Bevacizumab-induced tumor calcifications can be elicited in glioblastoma microspheroid culture and represent massive calcium accumulation death (MCAD) of tumor endothelial cells.

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Bähr et al reported that 22 of 36 glioblastoma patients treated with bevacizumab showed tumor calcifications on 8 week post therapy follow up with MRI. Early tumor calcification strongly predicted for response, time to progression, and overall survival (Neuro-Oncology, 13:1020, 2011, doi: 10.1093/neuonc/nor099). The authors didn't understand the mechanism, but speculated that it was vascular in nature. At the 13th International Anti-Angiogenic Symposium (2011), we presented our discovery of the phenomenon of massive calcium accumulation death, wherein MCAD occurred in endothelial cells (tumor, circulating, and HUVEC), in response to VEGF depletion by bevacizumab and other putative anti-angiogenic agents, but not in response to non-specific cytotoxins http://precedings.nature.com/documents/6647/version/1 , J Intern Med, In Press. In subsequent work, we have documented marked MCAD to occur in primary microcluster cultures from 6 fresh human glioblastoma biopsies, following 96 hours of VEGF depletion in vitro by bevacizumab (see examples below). The presence and degree of MCAD is strikingly dependent on the type of serum in the culture medium (RPMI-1640 + 25% serum) -- typically most striking in (very low VEGF) fetal calf serum, but inhibited (often) or enhanced (rarely) by 25% human serum from different patients or normal donors containing variable quantities of VEGF. There was not a linear relationship between VEGF concentration and MCAD inhibition (or enhancement), suggesting that other pro-angiogenic (or anti-angiogenic) serum factors may play a role. In epithelial metastatic tumors, circulating peripheral blood endothelial cells may be easily tested, using our methods, and the serum inhibition (or, rarely, enhancement) is faithfully reproduced on circulating endothelial cells, in comparison with the tumor cluster-associated endothelial cells. We propose MCAD as the mechanism of glioblastoma calcification following bevacizumab and further propose that testing tumor microclusters and/or circulating endothelial cells, in the presence of autologous serum, could be a useful predictive biomarker and research tool.

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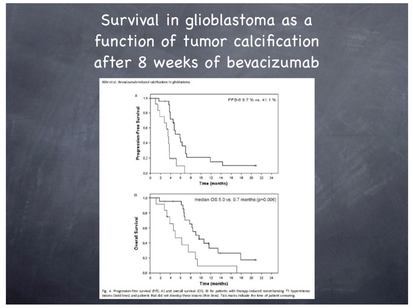
We have discovered that human endothelial cells undergo two forms of cell death.

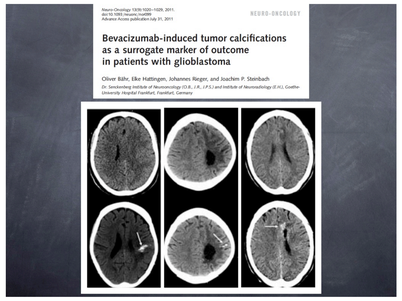
1. **A non-specific form of cell death, similar to that of other normal and neoplastic cells**
2. **A unique form of cell death, seen only in endothelial cells, associated with massive accumulation of calcium. We call this massive calcium accumulation death, or MCAD.**
3. **MCAD may be identified by cytochemical staining with:**

a. Fast Green/Hematoxylin

b. Fast Green/Wright-Giemsa, or

c. Alizarin red S (most adevantageous)





Bibliography relevant to AngioRx/Microvascular Viability assay (MVVA)

1. Weisenthal, L. M, Patel,N., Rueff-Weisenthal, C. (2008). Cell culture detection of microvascular cell death in clinical specimens of human neoplasms and peripheral blood. J Intern Med 264(3): 275-287.

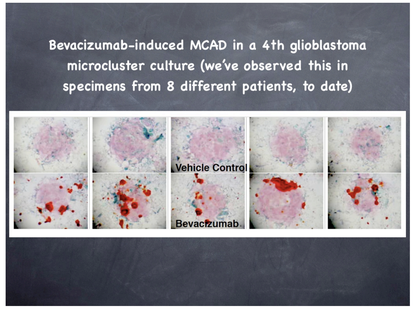
2. Weisenthal, L., Lee,DJ, and Patel,N. (2008). Antivascular activity of lapatinib and bevacizumab in primary microcluster cultures of breast cancer and other human neoplasms. ASCO 2008 Breast Cancer Symposium. Washington, D.C.: Abstract # 166. Slide presentation at: http://tinyurl.com/weisenthal-breast-lapatinib

