Death of human tumor endothelial cells in vitro through a probable calcium-associated mechanism induced by bevacizumab and detected via a novel method.

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The chief goal of cancer therapeutics has been the discovery of exploitable differences between cancer and normal cells, to allow for selective toxicity. Sixty years of intensive research have largely been an exercise in futility. However, cancer cannot exist without a blood supply and there is ample evidence of very important differences between embryonic and tumor vasculature, on one hand, and the vasculature of mature, adult tissues, on the other hand[1]. Better tools are needed for studying, understanding, and exploiting these differences.

Calcium influx and intracellular calcium release are felt to modulate endothelial cell proliferation, but sustained calcium influx has also been shown to be associated with endothelial cell death[2-5]. We previously described a novel method for detecting endothelial cell death in primary cell cultures prepared from fresh biopsy specimens of human tumors and from peripheral blood[6]. We report here that the distinctive cytological staining of dead endothelial cells observed in our method is the result of calcium accumulation and that staining for calcium (in the present case, with Alizarin red S) affords a sensitive method for detecting endothelial cell death in vitro.

Figure 1 shows the distinctive cytologic appearance of of fresh human tumors which were cultured without and with bevacizumab (2.5 mg/ml, which is a concentration sufficient to remove all detectable VEGF from cell culture supernatants[6)]. Dead endothelial cells have a distinctive hyperchromatic, blue-black appearance following the addition of the dye Fast Green to the culture medium, followed by Cytocentrifugation onto microscope slides, followed by counterstaining with Hematoxylin-eosin. Hematoxylin is known to form a blue-black “lake” effect, in the presence of calcium[7]. This staining, at least in dead endothelial cells, is markedly accentuated by pre-incubation with Fast Green, in our experience.

In the earlier publication[6], we speculated that the distinctive dead endothelial cell staining was owing to the dye exclusion principle, wherein living cells, with intact cell membranes, would exclude Fast Green, but that Fast Green penetrated dead cells, with incompetent cell membranes, thereby staining the dead endothelial cells. However, this explanation did not entirely explain the fact that the dead endothelial cells stain much more hyperchromatically and appeared to be refractile, in some cases, and even to have a crystalline appearance, in other cases (Figure 2). We suspected that the distinctive staining of the dead endothelial cells might be owing to a calcium effect.

We then cultured fresh human tumors and normal peripheral blood (contains circulating endothelial cells) in the presence and absence of bevacizumab and prepared Cytospin

slides with Fast Green/Hematoxylin-eosin, Fast Green/Wright-Giemsa, Fast Green/ Alizarin red S (reasonably specific for calcium http://www.ihcworld.com/_protocols/special_stains/alizarin_red_s.htm), Alizarin red S alone, and Alizarin red S, followed by Wright-Giemsa.

Figure 3 shows the dramatic difference between control and bevacizumab-exposed tumor cultures. The distinction between Alizarin red S and background material was greatest (from the standpoint of image analysis) when cells were first sedimented onto microscope slides, air dried, and stained only with Alizarin red S, and then photographed, without coverslips, for image analysis (Figure 3C, Table 1). These slides could then be coverslipped, with or without subsequent counterstaining with Wright-Giemsa (counterstaining with Hematoxylin-eosin or Hematoxylin alone had the effect of bleaching out the Alizarin red S stain).

There are currently no convenient, sensitive, specific methods for testing the effect of putative anti-angiogenic agents on human tumor-associated human endothelial cells, either generally or on an individual patient basis. Our present method affords such a method. For the purpose of high-throughput studies, one could test circulating peripheral blood endothelial cells from cancer patients (in whom circulating endothelial cells are often increased[8-11] and which originate, presumably, from tumor-associated capillaries) compared to endothelial cells from peripheral blood from normal volunteers, using simple, public domain image analysis software (http://rsbweb.nih.gov/ij/), and the Octospot cytocentrifuge system (http://www.testlaboratories.co.uk/products.asp).

Furthermore, the present studies suggest that bevacizumab-induced endothelial cell death (almost certainly resulting from VEGF depletion of the cell culture medium[6]) occurs via a calcium-accumulation mechanism. In addition to bevacizumab, other agents, including protein kinase inhibitors, ethanol, and/or dimethylsulfoxide also produce endothelial cell death in some specimens of human neoplasms through a mechanism which also produces intense calcium staining (most likely signifying calcium accumulation)[6,12,13], with the ethanol/calcium-influx/endothelial death finding supported independently by other evidence, in a different experimental system [4]. Additional support for our findings is the report that VEGF can function as a specific calcium channel blocker[14]. Removal of VEGF (e.g. with bevacizumab) could then lead to calcium entry and endothelial cell death.

