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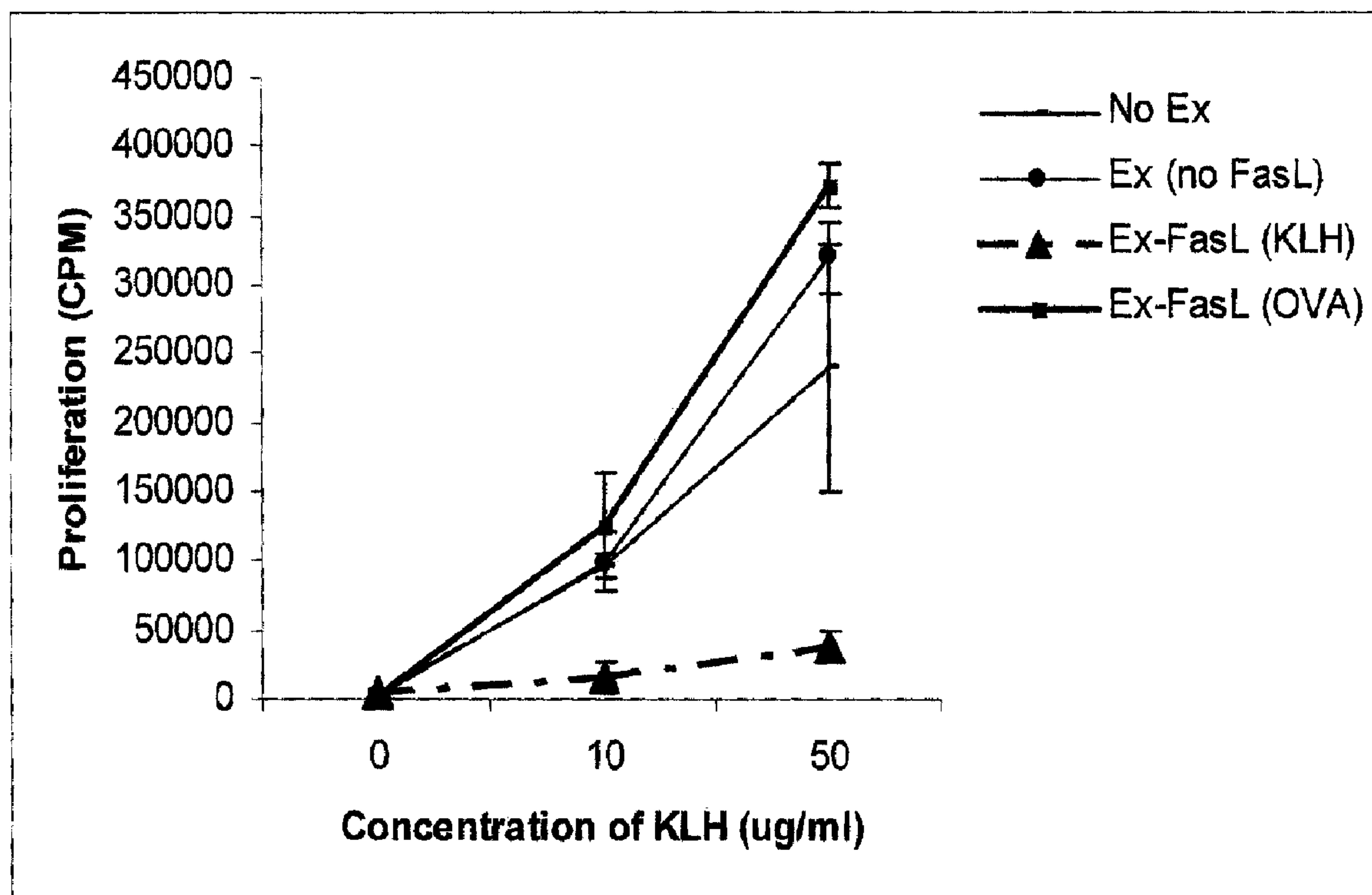
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(54) Titre : QUANTIFICATION ET PRODUCTION D'EXOSOMES IMMUNOSUPPRESSEURS

(54) Title: QUANTIFICATION AND GENERATION OF IMMUNE SUPPRESSIVE EXOSOMES



(57) Abrégé/Abstract:

Methods of identifying and quantifying immune suppressive factors in biological fluids of cancer patients comprising purification and assessment of small (60-250nm) microvesicles are disclosed. Said microvesicles can serve alone, or in combination with other immunological assays as a prognostic indicator for a cancer patient, or alternatively as a determinant for various immunological and non-immunological therapeutic interventions. Methods of generating immune suppressive exosomes are also described.

### **Abstract of the Disclosure**

Methods of identifying and quantifying immune suppressive factors in biological fluids of cancer patients comprising purification and assessment of small (60-250nm) microvesicles are disclosed. Said microvesicles can serve alone, or in combination with other immunological assays as a prognostic indicator for a cancer patient, or alternatively as a determinant for various immunological and non-immunological therapeutic interventions. Methods of generating immune suppressive exosomes are also described.

## DISCLOSURE

### Field of The Invention

The present invention relates to the field of immune modulatory factors, more specifically, it relates to the detection and synthesis of immune suppressive exosomes.

### Background of Invention

At present, little therapeutic options are available for patients with late stage cancer. This demands more sensitive diagnostic assays, as well as improvements in treatment available. An appealing method of treating cancer is through harnessing the power of the host's immune system. Although great progress has been made in the development of immune therapies, little clinical success has been achieved. A reason for this may be the profound immune defects found in advanced cancer patients. Soluble inhibitory molecules such as TGF- $\beta$ , IL-10, and VEGF [1] and surface-bound T cell killing molecules such as Fas ligand (FasL) have been reported in a wide variety of cancers [2]. Additionally, circulating FasL has been described as a T-cell apoptosis-inducing factor found in the plasma of cancer patients, being correlated with poor prognosis [2]. Although this type of immune suppression is antigen-nonspecific, specific T cell deletion is also seen in patients (reviewed in [3, 4]. Antigen specific deletion implies 2 things: a) that the T cell receptor interacts with tumor MHC and tumor antigen, and b) the tumor provides a death signal to the T cell. Such a signal could be an active death signal, such as tumor-expressed FasL, or absence of a survival signal, such as lack of costimulation [5, 6]. In light of a recent report that serum from tumor-bearing patients induces T apoptosis in TCR-specific manner [7], we questioned whether MHC-dependent T cell-killing factors may be circulating in the plasma of patients with advanced cancer.