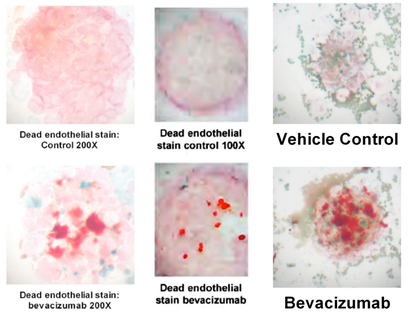
3. Weisenthal, L. M. (2010). Antitumor and anti-microvascular effects of sorafenib in fresh human tumor culture in comparison with other putative tyrosine kinase inhibitors. J Clin Oncol 28, 2010 (suppl; abstr e13617)

4. Weisenthal, L., H. Liu, Rueff-Weisenthal, C. (2010). Death of human tumor endothelial cells in vitro through a probable calcium-associated mechanism induced by bevacizumab and detected via a novel method. Nature Precedings 28 May 2010. from http://hdl.handle.net/10101/npre.2010.4499.1.

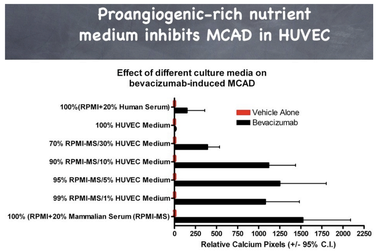
5. Weisenthal, L, Williamson, S, Ryan, K, Brunshwiler, C, and Rueff-Weisenthal, C. Massive calcium uptake in human endothelial cells, J Intern Med, In Press

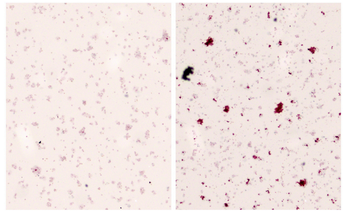
6. www.google.com/patents/US20070190648
7. www.google.com/patents/US20110171214
8. www.google.com/patents/US20110275115

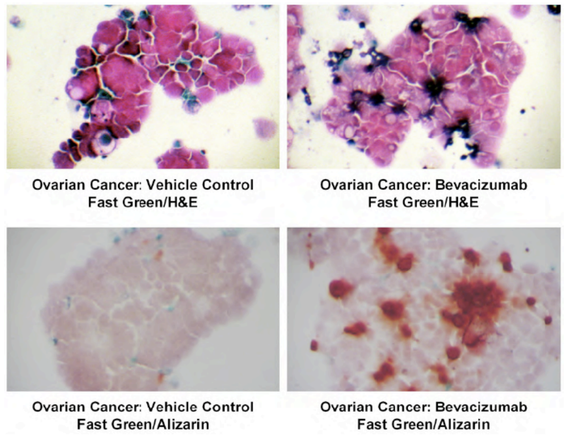




Massive calcium accumulation death induced by bevacizumab in microcluster endothelial cells in 3 fresh human primary glioblastoma cultures





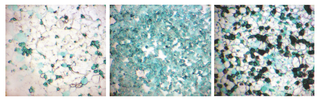


In the presence of Fast Green/ Hematoxylin, calcium is identified by a blue-black "lake" staining

In the presence of Alizarin red S, calcium is identified by an orange-red "lake" staining

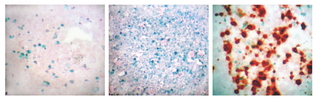
Human Umbilical Vein Endothelial Cells ImageJ Color (RGB) image quantification

Fast Green



	Area	Area	Area
	480,482	459,222	470,387
Living cells – clear or light pink	387,329	53,720	230,820
Non-specifically dead cells – light green	8,660	393,636	139,976
MCAD cells – dark green/black	6,493	11,866	99,591

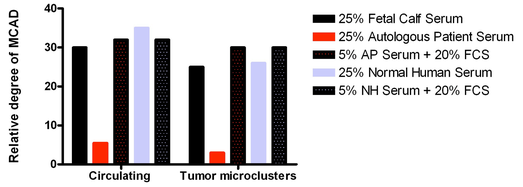
Fast Green/Alizarin



	Area	Area	Area
	484,959	489,231	459,988
Living cells – clear or light pink	460,509	92,264	222,712
Non-specifically dead cells – light green	24,246	396,945	110,468
MCAD cells – dark orange/red	204	22	126,808

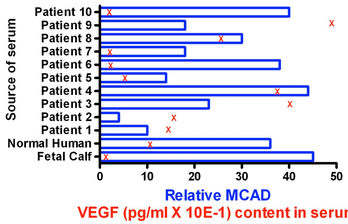
Total area per frame: 494,592

Bevacizumab-induced MCAD inhibition by different sera



Source of endothelial cells
(Both derived from the same patient with metastatic thymic carcinoma)

Effect of different sera on MCAD induced by bevacizumab in glioblastoma microclusters



Conclusions: Bevacizumab induces massive calcium accumulation death in endothelial cells in situ in tumor microcluster culture and in circulating endothelial cells. Bevacizumab resistance is a function of both intrinsic susceptibility to VEGF depletion and to the apparent presence of angiogenic modulators (other than VEGF) circulating in human serum.