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Tumor exosomes expressing Fas ligand mediate CD8$^+$ T-cell apoptosis

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Abstract

Tumor-derived immune suppression is considered to be a major mechanism of tumor evasion from the immune system destruction, however, little is known regarding the induction of T-cell functional suppression by tumor-derived exosomes. Herein, we investigate tumor-derived exosomes involved in normal immunological communications as means of inhibiting an antitumor T-cell response. Exosomes derived from LNCaP, a human prostate cancer cell line, were visualized by FACS and identified based on size (80–200 nm) in comparison to marker beads. Exosomes from tumor cell line inhibited T-cell proliferation. Dose-dependent apoptosis of T cells was induced by co-culture with tumor exosomes. Addition of anti-FasL antibody blocked the apoptosis induction by tumor exosomes. This study suggests that induction of T-cell apoptosis by tumor-derived exosomes appears to be a novel mechanism of tumor immune evasion.

Keywords: Cancer immune evasion; Exosome; FasL; Apoptosis; CD8$^+$ T cells

Introduction

Microvesicles, termed exosomes, have been demonstrated to possess powerful immune stimulatory functions both in vitro and in vivo [1]. Dendritic cell (DC) and B-cell-derived exosomes contain high quantities of MHC-I, MHC-II, and CD86, allowing potent activation of T cells [2,3]. Administration of exosomes from DC pulsed with tumor antigen into cancer-bearing mice induces antitumor T-cell responses and tumor regression [1]. In contrast, exosomes can also inhibit T-cell responses; for example, exosomes secreted by intestinal cells have been shown to induce antigen-specific inhibition of T-cell function during induction of oral tolerance [4]. Tumor cells have been reported to secrete exosomes possessing both MHC-I and tumor antigen [5]. This observation can also be seen in melanoma [6] and leukemia [7] exosomes which also express bioactive FasL.

Immune escape by tumors appears to be a critical factor in preventing cancer destruction by the host immune system. T-cell killing molecules such as Fas ligand (FasL) have been reported in a wide variety of cancers [8]. Circulating FasL has been described in the plasma of cancer patients and is correlated with poor prognosis [9]. Although this type of immune suppression is antigen-nonspecific, specific T-cell deletion has also been seen in cancer patients [10,11]. Recently, cancer patients' serum was found to induce T-cell apoptosis in a TCR-specific manner [12].

While it is established that immune cells secrete exosomes [13], it was uncertain if cancer cells possess the ability to do so. In light of the recent reports describing

Abbreviations: FasL, Fas ligand; VEGF, vascular endothelial growth factor; DC, dendritic cells.

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tumor-derived exosomes [1,6,7], we investigated the role of FasL on the tumor-secreted exosomes. We found that secreted and FasL-expressing exosomes suppressed T-cells response through induction of apoptosis. This study highlighted a new mechanism of tumor evasion from immune privilege though secreting circulating membrane-bound immune suppressive molecules.

Materials and methods

Cell lines

The human prostate cancer cell line LNCaP was purchased from American Type Culture Collection (ATCC, Rockville, MD). LNCaP cells were cultured in alpha-MEM media supplemented with 10% fetal calf serum (FCS, GIBCO, Invitrogen Life Technologies, Burlington, ON).

Exosome purification

LNCaP cell line-derived exosomes were purified from the supernatant of incubated confluent cell lines using a modification of the sequential ultracentrifugation procedure described by Zitvogel et al. [1]. Separation of cellular debris was performed by centrifugation at 10,000 × *g* for 1 h followed by pelleting of the exosome through centrifugation at 100,000 × *g* for 3 h. Immunoelectron microscopy was used to confirm that equivalent structures are being studied. The protein concentration of exosomes was assessed by the Bradford assay (Bio-Rad Laboratories, Mississauga, ON).

Proliferation assays

Peripheral blood mononuclear cells were extracted using Ficoll gradient (Pharmacia) and subsequently washed in PBS. T cells were purified using a T-cell enrichment column (Cederlane, ON). Plating of T cells at 2×10^5/well in 96-well plates in complete RPMI 1640 media supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% FCS. Pre-selected concentrations of LNCaP and control exosomes were added to activated T cells (activation was induced with 10 μg/ml PHA; Sigma). T cells were incubated for 72 h with 18-h pulse of 1 μCi [^{3}H]-thymidine. T cells were subsequently harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was counted using a Wallac 1450 Microbeta liquid scintillation counter, and the data were analyzed with UltraTerm 3 software.