It is known in the art that microvesicles termed “exosomes” possess powerful immune stimulatory functions both in vitro and in vivo [8]. Dendritic cell (DC) and B cell-derived exosomes contain high quantities of MHC I, MHC II, and CD86, allowing potent activation of T cells [9, 10]. Administration of exosomes from DC pulsed with tumor antigen into cancer-bearing mice induces antitumor T cell responses and tumor regression. Tumor cells have been reported to secrete exosomes which possess MHC I and tumor antigen, thus allowing possible vaccination with these entities [11].

It is the purpose of this disclosure to describe a novel biological function of exosomes. This being immune suppression. With the exception of a publication by Karlsson *et al*, who reported intestinal-derived exosome-like particles termed “tolerosomes” [12], there has not been indication in the literature that exosomes may serve physiological immune suppressive functions. Prolongation of allograft survival using donor DC-derived exosomes was reported in a rat allogeneic cardiac transplant model [13]. Prolongation was associated with decreased interferon-gamma secretion and inhibited number of graft-infiltrating lymphocytes. Interestingly, allo-antibody responses were increased after exosome-treatment, indicating that true tolerance was not induced. It has however been observed that melanoma [14] and leukemic [15] exosomes express bioactive FasL suggest that a two step interaction may be occurring between the tumor exosome and the T cell in which tumor antigen in MHC I induce a T cell activation event causing upregulation of Fas on the T cell. However before this disclosure, such an interaction was never demonstrated clinically.

Since the tumor exosomes expresses FasL, subsequent T cell apoptosis should occur. Another putative method by which tumor-derived exosomes may possess an immunoregulatory function is suggested in a report that melanoma-derived cell lines secrete exosomes expressing the immune inhibitory molecule HLA-G [16]. However no evidence of immune suppressive activities was demonstrated. With exception to the

present disclosure, this model of antigen-specific immune suppression by tumor exosomes has never been demonstrated

Methods of generating exosomes from natural sources and synthetically have been previously described in Canadian Patent Application # 2296750. None of these methods describe generation of immune suppressive exosomes.

### **Brief Description of the Invention**

The basis of the present invention is that exosomes from cancer patients possess FasL or other immune suppressive molecules that contribute to specific killing/inactivation of tumor-reactive T cells. Through quantification of these immune suppressive exosomes, as well as qualitatively assessing their functional activity, one can assess the immunological status of a patient suffering from an immunological deficient state such as cancer.

Alternatively, immune suppressive exosomes can be used therapeutically to treat conditions associated with immune hyperactivation, or inappropriate activation. Said conditions can include, but are not limited to autoimmunity, transplant rejection, or immune mediated pathology such as septic shock.

Using the immune suppressive properties of exosomes as a basis for immunotherapy, exosome generated from cancer cells, or dendritic cells (DC) that have been manipulated to possess immune suppressive properties can be applied therapeutically for treatment of pathologies associated with hyperactivation of immune responses. The ability to antigen-specifically pulse DC such that said antigen becomes attached to exosomes allows the utilization of suppressive-DC exosomes as a treatment for specific autoimmune diseases.

### **Detailed Description of the Invention**

A novel teaching of this invention is to use patient-derived exosomes as a measure of immune suppression. Said immune suppression could consist of, but is not limited to: T cell dysfunction, decreased ability of T cells to proliferation, decreased ability of T cells to secrete tumor inhibitory cytokines, suppressed ability of T cells to produce antiangiogenic effects, and upregulation of T cell immune-inhibitory activities either through contact-dependent or independent mechanisms. Assessing the ability of a tumor, or another immunosuppressive pathology would be useful in evaluating the eligibility of patients for immunotherapy. Another impetus for identifying the state of a patient's immune suppression is to guide the amount, and/or frequency of immune stimulatory agents the patient should receive.

In one embodiment of the present invention, serum is extracted from a patient in whom immune suppressive activity is being assessed. Exosomes can be purified using a variety of methods well known in the art, such methods are hereby included for reference: 1) 10 ml of patient blood is extracted using a Vacutainer or similar apparatus. Plasma is separated by centrifugation for a period of time sufficient to allow fractionation of the cellular particulates from the plasma. A suitable centrifugation time is 30 min at 1000g; 2) Cell-free plasma is subsequently centrifuged at for a period of 4-24 hours at 40,000 to 100,000g; 3) Collected pellets are subsequently washed in saline or a physiological solution by centrifugation at 4-24 hours under a force of 40,000 to 100,000g; and 4) Protein content of the collected exosomes is assessed by Bradford assay. Variations of exosome purification procedures are well-described in the art. Said methods have been optimized for purifying exosomes from cord-blood [17], ascites fluid [18], tissue culture [19], and bronchoalveolar lavage fluid [20].

Upon purification and standardization of exosomal protein content immune suppressive activity can be measured using a variety of assays well known to the skilled artisan. For assessment of direct T cell apoptosis-inducing ability, exosomes are co-cultured with

allogeneic PHA-activated T cells for a period sufficient to induce apoptosis if the exosomes contained Fas-L. Such a co-culture can last from 4-72 hours depending on the type of patient, the purity of the exosome preparation, and the method of evaluating apoptosis induction. In one method a concentration of 0.1 $\mu$ g-20 $\mu$ g of exosomes are purified from patient plasma and incubated with PHA activated (10 $\mu$ g/ml for 24 hours) peripheral blood mononuclear cells from healthy person. The exosome-PBMC combination is incubated for 72 hours at 37 Celsius and apoptosis of the PBMC is quantified using Annexin-V staining and analyzed by flow cytometry. Using this system an increased level of PBMC apoptosis is indicative of patient immune suppression through induction of immune cell apoptosis by circulating exosomes.

Many variations of this technique can be performed by a person skilled in the art without departing from the spirit or essence of this invention. For example, instead of using patient plasma as an initial source of exosomes one could utilize various biological fluids such as ascites fluid, bronchoalveolar lavage, or intratumoral secretions.

