Amplification of oncolytic vaccinia virus widespread tumor cell killing by sunitinib through multiple mechanisms

Minah Kim, Maximilian Nitschké, Barbara Sennino, Patrizia Murer, Brian J. Schriver, Alexander Bell, Aishwarya Subramanian, Corry E. McDonald, Jiahu Wang, Howard Cha, Marie-Claude Bourgeois-Daigneault, David H. Kirn, John C. Bell, Naomi De Silva, Caroline J. Breitbach and Donald M. McDonald

Supplemental Methods

Tissue preparation, immunohistochemistry, and imaging

Cryostat sections 80-μm in thickness were stained with combinations of two or three primary antibodies (1): (i) vaccinia virus: rabbit anti-vaccinia (1:500, Quartett, V-Biognostics); (ii) yellow fluorescent protein: goat anti-YFP (1:2000, Abcam); (iii) endothelial cells: rat anti-CD31 (clone MEC 13.3; 1:500; BD Pharmingen), hamster anti-CD31 (clone 2H8; 1:500; Pierce), or goat anti-VEGFR-2 (1:500, R&D AF644); (iv) fibrinogen/fibrin: rabbit polyclonal anti-fibrinogen/fibrin (1:2000; Dako); (v) erythrocytes: rat anti-TER119 (1:250, BD Biosciences); (vi) apoptotic cells: rabbit anti-activated caspase-3 (1:1000, R&D Systems); (vii) RIP-Tag2 tumor cells: guinea pig anti-swine insulin (1:100, Dako), or rabbit anti-SV40 T-antigen (1:500, Santa Cruz Biotechnology); (viii) pancreatic acinar cells: rabbit anti-amylase (1:500, Sigma-Aldrich); (ix) immune cells: rat anti-CD45 antibody (1:500, BD Pharmingen) or rat anti-CD8 (1:500, clone YTS169.4, UCSF Monoclonal Antibody Core).

Secondary antibodies were FITC-, Cy3- or Cy5-labeled donkey anti-goat, donkey/goat anti-rabbit, donkey/goat anti-rat, or donkey/goat anti-guinea pig IgG antibody (Jackson ImmunoResearch; all diluted 1:400). Cell nuclei were stained with Vectashield mounting medium containing DAPI (Vector Laboratories).

Measurements of tumor sections

Fractional area (area density) of vaccinia, CD31, activated caspase-3, extravasated microspheres, fibrin, or erythrocytes in digital fluorescence microscopic images of 80-µm sections of tumor was measured as the amount of immunoreactivity above a predetermined fluorescence intensity threshold (1). Tumor invasiveness was measured as the abundance of pancreatic acinar cells (amylase) surrounded by tumor cells (SV40 T-antigen) and expressed as a fraction of tumor area (2).

RIP-Tag2 tumor burden was calculated as the sum of areas of tumors visible in a montage of digital images of a pancreas section from each mouse (5x objective, 1x Optovar, section dimensions 1,920 by 2,560 µm) (2). Mean tumor area was also calculated (Supplemental Figure 7H).

Liver micrometastases were identified in images of 80-µm-thick sections of liver of RIP-Tag2 mice at age 17 weeks by the presence of two or more adjacent SV40 T-antigen-positive cells (3). Incidence was expressed as proportion of mice with liver micrometastases. Number of micrometastases was expressed per 10 mm^2 of liver section (3).

Flow cytometry

RIP-Tag2 mice were anesthetized, blood was removed by vascular perfusion of PBS for 2 minutes, and tumors were removed, weighed and digested in collagenase II and IV solution (625U/ml, Gibco) with DNase (60U/ml, Roche) for 30 minutes at 37°C, and erythrocytes were lysed. Total cells per tumor were counted with a Muse Cell Analyzer (Millipore Sigma, Billerica, MA). Cells were stained with anti-CD45-APC-Cy7, anti-CD3e-PE-Cy7 or anti-CD3e-Alexa 647,

anti-CD4-Alexa 647, anti-CD8-PerCP-Cy5.5 or anti-CD8-Alexa 700 (all BioLegend). Viable cells were identified by live-dead staining (Zombie Yellow, BioLegend). Some preparations were then permeabilized (Foxp3 staining buffer set, eBioscience) and stained with anti-Foxp3-eFluor 450 (eBioscience) for regulatory T-cells, with anti-granzyme B-FITC (Biolegend) for $CD8^+$ T-cell activation, or with non-specific control immunoglobulin of the same isotype (mouse IgG1, both BioLegend) as a control. Cells were sorted by FACS (BD Fortessa) and analyzed with FlowJo software (8.8.6 and X). All staining and washing steps were performed in the presence of GolgiPlug (1:1000, BD Biosciences). Cells per mg tumor were calculated from total acquired cells (gated by FSC/SSC, excluding debris).

1. Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol 2004;165:35-52.
2. Sennino B, Ishiguro-Oonuma T, Wei Y, Naylor RM, Williamson CW, Bhagwandin V, Tabruyn SP, You WK, Chapman HA, Christensen JG, Aftab DT, McDonald DM. Suppression of tumor invasion and metastasis by concurrent inhibition of c-Met and VEGF signaling in pancreatic neuroendocrine tumors. Cancer Discov 2012;2:270-87.
3. Moen I, Gebre M, Alonso-Camino V, Chen D, Epstein D, McDonald DM. Anti-metastatic action of FAK inhibitor OXA-11 in combination with VEGFR-2 signaling blockade in pancreatic neuroendocrine tumors. Clin Exp Metastasis 2015;32:799-817.
4. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol 2000;156:1363-80.