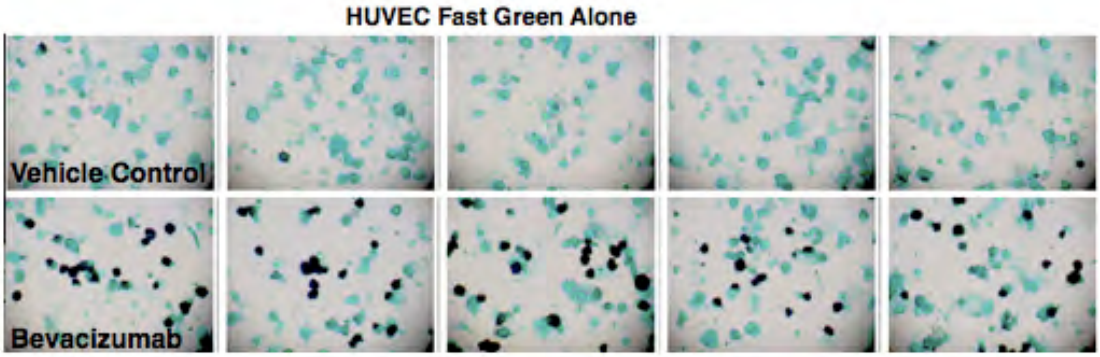
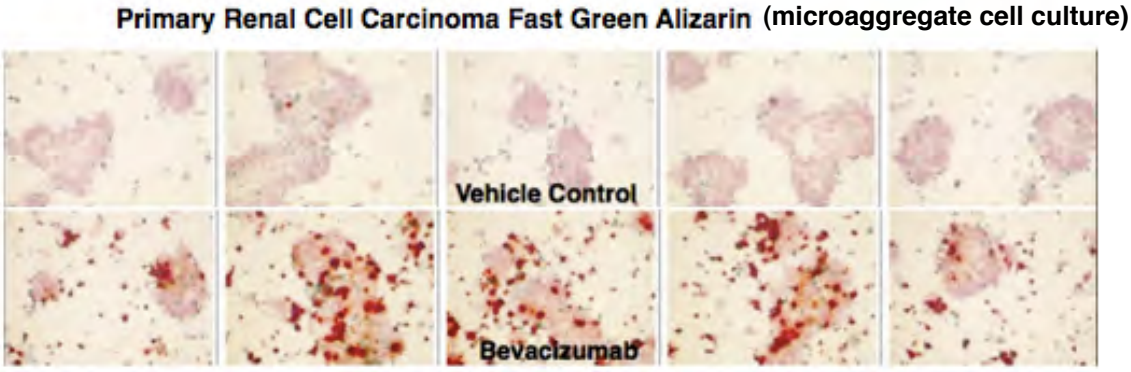
Endothelial calcium accumulation death (MCAD): mechanism, target, and predictive biomarker for anti-angiogenic (AA) therapy. L. Weisenthal, S. Williamson, H. Liu, K. Ryan, C. Sanchez, and C. Reuff-Weisenthal. Weisenthal Cancer Group; Huntington Beach, CA http://medpedia.com/users/110

We cultured human umbilical vein endothelial cells with bevacizumab, tyrosine kinase inhibitors known to be AA, and traditional cytotoxic drugs. The images below show that, in the presence of physiological saline and non-favorable culture conditions, the vast majority of the endothelial cells undergo a non-specific type of cell death (NSCD), not associated with calcium uptake, but with loss of cell membrane integrity, allowing uptake of the Fast Green dye, staining these dead dells a pale blue green. In the presence of known AA agents (e.g. bevacizumab, some TK inhibitors) a large percentage of the endothelial cells undergo death associated with massive calcium accumulation (MCAD), with these cells staining hyperchromatic, refractile, blue-black, precisely as reported in http://www.ncbi.nlm.nih.gov/pubmed/18793333 and http://meeting.ascopubs.org/cgi/content/abstract/28/15_suppl/e13617 and http://tinyurl.com/weisenthal-breast-lapatinib. MCAD is strikingly demonstrated by Fast Green/Alizarin staining as reported in http://precedings.nature.com/documents/4499/version/1. Traditional cytotoxic drugs (e.g. cisplatin) produce only GVCD and inhibit MCAD. We propose that MCAD is a cell death mechanism unique to endothelial cells and provides a practical biomarker to predict for AA activity in clinical oncology and drug development, as well as a potential drug target.