Alternatively, primary tumour tissue can be extracted by biopsy, cultured in vitro, and exosomes from culture supernatant can be purified and admixed with PBMC in order to assess apoptosis-inducing capacity.

A potential drawback of using healthy volunteer PBMC as the targets for exosome-induced apoptosis is variability of healthy volunteer PBMC populations, and the fact that PBMC constitute a plethora of cell types. In order to rule out such variability as standardized cellular target population can be employed. More specifically, exosomes purified from the cancer patient can be co-cultured with a standard cell line sensitive to exosome-induced killing. One such cell line is the human T cell leukemia cell line Jurkat. It is known in the art that exosomes released by activated T cells can induce apoptosis of the Jurkat cell line [21]. This is believed to be due to the expression of bioactive Fas receptor on this cell line. In agreement with our data that exosomes derived

from prostate cancer patients express FasL, the utilization of Jurkats as a standard target cell line for patient-exosome immunosuppressive activity is feasible.

Within the scope of the present invention lies the utilization of purified sub-population of healthy volunteer cells as a target for exosome-mediated killing. For example, some types of cancers may be more immunosuppressive to CD4, CD8, or CD56 immune cells. Thus purified exosomes from plasma of cancer-bearing patients can be co-cultured with sub-populations of immunological cells and apoptosis of said cells can be assessed. Methods of purifying subpopulations of immune cells are well-known in the art and include flow sorting, magnetic activated cell sorting, cell panning, and purification using column methods.

The immunosuppressive aspects of cancer-derived exosomes do not have to be limited to induction of apoptosis but may also induce cellular changes that render said immune cell unresponsive or hyporesponsive to further immunological stimuli. Such a state, termed “anergy” can result from inappropriate activation of the T cell receptor (TCR), activation of the TCR in absence of co-stimulatory molecules, and presentation to the T cell of inhibitory molecules such as HLA-G. The observation that exosomes possess HLA-G [16] suggests that cancer-immune evasion via exosomes does not have to strictly occur through induction of apoptosis but could also function via anergy induction. Thus, within the scope of the disclosed invention, patient-purified exosomes can be admixed with a population of immunological cells derived from a healthy volunteer and various functional aspects of the immune cells can be assessed instead of, or in conjunction with apoptosis evaluation. For example, exosomes from a cancer patient can be mixed with T cells purified from a cancer patient and stimulated with anti-CD3. A reduction in the proliferation, cytokine production, or upregulation of immune suppressive cytokines, would be indicative of the exosomes possessing immunosuppressive activity. Cytokines known in the art to be reduced in cancer patients include IFN- $\gamma$ , IL-2, IL-12, and TNF- $\alpha$ . In contrast, cytokines that are associated with cancer-induced immune suppression include TGF- $\beta$ , IL-10, VEGF, and IL-13.

Utilization of healthy volunteer cells as an immunological target for assessing suppressive capabilities of cancer-derived exosomes is performed since the immunological cells of the cancer patient are often suppressed as a result of past treatments (ie chemotherapy, radiation, surgery), or as a result of the cancer itself. It may be, however, desired by the practitioner of the invention to determine the extent to which exosome-derived immune suppression is responsible for the overall decrease in the patient's immune response. In this situation the practitioner of the invention may choose to utilize autologous immune cells as the source of exosome-targets. In this situation, an antigen-specific approach may be used. Patient T cell lines can be established in vitro using commonly known antigens such as KLH or ovalbumin. These cell lines can then be admixed with patient exosomes and inhibition of viability or immunological functions can be assessed.

In addition to determining the extent of immune suppression in a patient by assessing exosomal immune activity, the therapeutic utilization of immune suppressive exosomes is also possible. Generation of exosomes with ability to kill T cells can be performed by transfection of the DC with various membrane-bound killer molecules such as FasL. Within the disclosed invention are artificially generated exosomes that possess the ability to induce apoptosis of T cells in an antigen-specific manner. Such exosomes can be used therapeutically for silencing antigen-specific immune responses. Within the present disclosure we demonstrate that administration of exosomes from DC that are transfected with FasL and pulsed with the antigen KLH can inhibit the KLH-specific but not 3<sup>rd</sup> party responses (ie anti-ovalbumin) responses.

## **Examples**

### **1. Time-dependent Increase In Exosome Production by Human Prostate Cancer Cell Lines**

The human prostate cancer cell lines DU-145, PC-3 and LNCaP were allowed to grow to 50% confluence in OptiMEM media supplemented with 10% fetal calf serum. 10 ml samples of media were extracted at the indicated timepoints. Exosomes were collected by centrifugation at 7,000 g for 30 minutes, followed by a second centrifugation at 100,000g for 3 hours.. Exosomal proteins were assessed using the Bradford Assay (Biorad). A time-dependent increase in the production of exosomal proteins indicates exosomes were actively produced by the cell lines and rules out the possibility that non-specific exosomal contamination was being detected (Figure 1).

### **2. Detection of PSMA Expressing Exosomes From LNCaP Cells**

Exosomes were purified as described above from LNCaP cells at 75% confluency. The anti-prostate specific membrane antigen (PSMA) antibody clone 1G3, mouse IgG2a (Northwest Biotherapeutics, Seattle) was used for staining exosomes in combination with a FITC labelled secondary antibody. This antibody targets the extracellular domain of PSMA, increasing the likelihood of staining exosomes. As can be seen, a distinct population of LNCaP-derived exosomes specifically stains positive for expression of this tumor antigen (Figure 2).

### **3. Cancer Cell line Exosome-Induced Apoptosis is FasL- and MHC I-Dependent**

Exosome-induced apoptosis is FasL-dependent. PC-3 human prostate cancer cell exosomes were purified by ultracentrifugation. Exosomes (10µg/ml) were added to PHA (10µg/ml)-activated T cells for 48 hours in the presence of the indicated concentration of anti- HLA class-I antibody, w6/32. Apoptosis was assessed using annexin-V staining and

analyzed by flow cytometry. As seen in Figure 3A, cancer-exosome induced apoptosis was inhibited by addition of anti-MHC I antibody. Exosome-induced apoptosis is FasL-dependent. Increasing concentrations of the anti-human Fas ligand antagonistic antibody, NOK-2 (Pharmingen, San Diego, CA, USA) were added to a 72 hour coculture of PC-3 exosomes and activated T cells as described above. Apoptosis was assessed using annexin-V staining and analyzed by flow cytometry. As seen in Figure 3B, cancer-exosome induced apoptosis was significantly inhibited by addition of anti-FasL antibody.