In summary: 1.) The mechanism of endothelial cell death in human tumors exposed to bevacizumab and some other agents appears to involve abundant accumulations of calcium. These accumulations occur at levels vastly exceeding those observed in other types of normal and neoplastic cells, making calcium accumulation a sensitive and relatively specific marker for dead endothelial cells in primary human cell culture. Furthermore, perturbation of endothelial calcium influx affords a potential mechanism to augment the effect of agents such as bevacizumab. 2.) Calcium staining using our methodologies provides a convenient means for identifying and studying therapeutic agents targeting endothelial cells.

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Figure Legends:

Figure 1 Effect of bevacizumab (Avastin) on three dimensional primary cell culture of human solid tumors (post-culture stained with Fast Green/Hematoxylin&Eosin). Shown are assays performed on 8 different solid tumor specimens. Fresh solid tumor biopsies were minced and enzyme-digested. Three dimensional tumor clusters were enriched and cultured for 96 hours in anchorage-independent conditions in the absence (not shown) and presence of bevacizumab, 2.5 mg/ml, (which removes all detectable VEGF from the culture medium[5]), and stained with Fast Green/Hematoxylin&Eosin (methods described and referenced previously[5]). Living cells stain pink-red with H&E. Dead tumor and normal, non-endothelial cells stain blue green. Dead endothelial cells stain blue-black and produce surrounding blue-black "lakes." These cells correspond in size and location to CD31(endothelial antigen)-stained cells[5]. Control cultures virtually never exhibit these blue-black features, as documented in the previous publication[5].

Figure 2 Effect of bevacizumab (Avastin) and other agents on primary cell culture of human non-Hodgkin's lymphoma (panel A), myeloma (panel B), and normal peripheral blood cells (panel C). Lymphatic cells and associated endothelial cells were isolated from a lymph node biopsy (panel A), a bone marrow aspirate (panel B), and a peripheral blood specimen (panel C). Cells were cultured for 96 hours in anchorage-independent conditions in the presence and absence of test articles, including bevacizumab (Avastin), bortezomib, epirubicin, fludarabine, and 0.5% DMSO + 0.5% ethanol. Following culture, cells were stained with Fast Green/Wright-Giemsa, according to previously-described methods[5], or were stained with Alizarin red S (http://www.urmc.rochester.edu/path/zqu/StainsManual/index.html?DAHLSMETHODFORCALCIUM). Bevacizumab, bortezomib, and DMSO/ethanol produced blue-black stained,often highly refractile features and surrounding blue-black "lakes" which are thought to represent dead endothelial cells. Traditional cytotoxic agents (e.g. epirubicin, fludarabine) kill neoplastic cells, generally without producing the blue-black refractile features and lakes. Staining with Alizarin red S, followed by counterstaining with Wright-Giemsa, reveals that the blue-black, refractile features contain heavily concentrated calcium.

Figure 3 Effect of bevacizumab (Avastin) on three dimensional primary cell culture of human bladder (transitional cell) cancer: Panel A, stained with Fast Green/ Hematoxylin&Eosin; Panel B, stained with Alizarin red S; Panel C, stained with Alizarin red S and raw images subsequently processed with public domain ImageJ software (http://rsbweb.nih.gov/ij/). In panel C, shown on left side are raw photomicrographic images (40X original magnification) of cells which were cultured in the absence (control) or presence of bevacizumab (Avastin). These cells were stained with Alizarin red S, alone. The middle images of panel C show ImageJ-processed images, which were threshold gated to display all of the cellular material on the slide. The right sided images of panel C show ImageJ-processed images, which were threshold gated to display only the Alizarin red S stained features. Quantitative analysis of these slides was performed, using ImageJ software, with representative results shown in Table 1.

