Detection of Tumor Cell Surface-reactive Antibodies

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[Abstract] Vaccine-based immunotherapy is being used to treat dogs with primary brain tumors. The vaccines are composed of a lysate of autologous tumor cells, which stimulate an immune response producing tumor specific antibodies that are capable of inducing antibody-dependent cell-mediated cytotoxicity to allogeneic, as well as autologous, tumor cells. This protocol will describe the tumor cell serum antibody-binding assay to measure the tumor-reactive IgG antibody response. Key features of this assay are that it is performed with sera collected from the canine patient prior to and following vaccination as the source of antibodies and canine brain tumor cells used as the target cells.

Materials and Reagents

1. Tumor cells
   Note: Tumor sample is harvested from pet dogs during brain surgery.
2. BD Matrigel™ (BD Biosciences, catalog number: 354230)
3. DMEM/F12 Medium with L-glutamine (Life Technologies, Gibco®, catalog number: 11320-033)
4. 50x B-27 supplement without vitamin A (Life Technologies, Gibco®, catalog number: 12587-010)
5. 100x N2 supplement (Life Technologies, Gibco®, catalog number: 17502-048)
6. Recombinant human Epidermal Growth Factor (Pepro Tech, catalog number: AF-100-15)
7. Recombinant human fibroblast growth factor-basic (Pepro Tech, catalog number: 100-18B)
8. 50x Penicillin-streptomycin solution (Mediatech, Cellgro®, catalog number: 30-001-CI)
9. Dispase (BD Bioscience, catalog number: 354235)
10. 1x Phosphate-Buffered Saline (Mediatech, Cellgro®, catalog number: 21-040-CV)
11. MACS BSA stock solution (10% BSA) (Miltenyi Biotec, catalog number: 130-091-376)
12. 1x TrypLE™ Express (Life Technologies, Gibco®, catalog number: 12605-010)
13. 0.4% Trypan Blue Solution (Life Technologies, Gibco®, catalog number: 15250-061)

14. Goat anti-canine IgG (H&L) f(ab')2 - fluorescein isothiocyanate (American Qualex, catalog number: F145FN)
15. Neural Stem Cell (NSC) medium (see Recipes)
16. Matrigel Coating solution (see Recipes)
17. rh-EGF stock solution (see Recipes)
18. rh-FGF basic stock solution (see Recipes)

Equipment

1. 10 mm culture plates (Sigma-Aldrich, Corning® Costar®, catalog number: CLS430165)
2. Scalpel blade
3. 100 µm filter (Corning Incorporated, catalog number: 352360)
4. BD FACS tubes (12 x75 mm) (BD Biosciences, Falcon®, catalog number: 352235)
5. 15 ml tubes (BD Biosciences, Falcon®, catalog number: 352096)
6. Culture dish (100 x 20 mm) (Sarstedt AG, catalog number: 83.1802)
7. 1.5 ml Eppendorf microcentrifuge tubes (Sigma-Aldrich, catalog number: T9661-500EA)
8. Neubauer hemocytometer (Sigma-Aldrich, catalog number: Z359629)
9. Incubator (5% CO_2, 5% O_2, 37 °C) (BioSpherix)
10. Shaker table (Eppendorf, New Brunswick Excella® E25 incubator shaker)
11. Centrifuge (Thermo Fisher Scientific, model: ST40R)
12. Microcentrifuge (Thermo Fisher Scientific, model: MicroCL 17)
13. BD FACSCanto three-laser flow cytometer (BD Biosciences)

Procedure

A. Culture of tumor cells

1. Coat the 10 mm culture plates with 3 ml of matrigel solution, gently rock to completely cover the plate and set aside for 30 min for gel polymerization.
2. Mince canine tumor samples (2-5 cc depending on the size of the tumor) with a scalpel blade into 1 mm pieces, digest with 10 ml of TrypLE Express for 15 min at 37 °C on a shaker table, pass the suspension through a 100 µm filter, wash with 20 ml NSC medium centrifuge at 500 *x g* for 5 min, and resuspend with 1 ml of NSC medium.
3. Add 1 ml of canine tumor cell suspension and 6 ml of NSC medium to the plate and place in a 37 °C humidified incubator with 5% O_2 and 5% CO_2.
4. Supplement the NSC media plates with 10 µl each of rh-EGF stock solution and rh-FGF basic stock solution every Monday, Wednesday and Friday, and the media should be

changed at least once a week or if the medium indicator changes to a orange-yellow color due to rapid cell proliferation.

B. Harvest of tumor cells

1. When the tumor cells have grown to 100% confluency, the cells should be harvested.
2. Aspirate all of the remaining NSC medium, then add 3 ml of medium back to the plate.
3. Add 500 µl of Dispase to the plate and gently rock to spread over the cells to solubilize the matrigel coating and detach the cells. Prepare 1 ml aliquots of the Dispase to prevent excessive freeze-thaw cycles.
4. Incubate the plate at 37 °C for 20-30 min or until all cells are detached.
5. Decant the tumor cell suspension into a 15-ml conical tube and add 1x PBS to a final volume of 10 ml.
6. Centrifuge the cells at 500 *x g* for 5 min, decant supernatant, resuspend in 1x PBS (1 ml).
7. The cells are counted by placing 10 µl of cell suspension into an Eppendorf microcentrifuge tube and adding 10 µl of Trypan Blue.
8. Count cells using a Neubauer hemocytometer.

C. Serum antibody: tumor cell binding experiment

1. Place 10^5 tumor cells into a FACS tube and add PBS to a total volume of 200 µl (produce triplicate samples).
2. Add 2 µl of serum (pre or post –treatment sera from the canine patient) into the cell suspension (1:100 dilution of serum to cells).
3. Incubate at 4 °C for 30 min.
4. After incubation, dilute cell suspension with 2 ml of FACS buffer, vortex, and centrifuge at 1,000 rpm for 5 min.
5. Decant the supernatant, repeat the wash with FACS buffer and decant.
6. Add 1 µl of the conjugated 2° antibody [goat anti-canine IgG (H&L) f(ab')2-fluorescein isothiocyanate] and incubate at 4 °C for 30 min.
7. After incubation, add 2 ml of FACS buffer, vortex, and centrifuge at 1,000 rpm for 5 min, decant, repeat the wash and decant.
8. Resuspend the labeled cells in 200 µl FACS buffer and analyze using flow cytometer.

D. Observations

Use as controls tubes:

1. 2° Ab only - tumor cells + conjugated 2° antibody (no serum)
2. Unstained - tumor cells + serum (no 2° conjugated Ab)
3. Normal dog serum - tumor cells + serum from normal dog + 2° conjugated Ab

Recipes

1. Neural Stem Cell medium
   500 ml DMEM/F12
   100x 5 ml N2
   50x 10 ml B27
   5 ml Penicillin-streptomycin solution
2. Matrigel Coating solution
   2.7 ml Neural Stem Cell medium
   0.3 ml BD Matrigel™ (0.3 ml aliquots in Eppendorf tubes stored at -20 °C)
3. rh-EGF stock solution (20 µg/ml)
   20 µg recombinant human EGF
   1 ml 0.1% BSA in PBS stock solution
   Stored at -20 °C
4. rh-FGF basic stock solution (20 µg/ml)
   20 µg recombinant human FGF basic
   1 ml 0.1% BSA stock solution
   Stored at -20 °C

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