#### **4. Prostate Cancer Patient Exosomes Induce Apoptosis Through a FasL- and MHC I-Dependent Mechanism**

Apoptosis induced by exosomes isolated from prostate cancer patients. Plasma was purified from peripheral blood of healthy controls or 3 prostate cancer patients by centrifugation at 500g for half-hour. Separation of cellular debris was performed by centrifugation at 7,000 g for ½ hour followed by pelleting of the exosome through centrifugation at 100,000g for 3 hours, followed by one wash in PBS. Protein concentration of exosomes was detected by the Bradford assay (Biorad). Exosomes were added at the indicated concentrations to PHA (10µg/ml)-activated T cells for 48 hours. Apoptosis was assessed using annexin-V staining and analyzed by flow cytometry. This figure is representative of 3 other prostate cancer patients whose exosomes were analyzed. Has seen in Figure 4A exosomes purified from plasma of cancer patients but not healthy controls induced a dose-dependent apoptosis in healthy T cells. Prostate cancer patient derived exosomes induced apoptosis is MHC I and FasL-dependent. Exosomes were purified from prostate cancer patient M142 as described in Figure 4. Increasing concentrations of anti- HLA class-I antibody, w6/32, or anti-human Fas ligand antagonistic antibody, NOK-2 (Pharmingen, San Diego, CA, USA) were added to a 48 hour coculture of exosomes (5 µg/ml) and activated T cells (PHA 10µg/ml). Apoptosis was assessed using annexin-V staining and analyzed by flow cytometry. As seen in Figure 4B apoptosis was dependent on MHC I and FasL.

## **5. Detection of FasL-bearing Exosomes From Plasma of Prostate Cancer Patients Using Cancer-Exosome ELISA**

ELISA plates were coated with human anti-MHC I monoclonal antibodies (w6/32) and incubated overnight. Serum was collected from healthy controls and advanced prostate cancer patients and added to the coated plates. After washing, biotinylated anti-human Fas ligand antibody (NOK-2) was added to the plates. Following an additional wash, Streptavidin-conjugated Horse Radish Peroxidase was added and after a half hour incubation ABTS (3-ethylbenzthiazoline-6-sulfonic acid) was used as a developing agent. The reaction was stopped with H<sub>2</sub>O<sub>2</sub> and fluorescence was assessed by spectrophotometry. A dose-dependent increase in fluorescence was observed in serum samples from prostate cancer patients but not controls.

## **6. Induction of Antigen-Specific Immune Modulation Using FasL-bearing Exosomes**

DC were generated as follows: bone marrow cells were flushed from femurs and tibias of C57BL/6 mice, washed, and cultured in 6-well plates ( $2 \times 10^6$ /ml) in 4 ml RPMI 1640 containing rGM-CSF (10 ng/ml; Peprotech, Rocky Hill, NJ) and mouse rIL-4 (10 ng/ml; Peprotech). Nonadherent granulocytes were removed after 48 h of culture, and fresh media added every 48 h. By day 4 to 6 of culture, proliferating clusters of cells with typical dendritic morphology were seen, and by day 7 to 9 more than 90% of the cells expressed the DC cell surface marker CD11c. On day 6 of culture DC were pulsed with 50 µg/ml of KLH, ovalbumin, or left untreated. On day 7, DC were transfected with the pBK-CMV phagemid vector (2 µg) containing full-length human FasL cDNA or empty control vector. Transfection reagent was prepared by incubating 8 µl Lipofectin (Life Technologies, Gaithersburg, MD) in a volume of 100 µl of PBS at room temperature for 45 min with the plasmid DNA. This mixture was added to 7-day cultured DC in a final volume of 1 ml of serum-free medium. After 4-h incubation at 37°C with 5% CO<sub>2</sub>, the cells were washed and cultured in RPMI 1640 with 10% FCS for 48 h. Subsequent to

transfection, supernatants were collected at 48 hours and exosomes were purified by centrifugation at 7,000 g for ½ hour, pelleting, and then centrifuging at 100,000g for 3 hours, followed by one wash in PBS. Protein concentration of exosomes was determined by Bradford assay. In order to test the ability of FasL-transfected and antigen-pulsed exosomes to suppress immune response in an antigen-specific manner we immunized adult C57/BL6 mice with 50µg/ml KLH in complete Freund's adjuvant. At the same time mice were administered various types of exosomes. 10 µg of exosomes per mouse were injected intravenously using the following groups of C57/BL6 mice: 1). Exosomes from untransfected DC; 2). Exosomes from FasL-Transfected DC with no antigen pulse; 3) Exosomes from FasL-transfected DC pulsed with ovalbumin (OVA); and 4) Exosomes from FasL-transfected DC pulsed with KLH. After a period of 2 weeks lymph node cells were purified and stimulated in vitro with 10µg/ml KLH for 72 hours with a pulse of tritiated thymidine (1 µCi/well) for the last 16 hours of culture. Proliferation was assessed by scintillation counting. As seen in Figure 5 only the KLH-FasL-exosome caused a specific inhibition of KLH-reactive responses. In contrast the co-administration of FasL exosomes, OVA-pulsed-FasL-exosomes, or non-transfected, non-antigen-pulsed exosomes did not affect the KLH-specific response.

#### References:

1. Ohm, J.E. and D.P. Carbone, *VEGF as a mediator of tumor-associated immunodeficiency*. Immunol Res, 2001. **23**(2-3): p. 263-72.
2. Whiteside, T.L., *Tumor-induced death of immune cells: its mechanisms and consequences*. Semin Cancer Biol, 2002. **12**(1): p. 43-50.
3. De Visser, K.E., T.N. Schumacher, and A.M. Kruisbeek, *CD8+ T Cell Tolerance and Cancer Immunotherapy*. J Immunother, 2003. **26**(1): p. 1-11.
4. Parmiani, G., *Tumor immunity as autoimmunity: tumor antigens include normal self proteins which stimulate anergic peripheral T cells*. Immunol Today, 1993. **14**(11): p. 536-8.

