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Immunogenic potential of three transmissible venereal tumor cell lysates to prime canine-dendritic cells for cancer immunotherapy

Alex Jesús Hernández-Granados, Moisés Armides Franco-Molina*,[1], Erika Evangelina Coronado-Cerda, Pablo Zapata-Benavides, Edgar Mendoza Gamboa, Yareellys Ramos-Zayas, Silvia Elena Santana-Krímskaya, Cristina Rodríguez-Padilla

Laboratorio de Inmunologia y Virologia, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León (UANL), PO BOX 46, "F" Zip Code 66455, San Nicolás de los Garza, N. L., Mexico

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ABSTRACT

Whole tumor cell lysates consist of a mixture of tumor antigens and danger associated molecular patterns (DAMPs) that can be used for dendritic cell maturation and consequently for the activation of a polyclonal T cell-specific tumor response. We evaluated the *in vitro* efficacy of three different preparations of canine transmissible venereal tumor (TVT) cell lysates: hypochlorous acid-whole tumor cell lysates (HOCl-L), heat shock-whole tumor cell lysates (HS-L), and freeze-thaw cycles-whole tumor cell lysates (FT-L) for the maturation of canine-derived dendritic cells. Our results showed calreticulin, HSP70, and HSP90 release in the three tumor lysates preparations (HOCl-L, HS-L, and FT-L); however, HMGB1 was detected only in HOCl-L and FT-L. Additionally, the uptake by HOCl-L pulsed dendritic cell (DC) increased compared to HS-L and FT-L pulsed DC; and dendritic cell maturation was confirmed by the appropriate cell surface markers (CD11c, CD80, CD83, and MHCII). Furthermore, dendritic cells pulsed with HOCl-L, HS-L or FT-L were cultured with canine lymphocytes. There was an increase of Th1-type cytokines (IL-12, TNF-α, and IFN-γ), in all the tumor cell lysates co-cultures, this correlates with T lymphocyte activation and cytotoxic response. Our data confirm that TVT cell lysates can induce functional canine-DC and that HOCl-L is the most effective one. This preparation of TVT cell lysates with HOCl is an attractive approach that allows the recognition of neoantigens as potential tumor targets and DC priming and therefore could be used for cancer immunotherapy against TVT.

1. Introduction

Canine transmissible venereal tumor (TVT) is a transmissible tumor distributed around the world affecting mostly dogs and has the capacity of being transmitted as an allograft (Murgia et al., 2006). It has been used to evaluate cancer therapies, including some related to cancer immunotherapy (Pai et al., 2011). Experimental evidence has demonstrated the active involvement of the immune system in the control and elimination of tumors by recognition and uptake of tumor antigens by dendritic cells that can prime a T cell-specific antitumor response (Rainone et al., 2016). For this reason, DC-based vaccines have been used to treat different types of cancer including renal cell carcinoma, carcinomas of the breast, ovary, prostate as well as melanoma and glioma, resulting in clinical benefits for some patients (Markov et al., 2017).

Unfortunately, many patients do not respond to DC-based vaccines. One of the probable reasons is the lack of tumor neo-antigens that could elicit a strong cytotoxic T-lymphocyte (CTL) response (Mantia-Smaldone & Chu, 2013). Another reason is the high mutation rate of some tumors, that results in loss or change of single or multiple epitopes since most DC-based vaccines are generated against one or few antigenic targets (HER2-Neu, Wt1, gp100 or its combinations) (Abe et al., 2013).

Whole tumor cell lysates are a mixture of damage-associated molecular patterns (DAMPs) and tumor antigens. DAMPs can interact with dendritic cell receptors (CD91, Toll-like receptor 4, LRP1, NOD1 and NOD2), promoting their maturation and increasing antitumor activity (Dalod et al., 2014). Thus, whole tumor cell lysates can be used to induce an immunogenic response from dendritic cells against multiple tumor antigens for the activation of a polyclonal tumor-specific T cell response (Rainone et al., 2016).