Apoptosis assay

The activated T cells were incubated with the pre-selected concentrations of cell line and control exosomes for 48 h followed by harvesting. Annexin-V (Oncogene, Cambridge, CA) staining was used to assess apoptosis. Staining was determined by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA) and analyzed using CellQuest software (Becton Dickinson).

Antibody blockade of apoptosis

The human FasL antagonistic antibody NOK-2 (BD PharMingen, San Diego, CA) was added into the co-cultures of exosomes and T cells, then apoptosis was re-evaluated with Annexin-V, as described previously [12].

Results

Characterization and secretion of tumor-derived exosomes

To determine if tumor cells secrete exosomes actively, we cultured LNCaP cell line and collected the supernatants at various time points (24, 48, and 72 h) followed by exosome purification and quantification, as described above, A time-dependent increase in exosome concentration was observed (data not shown). To confirm that prostate cancer exosomes were secreted under in vitro culture systems, we used a flow cytometric detection method previously described by Martinez-Lorenzo et al. [14]. The flow cytometric profile on size versus forward scatter indicated that the exosomes were slightly larger than 60 nm marker beads (Figs. 1A and B) and smaller than the 500 (Fig. 1C) and 1000 nm (Fig. 1D) markers. This observation suggests that the exosome purification

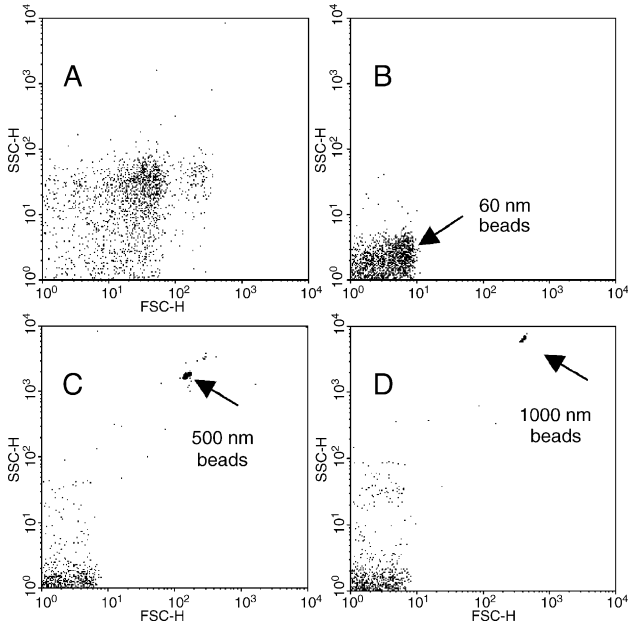

Fig. 1. Physical detection of exosomes from cell lines. Prostate cancer exosomes were purified from LNCaP as described in Materials and methods. Flow cytometry was performed using a FACScan to detect microvesicles present in the pellet obtained from ultracentrifugation. (A) The forward and side scatter profile of tissue culture exosomes. (B) 60 nm marker beads. (C) 500 nm marker beads. (D) 1 μm marker beads.

methodology used in this study is effective and collects little residual debris.

Inhibition of T-cell proliferation by tumor exosomes

LNCaP and control exosomes were added to PHA-activated T cells in pre-selected concentrations then co-cultured for 72 h. Assessment of proliferation revealed a classical dose–response inhibition of T-cell proliferation caused by the cancer-derived exosomes (Fig. 2A). On the other hand, control exosomes failed to show significant inhibitory effects at any concentration (Fig. 2A). To rule out that FCS in culture media was a source of exosomes, we used the exosome purification procedure on cell-free complete culture media. T-cell proliferation was not affected