5. Johnson, J.G. and M.K. Jenkins, *Accessory cell-derived signals required for T cell activation*. Immunol Res, 1993. **12**(1): p. 48-64.
6. Antonia, S.J., M. Extermann, and R.A. Flavell, *Immunologic nonresponsiveness to tumors*. Crit Rev Oncog, 1998. **9**(1): p. 35-41.
7. Maccalli, C., et al., *Differential loss of T cell signaling molecules in metastatic melanoma patients' T lymphocyte subsets expressing distinct TCR variable regions*. J Immunol, 1999. **163**(12): p. 6912-23.
8. Zitvogel, L., et al., *Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes*. Nat Med, 1998. **4**(5): p. 594-600.
9. Wubbolts, R.W., et al., *Proteomic and biochemical analyses of human B cell-derived exosomes: potential implications for their function and multivesicular body formation*. J Biol Chem, 2003.
10. Lamparski, H.G., et al., *Production and characterization of clinical grade exosomes derived from dendritic cells*. J Immunol Methods, 2002. **270**(2): p. 211-26.
11. Wolfers, J., et al., *Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming*. Nat Med, 2001. **7**(3): p. 297-303.
12. Karlsson, M., et al., *"Tolerosomes" are produced by intestinal epithelial cells*. Eur J Immunol, 2001. **31**(10): p. 2892-900.
13. Peche, H., et al., *Presentation of donor major histocompatibility complex antigens by bone marrow dendritic cell-derived exosomes modulates allograft rejection*. Transplantation, 2003. **76**(10): p. 1503-10.
14. Andreola, G., et al., *Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles*. J Exp Med, 2002. **195**(10): p. 1303-16.
15. Monleon, I., et al., *Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells*. J Immunol, 2001. **167**(12): p. 6736-44.
16. Riteau, B., et al., *Exosomes bearing HLA-G are released by melanoma cells*. Hum Immunol, 2003. **64**(11): p. 1064-72.

17. Gansuud, B., et al., *Umbilical cord blood dendritic cells are a rich source of soluble HLA-DR: synergistic effect of exosomes and dendritic cells on autologous or allogeneic T-Cell proliferation*. Hum Immunol, 2003. **64**(4): p. 427-39.
18. Andre, F., et al., *Malignant effusions and immunogenic tumour-derived exosomes*. Lancet, 2002. **360**(9329): p. 295-305.
19. de Gassart, A., et al., *Lipid raft-associated protein sorting in exosomes*. Blood, 2003. **102**(13): p. 4336-44.
20. Admyre, C., et al., *Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid*. Eur Respir J, 2003. **22**(4): p. 578-83.
21. Blanchard, N., et al., *TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex*. J Immunol, 2002. **168**(7): p. 3235-41.

**Claims:**

1. A method of detecting microvesicular particles hereafter referred to as exosomes comprising the steps of:
  - a) Attaching an antibody that binds specifically to an epitope of a known protein found on the exosome whose detection is sought, to a surface;
  - b) Performing a washing step to remove antibody that is not attached to the said surface;
  - c) Adding to the said antibody a fluid in which exosomes are believed to be found;
  - d) Performing a washing step to remove matter that is unbound to the said surface-bound antibody;
  - e) Adding a secondary antibody, that binds specifically to an epitope found on the exosome, said secondary antibody labelled with a detection marker;
  - f) Performing a washing step in order to remove secondary antibody that is not bound to the exosome;
  - g) Adding a chemical composition to the said mixture of antibody, exosome containing fluid and secondary antibody, in order to indicate amount of said secondary antibody bound to the exosome.
2. The method of claim 1 wherein the plate-bound antibody detects epitopes selected from a group of proteins comprising MHC I, MHC II, HLA-G, CD9, CD81, CD82, or FasL.
3. The method of claim 1 wherein the washing step is performed with physiological saline constituted with an agent for maintaining protein shape and structure.
4. The method of claim 3 wherein said agent is albumin.

5. The method of claim 1 wherein the fluid assessed for exosomal content is selected from a group comprised of: blood, plasma, serum, urine, lacrimal secretions, sweat, or saliva.
6. The method of claim 1 wherein the secondary antibody recognizes a different epitope found on the exosome than the first, plate bound antibody, said different epitope being chosen from a group of proteins comprising MHC I, MHC II, HLA-G, CD9, CD81, CD82, or FasL.
7. A method of assessing the relative amount of cancer-induced immune suppression through quantification of FasL content on exosomes derived from biological fluids of cancer patients.
8. A method of assessing the relative amount of cancer-induced immune suppression through quantification of HLA-G content on exosomes derived from biological fluids of cancer patients.
9. A method of assessing the relative amount of cancer induced immune suppression through co-culture of cancer-derived exosomes with activated lymphocytes and detecting the amount of exosome-induced apoptosis, and exosome-induced suppressor cytokine production.
10. The method of claim 9 wherein T-cell apoptosis is detected by decreased mitochondrial potential, increased stain with Annexin-V or alterations in T cell morphology.
11. The method of claim 9 wherein suppressor cytokine production is assessed by relative levels of interleukin-4, 10, 13, or 20, transforming growth factor-beta, and other soluble agents that inhibit the proliferation of T cells.

12. A method of identifying immune-suppressive exosomes from the serum of a cancer patient comprising of:

- a) Extracting a sample of patient blood;
- b) Purifying plasma or serum from said patient blood;
- c) Staining with an antibody directed against a surface molecule found on exosomes;
- d) Staining with another antibody directed against an immune suppressive molecule found on the patient exosome;
- e) Analyzing said stained exosomes through flow cytometry for expression of both the exosome marker and the immune suppressive molecule.