FIGURES

Figure 1 Effect of bevacizumab (Avastin) on three dimensional primary cell culture of human solid tumors (post-culture stained with Fast Green/Hematoxylin&Eosin)

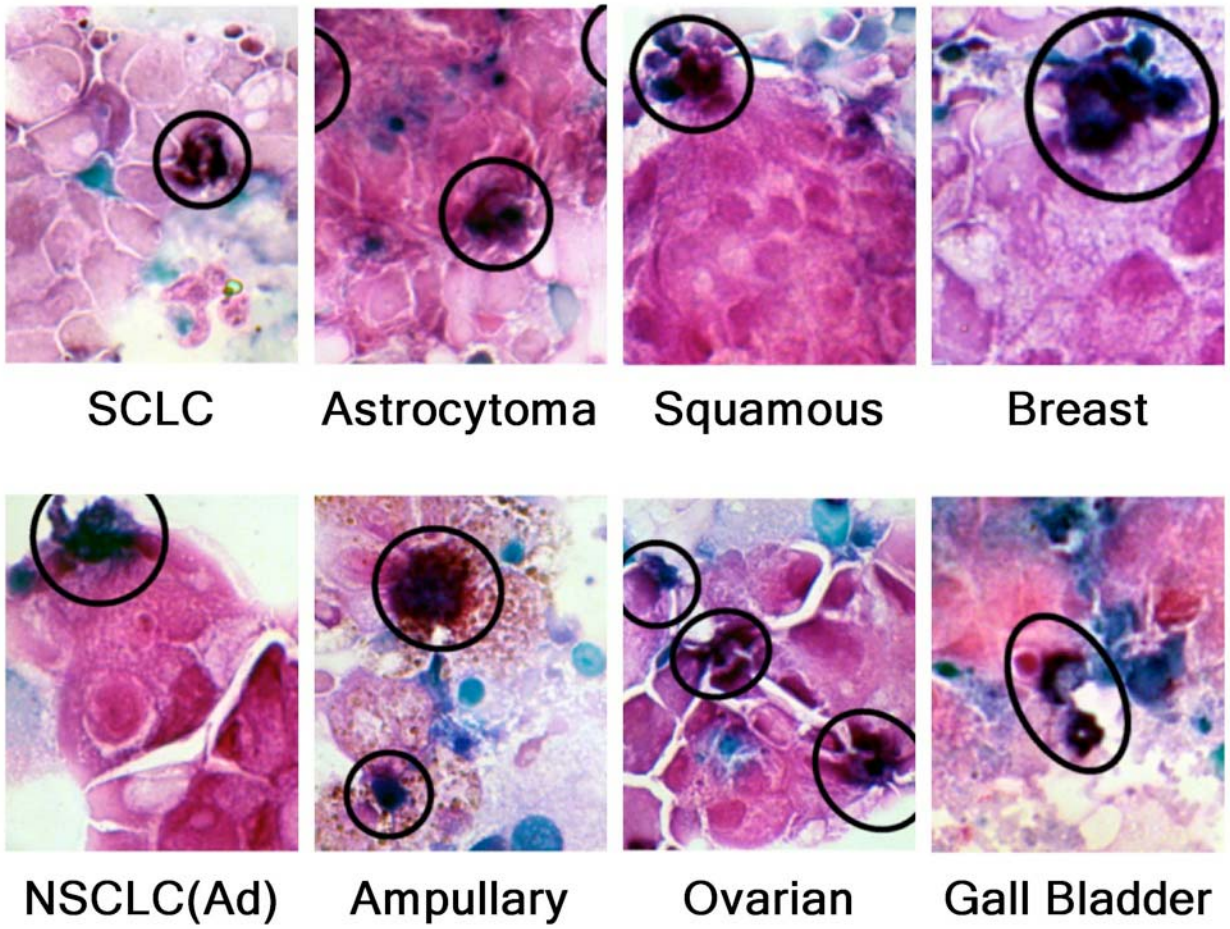


Figure 2 Effect of bevacizumab (Avastin) and other agents on primary cell culture of human lymphoma (panel A), myeloma (panel B), and normal peripheral blood cells (panel C) (post-culture stained with Fast Green/Wright-Giemsa, and, in the case of the normal cells, also with Alizarin red S - panel C, bottom)