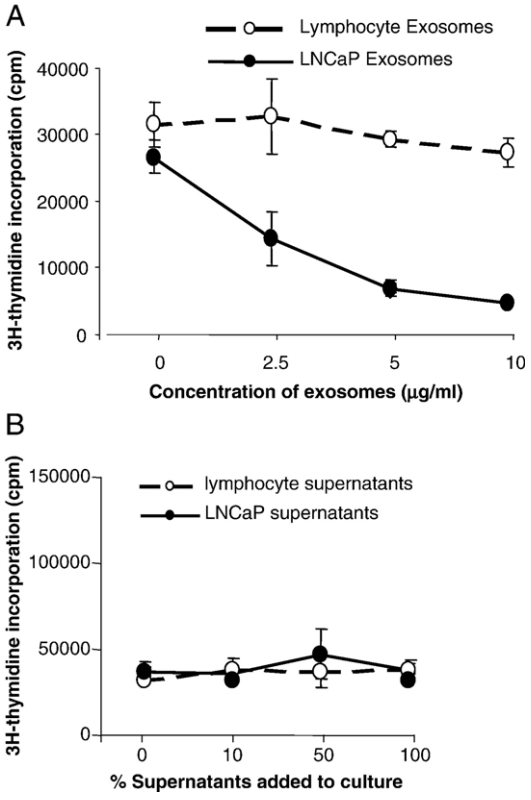

Fig. 2. Inhibition of T-cell proliferation by prostate-cancer-derived exosomes. (A) Exosomes from LNCaP inhibit proliferation of human peripheral blood T cells. Exosomes were purified as described in Materials and methods. Protein concentration of exosomes was detected by the Bradford assay. Exosomes were added at the indicated concentrations to PHA (10 μg/ml)-activated T cells for 72 h. ^{3}H-thymidine was added for the last 18 h of culture at 1 μC/well. Cells were subsequently harvested, and thymidine incorporation was assessed by scintillation counting. (B) Exosome-free supernatants from the prostate cancer cell line did not inhibit T-cell proliferation. Supernatants of 48-h incubated confluent LNCaP cell line or control human T cells were collected and ultracentrifuged as described in Materials and methods. The exosome-free supernatants were added to cultures of PHA-activated T cells for 48 h. ^{3}H-thymidine was added for the last 18 h of culture at 1 μC/well. Cells were subsequently harvested, and thymidine incorporation was assessed by scintillation counting.

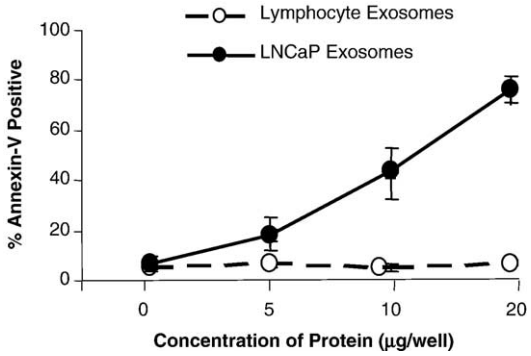

Fig. 3. Exosomes from prostate cancer cell line induce apoptosis in human peripheral blood T cells. Exosomes were purified as described in Materials and methods. Protein concentration of exosomes was detected by the Bradford assay (Bio-Rad). Exosomes were added at the indicated concentrations to PHA (10 μg/ml)-activated CD8^{+}T cells for 72 h. Apoptosis was assessed using Annexin-V staining and analyzed by flow cytometry.

by the proteins purified (data not shown). To exclude the possibility of unidentified immune-inhibitory soluble factors being responsible for the observed suppression of T-cells proliferation, supernatants from cell line exosome purification procedure were added to activated T cells. The exosome-free supernatants possessed no suppressive effects (Fig. 2B).

Confirmation that tumor exosomes were the de facto suppressants of proliferation was provided by our observation that the T-cell inhibitory activity was lost after passing the exosomes through microfilters (data not shown).

Tumor exosomes induce CD8^{+} T-cell apoptosis

The previously observed inhibition of T-cell proliferation (Fig. 2A) led us to hypothesize that induction of T-cell apoptosis was the underlying mechanism. To examine this hypothesis, various concentrations of exosomes from LNCaP and lymphocytes (controls) were co-incubated with PHA-activated T cells for 48 h. Subsequently, Annexin-V-FITC staining for apoptosis was analyzed by flow cytometry. A dose-dependent induction of apoptosis was observed in CD8^{+} T cells treated with exosomes derived from LNCaP cell line, but not in the T cells treated with control exosomes (Fig. 3). At the highest exosome concentration tested (10 μg/ml), 87% of CD8^{+} T cells underwent apoptosis; while control lymphocyte exosomes only induced 2% apoptosis. CD4^{+} T cells underwent negligible levels of apoptosis (data not shown).

Exosome-induced apoptosis is FasL-dependent

FasL has been studied as a contributor to the spontaneous T-cell apoptosis described in cancer patients [14–17]. Since it has been previously demonstrated that exosomes may possess FasL [6], we hypothesized that FasL is responsible for the exosome-induced CD8^{+} T-cell