Three of the most used cell lysate preparation methods are: 1) heat

* Corresponding author.
E-mail addresses: moyfranco@gmail.com, moises.francoml@uanl.edu.mx (M.A. Franco-Molina).
[1] Contributed equally.

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shock that induces release of heat shock proteins 70 and 90 (HSP70 and HSP90, respectively) and non-histone chromatin binding protein high-mobility group Box 1 (HMGB1) (González et al., 2014); 2) freezing-thawing cycles that promote cell-surface exposure of calreticulin (CRT) (Rainone et al., 2016); and hypochlorous acid (HOCl) treatment that induces the oxidative modification of the proteins and release HMGB1 (Grant et al., 2017).

The aim of the present study was to compare the efficiency of TVT derived HOCl cell lysate (HOCl-L), heat shock cell lysate (HS-L) and freezing-thawing cycles cell lysate (FT-L) to induce canine dendritic cells maturation *ex vivo*. The results obtained in the present study could be used for the establishment of an alternative approach for the treatment and prevention of TVT.

2. Materials and methods

2.1. Transmissible venereal tumor

The tumor sample was obtained with the informed consent of the owner of a mongrel female dog with TVT. The dog was two-years old, weighted 10 kg, and presented a 5 mm^3 tumor mass inside the vulva. The tumor was surgically removed from the vulva under general anesthesia (xylazine hydrochloride (1.1 mg/kg PISA, México) and tiletamine hydrochloride/zolazepam hydrochloride (7.5 mg/kg Virbac, México). After anesthesia recovery, ketorolac tromethamine (0.5 mg/kg PISA, México) every 8 h during three days and ciprofloxacin (10 mg/kg PISA, México) every 12 h during seven days, were administered by oral route to prevent pain and infection. The tumor sample was donated to us by the "Small species clinic" of the Veterinary Sciences Faculty of the Autonomous University of Nuevo León were the procedure was performed.

For primary TVT cell culture, the tumor was disrupted by mechanical disaggregation and enzymatic digestion with trypsin (0.25% Trypsin-EDTA, Thermo Fisher Scientific Inc.) for 5 min at 37 °C. The resulting suspension was filtered through a 30 μM millipore membrane, and eight milliliters were taken and mixed with 4 mL of Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) and then centrifuged at 820 ×*g* for 25 min at 4 °C for separation and isolation of TVT cells and lymphocytes by density gradient.

After the purification and recovery procedures, cell viability (> 95%) was determined by trypan blue dye exclusion method.

Thereafter TVT cells were cultured in DMEM/F-12 medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island NY, USA) and 100 U/mL penicillin/streptomycin (Sigma, St. Louis, MO, USA), and incubated at 37 °C in a 5% CO_2 atmosphere for further use.

The care and use of experimental animals complied with Mexican federal animal welfare laws (NOM-062-ZOO-1999). All procedures were approved by the institutional ethics committee for research-animals welfare (CEIBA) of the Autonomous University of Nuevo Léon. The approved protocol number is CEIBA-2015-038.

2.2. Whole tumor cell lysate preparations

2.2.1. HOCl-L preparation

First, TVT cells (1×10^5 cells per mL) were incubated with a solution of HOCl (400 μM) in sterile 1× PBS (Sigma-aldrich) for 1 h at 37 °C and centrifuged at 400 ×*g* for 20 min. Followed by two centrifugation steps with sterile 1× PBS to remove excess of HOCl. Finally, both pellet and supernatant were stored at −80 °C until use.

2.2.2. HS-L preparation

1×10^7 TVT cells in 1 mL of PBS 1× were heated at 60 °C for 1 h in a heated circulating water bath (Heated Circulating Bath, Model PP07R-20 PolyScience, USA). Then the lysate was centrifuged at 400 ×*g* for 20 min at 18 °C. The supernatant was removed and stored at −80 °C and the pellet was washed twice (by centrifugation at 400 ×*g* for 20 min at 18 °C) using sterile 1× PBS and stored at −80 °C until use.