13. The method of claim 12 wherein said exosome-staining antibody is directed against MHC I.

14. The method of claim 12 wherein said immune suppressive molecule on exosomes is Fas ligand.

15. The method of claim 12 wherein said immune suppressive molecule on exosomes is HLA-G.

16. A method for suppressing immune responses in an antigen-specific manner.

17. The method of claim 16, wherein immune responses refers to activation of T cells, said T cells comprising CD8<sup>+</sup>, CD4<sup>+</sup>, or NKT cells

18. The method of claim 16, wherein activation refers to proliferation, cytokine secretion, and direct or indirect cytotoxic and cytolytic activities.

19. The method of claim 16 wherein antigen-specific means the immune response directed against a certain chemical entity, or class of chemical entities.
20. The method of claim 16 wherein where the method of suppressing immune responses involves:
- a) The extraction of antigen presenting cells with suppressive properties from a mammal and artificially endowing a population of antigen presenting cells with suppressive properties through manipulation;
  - b) Purification of exosome vesicles released from the suppressive cell population;
  - c) Administration of the exosome vesicles to the mammal in which immune suppression is desired.
21. The method of claim 20 wherein antigen presenting cells can include B cells, macrophages, and dendritic cells.
22. The method of claim 20 wherein the exosome is prepared by extracted supernatant from antigen presenting cells and centrifuging at 300 g for 5 minutes, then 1200 g for 20 minutes, then 10000 g for 30 minutes, and 60 minutes at 110 000 g, subsequently to which the remaining pellet is resuspended in a physiological mixture and used as a source of exosomes.
23. The method of claim 20 wherein extraction of antigen presenting cells with suppressive properties is accomplished by purification with antibody based approaches.
24. The method of claim 20 wherein antibody-based approaches include purification though antibody-coated magnetic beads using antibodies for markers found on immature or T cell suppressing DC
25. The method of claim 24 wherein said antibody is selected from a group targeting DEC-205, CD123, PD-1 ligand, or TGF-b.

26. The method of claim 20 wherein the induction of suppressive properties in the antigen presenting cell is accomplished by treatment of the cells with cytokines selected from a group comprising: IL-4, IL-10, VEGF, TGF-b or IL-13.

27. The method of claim 20 wherein the induction of suppressive properties in the antigen presenting cell is accomplished by transfection of the antigen presenting cell with molecules which will suppress T cell activation such as IL-4, IL-10, VEGF, TGF-b or IL-13 or indoleamine-2,3-deoxygenase.

28. The method of claim 20 wherein the induction of suppressive properties in the antigen presenting cell is accomplished by transfection of the antigen presenting cell with molecules that inhibit T cell viability such as Fas ligand.

29. The method of claim 20 wherein the induction of suppressive properties in the antigen presenting cell is accomplished by transfection of the antigen presenting cell with agents that specifically inhibit expression of immune stimulatory molecules such as short interfering RNA targeting CD40, CD80, CD86, Rel-b, Rel-c, NF-kB p50, NF-kB p65, STAT4 or T-bet.

Application number / numéro de demande: 2453198

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Documents reçu avec cette demande ne pouvant être balayés  
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Figure 2.

