transferred to an SDS gel. After treating it with 0.5 percent Tween-20 in PBS for two hours, it was left to form a membrane block. It was then treated with primary monoclonal antibodies directed against the target genes from Thermo Fisher Scientific Inc. and then subjected to 24 hours of dark curing before being stained with secondary antibodies from Thisromian Corp. that were HRP-conjugated for an additional hour. It was possible to see the protein bands thanks to an upgraded chemiluminescence kit. The bands were next visualized by an imaging device. The density of the bands was adjusted in relation to the band for β-actin (ACTB).

MTT assay

Following the guidelines provided by the

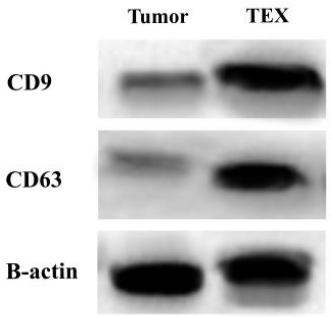

Fig. 1. Study of CD9 and CD63 expression in ACHN cell and derivative exosome (TEX) using western blotting.

MTT kit's manufacturer (Gibco, Thermo Fisher Scientific, US), we assessed the cytotoxicity of the ACHN-exosome treatment on human T cells using the MTT assay. A total of $1x10^5$ T cells/100 µL of RPMI-1640 medium was added to the wells in a 96-well plate. Afterward, the wells that contained the T cells were filled with the RCC-exosome at different concentrations (500-2000 ng/ml). 20 µL of 5 mg MTT/ml medium was added to the wells at four different time points: 24, 48, 72, and 96 hours after treatment. Cells were then incubated for the following four hours at 37°C. The optical density (OD) of the wells was measured at a wavelength of 570 nm following incubation using an ELISA reader. The viability of treated cells was calculated and presented as a percentage compared to the viability level of control cells. For all viability data, which were the results of three independent tests, the values were presented as means±SEM.

Flow cytometric analysis of apoptosis

Using the ApoptotestTM-FITC Kit from Dako, Glostrup, Denmark, the rate of apoptosis was measured. T cells received two doses (500 and 2000 ng/ml) of ACHN-exosome over the course of 96 hours. The addition of propidium iodide (PI) at 5 µL and fluorescein isocyanate (FITC)-conjugated Annexin-V at 10 µL to cell cultures resulted in the binding of any phosphatidyl serine-exposed area on the outer leaflet of the cell membrane to FITN. The amount of apoptosis was calculated based on the fluorescent signal emission from the phosphatidylserines attached to FITC-Annexin. Fluorescence emission was detected using a FACSCalibur device from BD Biosciences in Franklin Lakes, New Jersey.

Statistical analysis

Version 8.01 of GraphPad Prism was used for the statistical analyses. The findings were typical of the means and standard error of three separate experiments (triplicate). The Student's t-test was performed to compare the experimental groups statistically. Statistics were considered significant for P-values under 0.05.

RESULTS AND DISCUSSION

Characterizing ACHN-exosome

We explored the expression of CD63 and CD9 utilizing western blotting in ACHN cell line and derivate exosome. The expression of CD63 and CD9 is higher in exosome compared to parental ACHN cell (Fig. 1).

ACHN -exosome inhibited T cell proliferation

By using the MTT assay in accordance with the recommendations from the MTT kit manufacturer, we assessed the cytotoxicity of ACHN-exosome treatment in human T cells. In the T cell-containing wells, ACHN-exosome was added at various concentrations (500–2000 ng/ml). The results indicated that exosomes had a negative impact on T cell proliferation at four time-points: 24, 48, 72, and 96 hours after treatment (P>0.05). Due to this, T cell proliferation at 24, 48, and 72 hours after exposure to 500 ng/ml exosome was not significantly impacted. T cell proliferation was not significantly impacted by the presence of 750 ng/ml exosome within 24 and 48 hours, with a P>0.05 (Fig. 2).

The antitumor functions of immune cells are impacted by immunosuppressive factors carried by TEX, as evidenced previously. TEX control T cell activity by promoting CD8+ T cell death, inhibiting signaling and proliferation, and other mechanisms [19]. In contrast, immune cells produce exosomes that promote the growth of all T cells [20]. Additionally, there is evidence that cytotoxic CD8+

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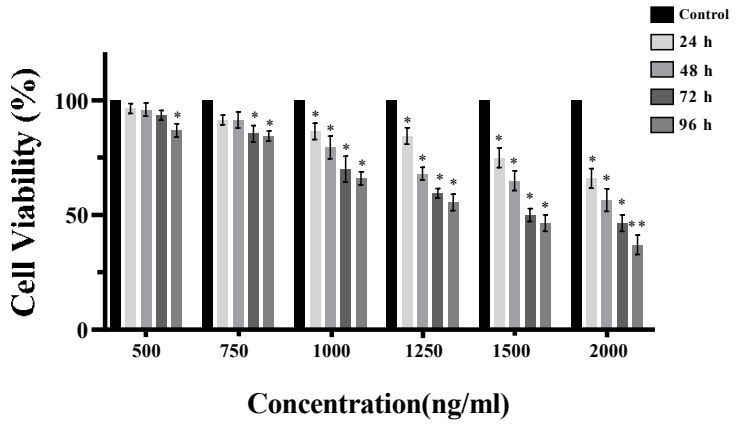

Fig. 2. Negative effects of ACHN-exosome on human T cell proliferation (MTT assay).