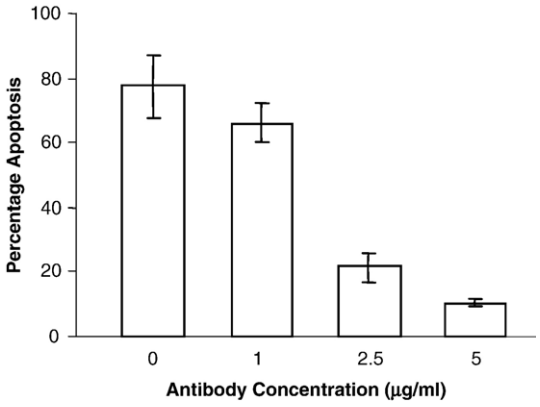

Fig. 4. Exosome-induced apoptosis is FasL-dependent. Exosome-induced apoptosis is FasL-dependent. LNCaP human prostate cancer cell exosomes were purified by ultracentrifugation. Exosomes (10 μg/ml) were added to PHA (10 μg/ml)-activated T cells for 72 h in the presence of increasing anti-human FasL antagonistic antibody concentrations. Apoptosis was assessed using Annexin-V staining and analyzed by flow cytometry.

apoptosis. To investigate this, Fas–FasL pathway was blocked on cancer-derived exosomes by adding various doses (0–5 μg/ml) of FasL-blocking antibody [18]. Dose-dependent inhibition of the Fas–FasL interaction resulted in a corresponding dose-dependent decrease in T-cell apoptosis (Fig. 4), suggesting that FasL on cancer exosomes triggered the previously observed apoptosis of T cells. In contrast, addition of isotype control IgG2a did not affect the exosomes-induced T-cell apoptosis (data not shown).

Discussion

Despite the great progress made in developing immunotherapeutic interventions, little clinical success has been achieved. This might be due to the profound immune defects found in advanced cancer patients. Overall, immune suppression is seen in cancer patients [19]. Attempts to enhance antitumor immunity have been performed by drugs, cytokines, and vaccines, but with little therapeutic efficacy in clinical trials. Among the mechanisms of tumor progression, tumor-mediated immune suppression appears to be a critical factor in preventing cancer destruction by the host immune system. In this report, we describe a mechanism by which cancer cells inhibit T-cell responses.

Soluble factors secreted by tumor cells, called “blocking factors”, have been described for more than 20 years in the serum of cancer patients. Hellstrom et al. showed that lymphocytes from cancer patients could inhibit the growth of autologous tumors only in absence of autologous serum [20,21]. Serum from healthy controls, or allogeneic cancer patients, would not block tumor inhibition. The authors postulated that the serum-blocking factor was comprised of MHC and tumor antigen. More recently, it was demonstrated that TCR-specific inhibition of T cells from healthy volunteers could be achieved by the addition of serum from melanoma patients. Interestingly, when blocking antibody to MHC-I was added to the serum, the suppressive effects were abolished [12]. In the present study, we demonstrated that tumor-derived exosomes may serve as mediators that inhibit the host immune response in a similar manner to the described blocking factors.

One of these mediators might be FasL expressed on tumor-derived exosomes. We demonstrated that cancer exosome-mediated apoptosis was dependent on the Fas–FasL pathway (Fig. 4). An elevated expression of FasL on cancers has been reported [8]. Apoptosis of tumor-reactive T cells through Fas–FasL pathway has also been well documented [9]. FasL is typically a membrane-bound surface molecule. Although a soluble form of FasL might exist in the circulation, the capacity of inducing apoptosis by soluble FasL is much weaker than membrane-bound FasL [22]. However, to date, it remains unclear how the circulating T cells are killed by local tumor FasL. The data shown in this study suggest that exosome-carried “membrane-bound” FasL exists in peripheral circulation. This tumor-derived circulating “membrane-bound” FasL may target circulating T cells distantly and induce apoptosis efficiently.

Exosomes have been demonstrated to possess powerful immune stimulatory functions both in vitro and in vivo [1]. DC and B cell exosomes contain high quantities of MHC-I, MHC-II, and CD86, allowing potent activation of T cells [2,3]. Administration of exosomes from DC pulsed with tumor antigen into cancer-bearing mice induces antitumor T-cell responses and tumor regression [1]. In contrast, exosomes can also inhibit T-cell responses, for example, exosomes secreted by intestinal cells, termed tolerosomes, induce antigen-specific inhibition of T-cell function during oral tolerance [4]. The importance of FasL in antigen-specific elimination of T cells was functionally demonstrated by our group in previous work where DC transfected with FasL were able to induce antigen-specific tolerance in a murine model of transplantation [23].

Our findings show that FasL-expressing exosomes suppressed T-cell responses through induction of apoptosis. This study highlighted a new mechanism of tumor immune evasion through secreting such circulating, membrane-bound, immune suppressive molecules as FasL. This novel mechanism of tumor-mediated immune suppression may have implications for the development of future anticancer immune therapies.

Acknowledgment

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