2.2.3. FT-L preparation

1×10^7 TVT cells in 1 mL of were frozen in liquid nitrogen at −196 °C for 4 cycles of 1 h each, with 15 min intervals at room temperature. Then, the lysate was suspended in sterile 1× PBS and centrifuged at 820 ×*g* for 25 min at 18 °C. Finally, the supernatant and pellet were collected and stored at −80 °C until use.

For all cell lysates preparations, cell death was corroborated by Annexin PE/7-AAD kit (BD Biosciences, San Jose, CA, USA) using the Accuri C6 flow cytometer (BD Biosciences, San Diego, CA, USA). Protein concentration was quantified by the Lowry protein assay (Bio-Rad Laboratories, Inc).

2.3. DAMPs determination

Pellets and supernatants of the three whole tumor cell lysates were analyzed by Western blotting for DAMPs determination. Briefly, the pellets were homogenized in the lysis buffer Tris-Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Equal amounts of soluble proteins (50 μg) were resolved by 12% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane.

Unspecific binding sites were blocked by incubating the membrane at room temperature for 1 h in blocking buffer (Tris-buffered saline with 0.5% Tween 20) supplemented with 5% non-fat milk temperature. Followed by overnight incubation at 4 °C with primary antibodies.

Calreticulin antibody (N-19) goat polyclonal IgG_1 (sc-6468), dilution 1:1000, HSP70 antibody (C92F3A5) mouse monoclonal IgG_1 (sc-66,048), HSP90 antibody (H-114) rabbit polyclonal IgG_1 (sc-6564) dilution 1:1000, and HMGB1 antibody (T-16) goat polyclonal IgG_1 (sc-2523), dilution 1:500. All antibodies were purchased from Santa Cruz Biotech (California, USA).

After incubation with primary antibodies, the membrane was washed three times (for 10 min each) with blocking buffer and incubated for 1 h with the corresponding secondary antibody (IgG_1 anti-mouse, IgG_1 anti-goat, or IgG_1 anti-rabbit) all conjugated with horseradish peroxidase; and used at a 1:5000 dilution.

Blots were developed with the RPN2109 western blotting detection reagent (GE Healthcare, Little Chalfont, UK) and revealed on high-performance chemiluminescence film (GE Healthcare, Little Chalfont, UK). β-actin antibody (sc-69,879) mouse monoclonal IgG1 (Santa Cruz Biotech, California, USA) was used as loading control for pellets, and Ponceau S solution (Sigma, St Lewis, MO) was used as loading control for supernatants. Non-immunogenic lysis with Triton 100× of TVT cells was used as negative control.

2.4. Generation of canine monocyte-derived immature dendritic cells

The blood (15–20 mL) was collected from female healthy dogs (3 years old) in tubes containing EDTA (Becton, Dickinson) and donated to us by the "Small species clinic" of the Veterinary Sciences Faculty of the Autonomous University of Nuevo León, with informed consent of the dog-owners. Blood was diluted in sterile 1× PBS, in a 1:1 dilution, and mixed with Ficoll-Paque PLUS (density 1.077 g/mL) (GE Healthcare Life Sciences). The blood-Ficoll-Paque PLUS mixture was centrifuged at 400 *g* for 30 min at 18 °C for density gradient separation of peripheral blood mononuclear cells (PBMC). After centrifugation, the PBMC layer was collected and treated with erythrocyte lysis buffer (150 mM NH_4Cl, 8 mM $KHCO_3$, 2 mM EDTA; pH 7), followed by centrifugation at 1200 rpm for 10 min with sterile 1× PBS, twice.

2×10^6 cells per well were seeded in a 12-well cell culture plate in 1 mL of RPMI 1640 (GIBCO, Grand Island, NY, USA) culture medium, supplemented with 10% FBS, and incubated for 2–3 h in an atmosphere at 37 °C at 5% CO_2. After the incubation period, the supernatant was