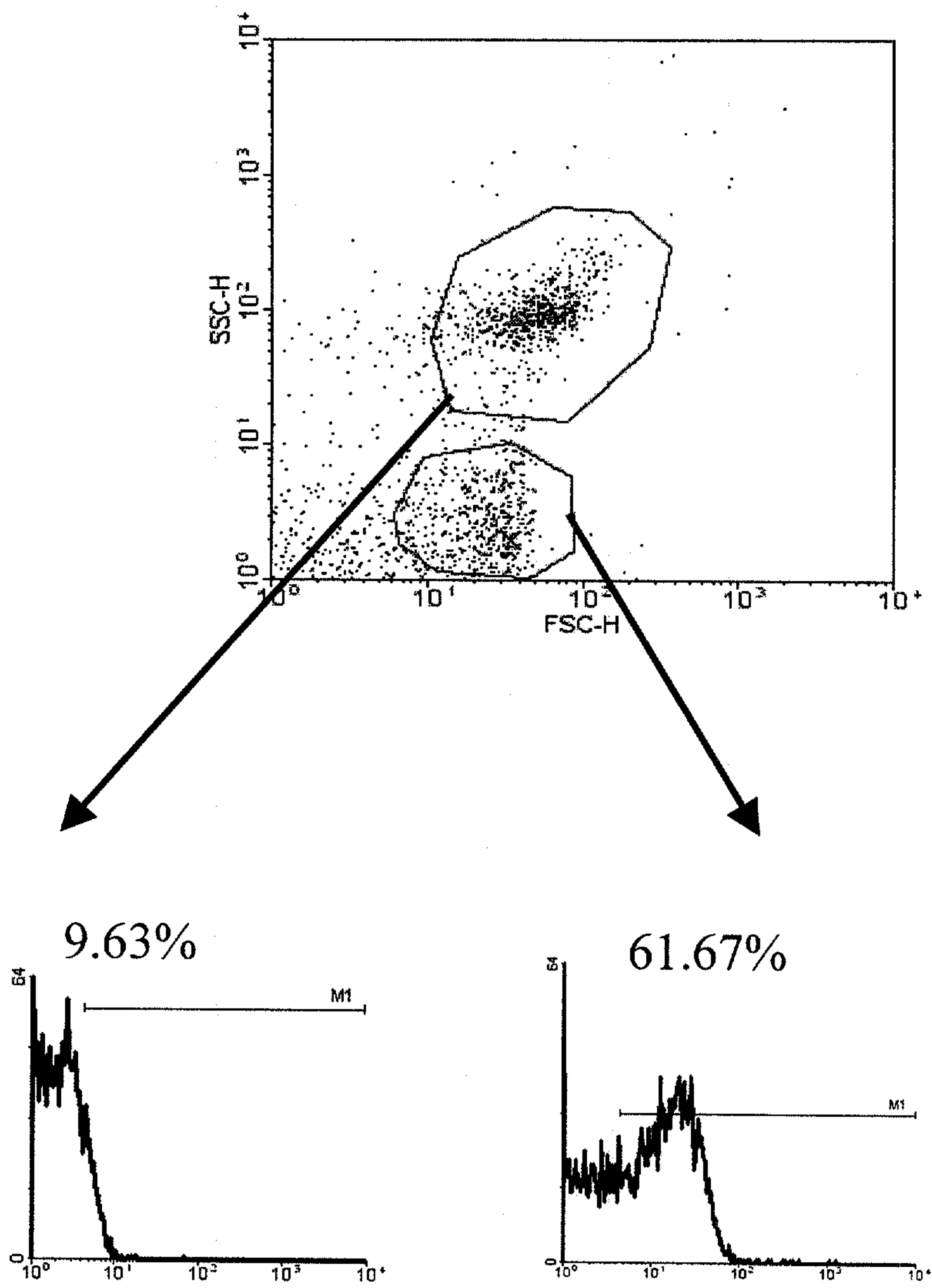


Figure 3

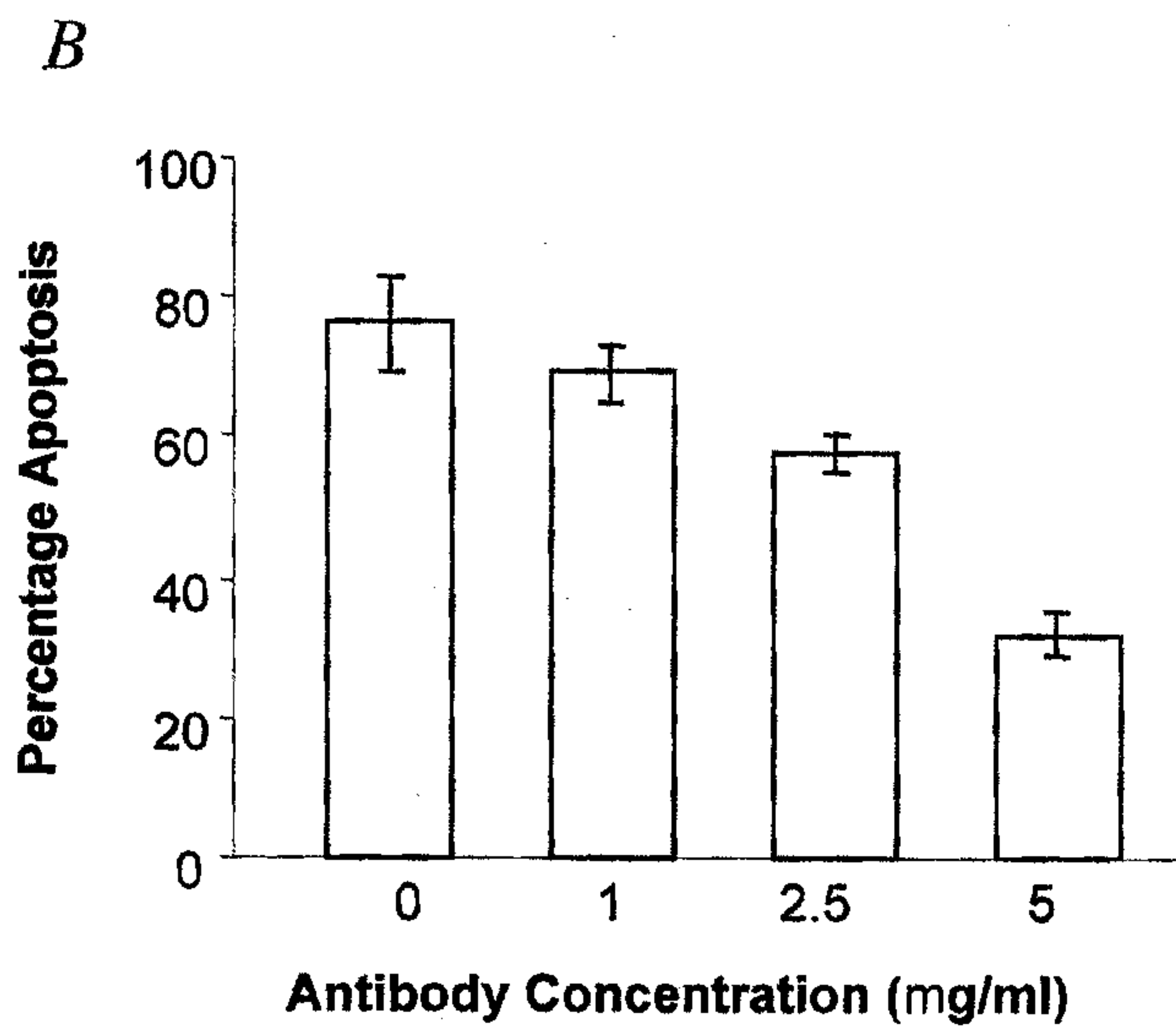
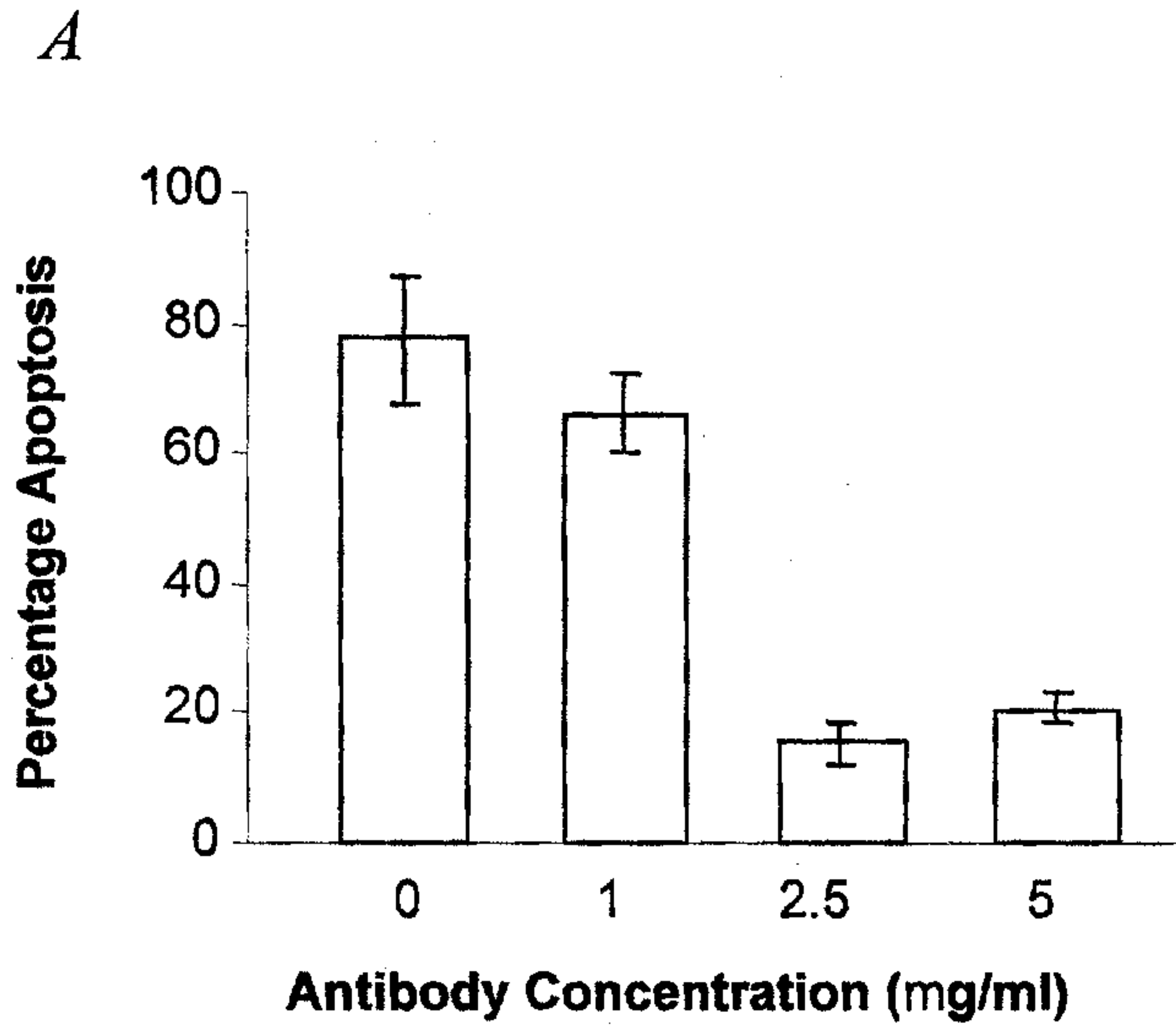