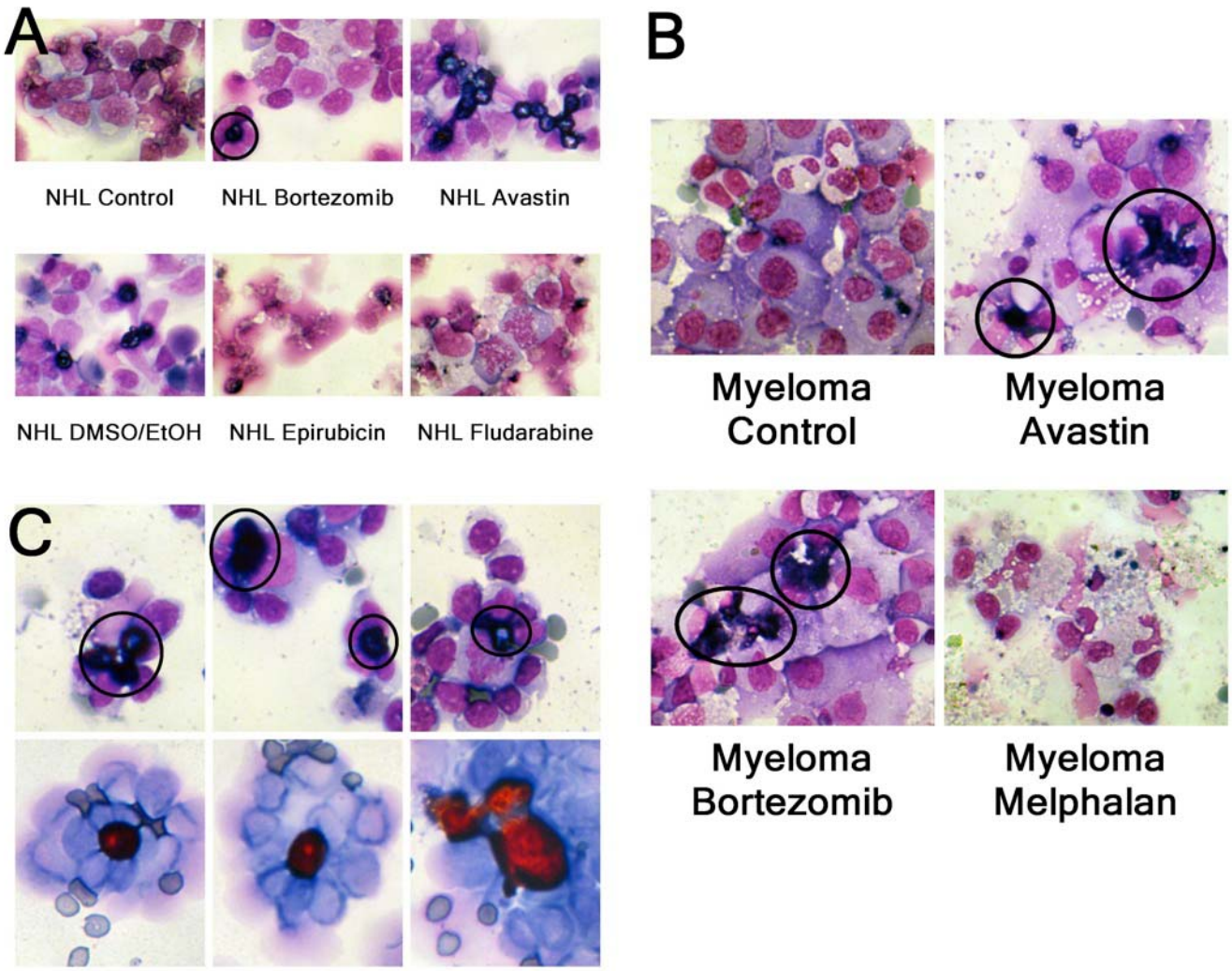
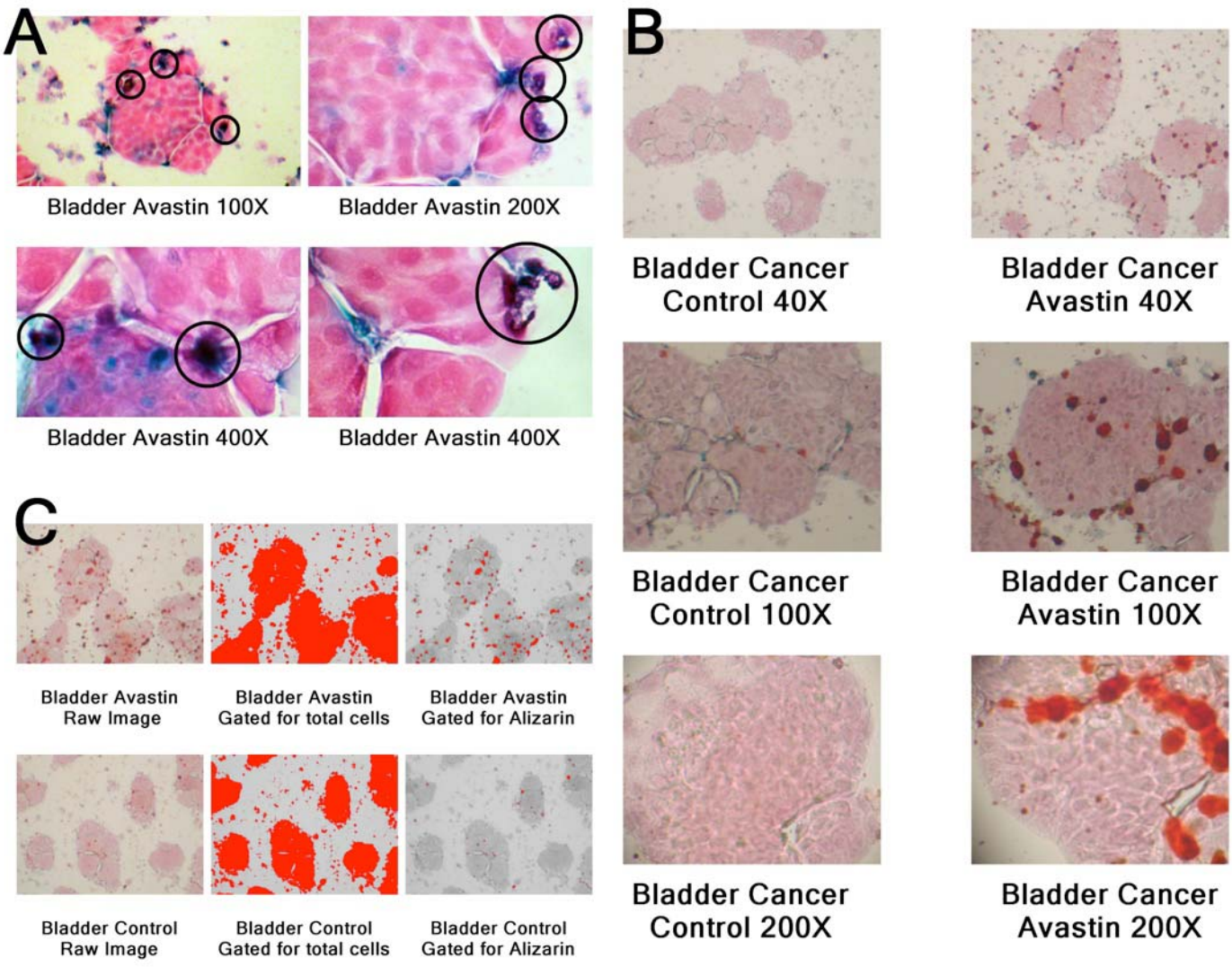


Figure 3 Effect of bevacizumab (Avastin) on three dimensional primary cell culture of human bladder (transitional cell) cancer: Panel A, stained with Fast Green/ Hematoxylin&Eosin; Panel B, stained with Alizarin red S; Panel C, stained with Alizarin red S and raw images subsequently processed with ImageJ software.



TABLE

Table 1. ImageJ analysis of bladder (transitional cell) cancer depicted in Figure 3.

40X field sampled	Count of individual alizarin-gated features divided by total pixels of cell-gated features (* 10E6)	Area (total pixels) of alizarin-gated features divided by total pixels of cell gated features (* 10E5)
Avastin #1	366	8629
Avastin #2	321	5353
Avastin #3	378	6663
Avastin #4	280	2859
Avastin #5	218	6262
Avastin #6	343	7292
Mean Avastin (95% C.I.)	**318 (218-378)**	**6176 (4120-8232)**
Control # 1	59	591
Control # 2	89	644
Control # 3	37	291
Control # 4	48	280
Control # 5	70	575
Control # 6	133	899
Mean Control (95% C.I.)	**73 (36-109)**	**547 (301-791)**
2-sided P Student's T	**<0.0001**	**<0.0001**