Endothelial massive calcium-accumulation death (MCAD): mechanism, target, and predictive biomarker for anti-angiogenic therapy.

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Bafferi, F, et al. Cellular changes in normal blood capillaries undergoing regression after inhibition of VEGF signaling. *Am J Physiol Heart Circ Physiol* 290: H547–H559, 2006.

- VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) is essential for blood vessel growth during embryonic development and in wound healing, macular degeneration, and cancer in the adult.
- VEGF acts as a survival factor for newly formed blood vessels in tumors and in the neonatal retina.
- Unlike the VEGF-dependent vasculature of the embryo, most blood vessels in the adult are thought to be stable and do not require VEGF for survival. The infrequency of serious side effects in preclinical studies and in patients receiving VEGF inhibitors is consistent with this.

These facts provide a basis for selective toxicity of antivascular drugs against tumors.

A predictive biomarker is needed for drug development and individualization of anti-angiogenic therapy

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

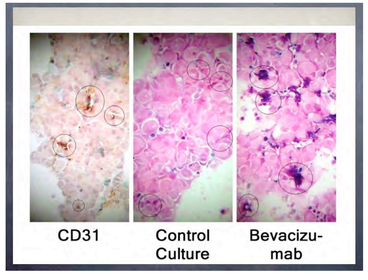
1. **A non-specific form of cell death, similar to that of other normal and neoplastic cells**
2. **A unique form of cell death, seen only in endothelial cells, associated with massive accumulation of calcium. We call this massive calcium accumulation death, or MCAD.**
3. **MCAD may be identified by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

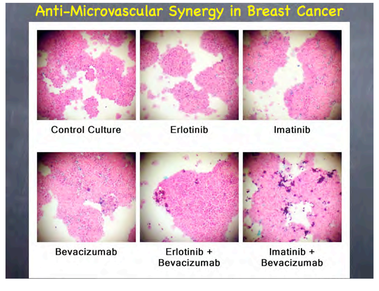
a. Fast Green/Hematoxylin

b. Fast Green/Wright-Giemsa, or

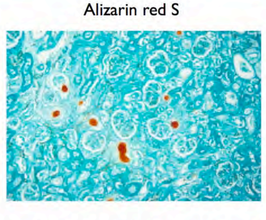
c. Alizarin red S (most adevantageous)

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

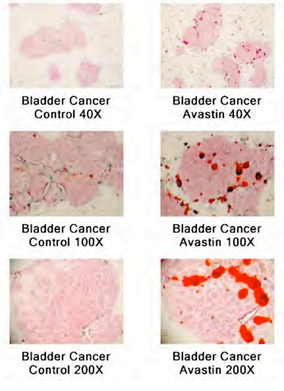


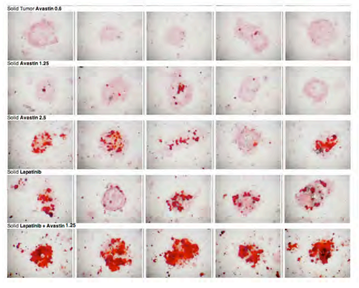


In the presence of Alizarin red S, calcium is identified by an orange-red "lake" staining effect