Figure 4

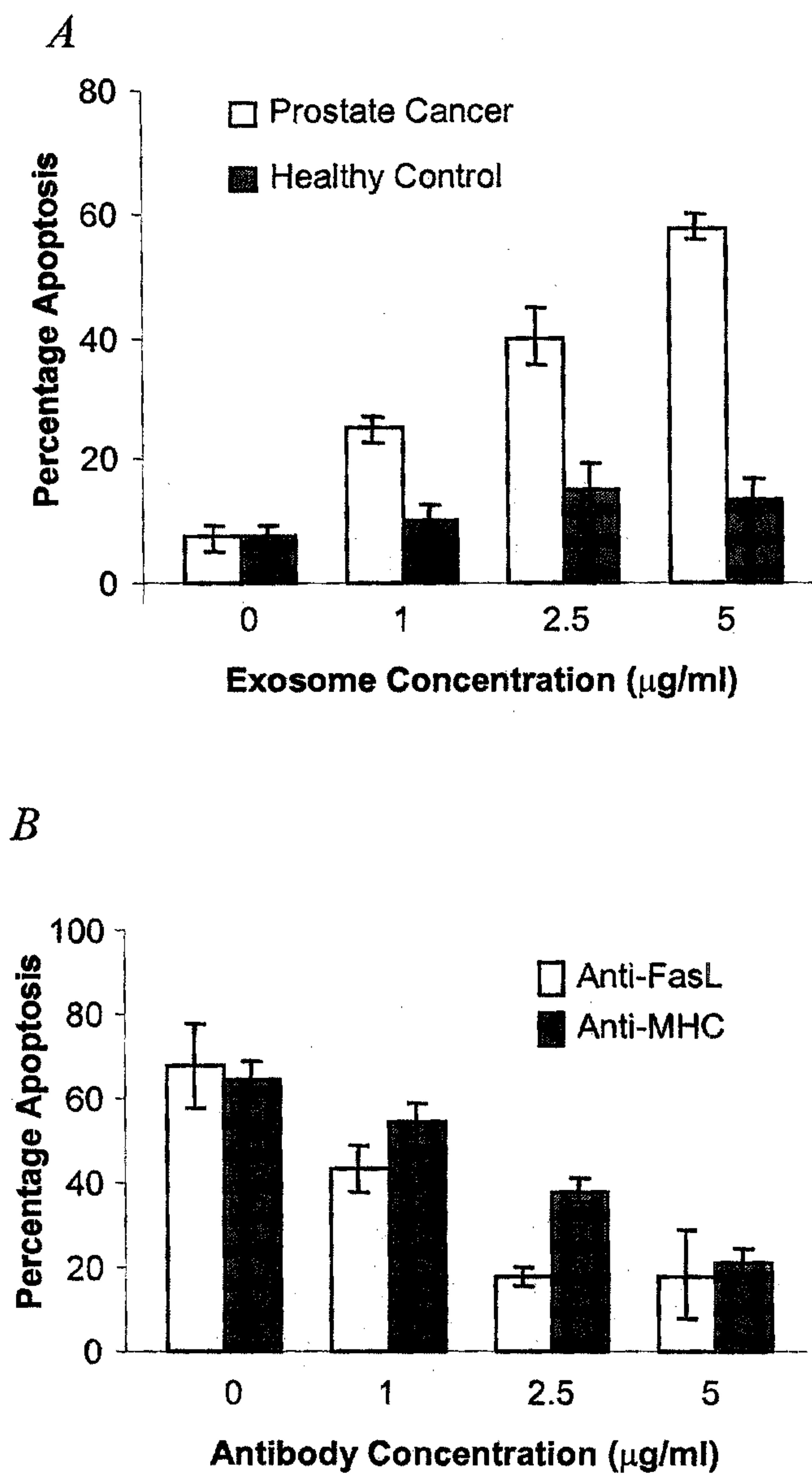


Figure 5.