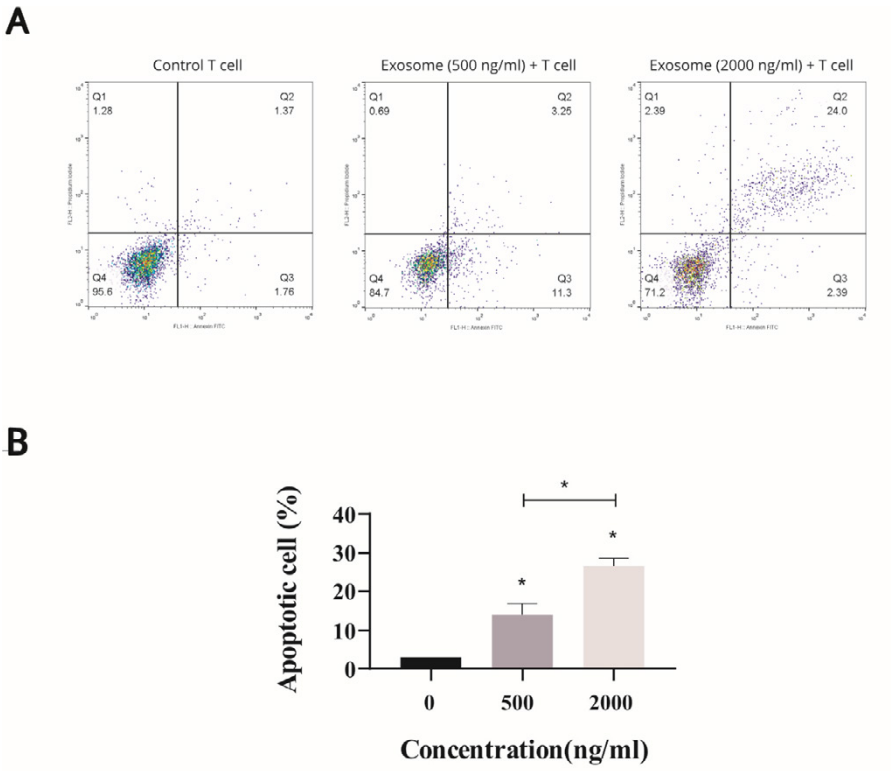

Fig. 3. Human T cell apoptosis upon exposure with ACHN-exosome (Annexin-V/PI staining and FACS).

T cells can be activated to suppress tumor activity by changing their antitumor properties [21]. TEX can cause the depletion of CD27/28 expression, leading to a distinct phenotype. These suppressive features were detected in tumor CD8+ T cells and tumor T cell in vitro coculture studies in head as well as neck cancer patients [22]. Furthermore, exosome-derived RNA can alter the phenotype of regular CD8+ T cells when it is introduced to them.

ACHN-exosome inspires T cell apoptosis

T cells were treated with ACHN-exosome at two doses (500 and 2000 ng/ml) for 96 hours. Then cell apoptosis was estimated using Annexin-V/PI staining and FACS. Results showed the noticeable enhancement in apoptosis rates of the T cells following treatment with 500 and 2000 ng/ml exosome within 96 hours of incubation ($P>0.05$) (Fig. 3A, B). Apoptosis percentages in T cells

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(control), cells treated with 500 ng/ml exosome and 2000 ng/ml exosome were 2.28±1.78, 15.48±3.05, and 27.89±2.7 of total cells, respectively (Figs. 3A, B).

FasL expression is a characteristic of tumor cells. Through its association with the receptor Fas, the transmembrane protein FasL promotes apoptosis, inhibits immunological response, and regulates self-antigen tolerance [23]. The ability of TEX with FasL expression to induce antitumor CD8+ T cell apoptosis has been demonstrated [24]. The production of MHC class I molecules in TEX may also contribute to this propensity to cause cytotoxic T cells to undergo apoptosis. The activation of the Fas/FasL signaling pathway is thought to be the mechanism by which the interaction of MHC class I with the CD8 receptor causes T lymphocytes to undergo apoptosis.

CONCLUSION

Recent research suggests that tumor-derived exosomes are crucial in helping tumors avoid immune surveillance. To accomplish immune suppression, tumor-derived exosomes can modify the actions of many immunological components in the TME. According to the research, tumor-derived EVs can impair T cell responses and proliferation in addition to other mechanisms to suppress T cell antitumor immunity. In this study, we demonstrated that exosomes from RCC suppressed T cell growth and caused these cells to undergo apoptosis in vitro.

CONFLICT OF INTEREST

There is no conflict of interest.

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