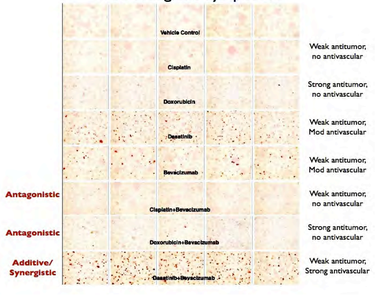


Breast Cancer

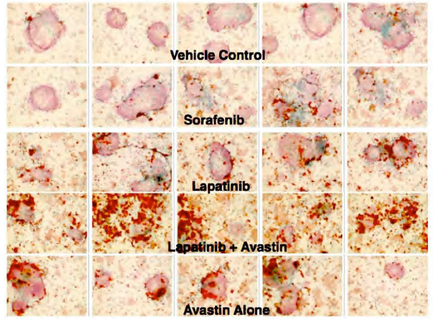




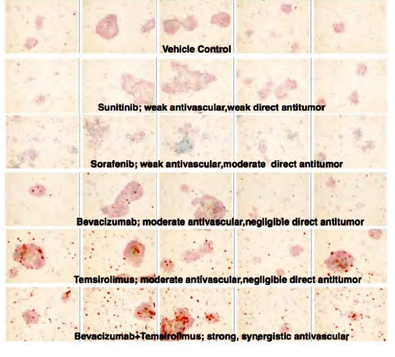
Non-Hodgkin's Lymphoma



Ovarian Cancer

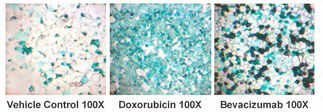


Renal Cell Carcinoma



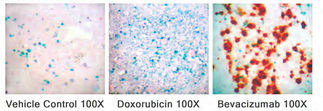
Human Umbilical Vein Endothelial Cells ImageJ Color (RGB) image quantification

Fast Green

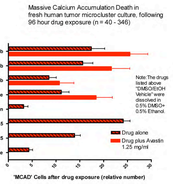


	Area	Area	Area
Living cells – clear or light pink	480,482	459,222	470,387
Non-specifically dead cells – light green	387,329	53,720	230,820
MCAD cells – dark green/black	8,660	393,636	139,976
	6,493	11,866	99,591

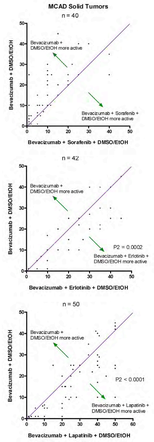
Fast Green/Alizarin

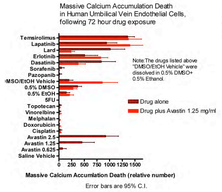


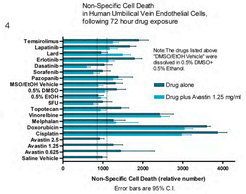
	Area	Area	Area
Living cells – clear or light pink	484,959	489,231	459,988
Non-specifically dead cells – light green	460,509	92,264	222,712
MCAD cells – dark orange/red	24,246	396,945	110,468
	204	22	

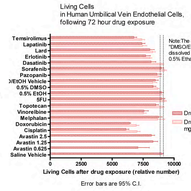


In general, tyrosine kinase inhibitors (exception: sorafenib) and mTor inhibitors are additive with bevacizumab, while traditional cytotoxic chemotherapy drugs inhibit the ability of bevacizumab to produce MCAD.









Example: relative effects of different agents on MCAD in HUVEC.

Lard is comprised of the following fatty acids: palmitic, stearic, myristic, oleic, palmitoleic, and linoleic. Saturated fatty acids and low density lipoprotein cholesterol and other lipids are known to produce apoptosis in cultured endothelial cells.

Lard (0.5 mg/ml or 50 mg/dl) increased MCAD in HUVEC cells and markedly inhibited the ability of bevacizumab to produce MCAD.

Potential approaches to increasing the activity of bevacizumab may be (1) avoid concurrent administration of cytotoxic chemotherapy, (2) reduce serum lipids, (3) co-administer DMSO and/or ethanol. Massive doses of DMSO have been safely administered with chemotherapy in cancer treatment (Fuks, JZ, et al Cancer Chemother Pharmacol (1981) 6:117-120)

This system offers a practical approach to identifying more effective anti-angiogenic agents and combination regimens -- both generally and in personalized therapy.