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Targeting anionic phospholipids on tumor blood vessels and tumor cells

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Vascular targeting agents for the treatment of cancer

Vascular targeting agents (VTAs) bind selectively to molecules that are present on tumor vasculature, and either recruit host effector cells or deliver agents that occlude or destroy tumor blood vessels [1]. This leads to an avalanche of tumor cell death through deprivation of oxygen and nutrients. VTAs have a number of advantages over other cancer therapies. First, a single vessel provides the nutrition and waste removal for hundreds or thousands of tumor cells, and only has to be damaged at a single point to block blood flow upstream and downstream. Second, the endothelial cell is in contact with the blood stream ensuring rapid drug delivery. Third, the target is unlikely to acquire genetic mutations that render it drug resistant. Fourth, VTAs act on existing as well as newly forming tumor vasculature. Finally, VTAs preferentially kill the hypoxic core regions of tumors, where the vasculature is most stressed and compromised, thus providing a complementary pattern of killing to chemotherapeutic drugs and irradiation, which are most effective against well-oxygenated peripheral regions of tumors.

The VTA described in the present article is a naked antibody, *bavituximab*, which employs host cells as the effectors. Other VTAs employ coupled toxins, procoagulants and pro-apoptotic drugs as effectors. The functionally-similar tumor vascular disrupting agents, such as combretastatin, also occlude tumor vasculature, but do so by exploiting pathophysiological differences between tumor vessels and normal ones, rather than by selectively binding to tumor vessels. The vascular disrupting agents also show improved efficacy when used together with irradiation [2]. Bavituximab stands apart from other VTA and vascular disrupting drugs in causing no significant toxicity by itself and no enhancement of toxicity when combined with other anti-cancer strategies [1].

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Phosphatidylserine (PS) is a universal marker of tumor vasculature

PS is normally tightly segregated to the internal surface of the plasma membrane in most cell types, including the vascular endothelium [3–5]. PS asymmetry is maintained by ATP-dependent aminophospholipid translocases (Mg^{2+}-ATPases) that catalyze the transport of aminophospholipids from the external to the internal leaflet of the plasma membrane [6]. Loss of PS asymmetry occurs during apoptosis [7], necrosis [8], cell activation [9] and transformation [10], resulting in the exposure of PS on the external surface of the cells. PS exposure occurs when the aminophospholipid translocases become inhibited or when transporters such as scramblases [11] and the ABC floppases become activated by Ca^{2+} fluxes into the cytosol [12]. Ceramide generation by activation of acid sphingomyelinase A on endothelial cells can also facilitate the transbilayer movement of PS [13].

We recently showed that anionic phospholipids, principally PS, are specifically exposed on tumor endothelial cells, likely in response to oxidative stresses present in the tumor microenvironment [3]. This is true of all tumors so far examined, including orthotopically grown, syngeneic and spontaneous tumors in mice and rats. Typically, fewer than half of the vessels in tumors are positive for exposed PS [14,15]. PS exposure is normally greatest on vessels in the core of the tumor and least in the outmost vessels. Chemotherapy, radiation and androgen deprivation therapy markedly increase PS exposure on tumor vessels, so that the large majority (70–95%) become positive.

The tumor microenvironment contains factors that activate and/or injure tumor EC: (a) tumor-derived interleukin-1 and tumor necrosis factor-α, which activate the endothelium and induce expression of cell adhesion molecules; (b) reactive oxygen species (ROS) generated by leukocytes that adhere to the tumor endothelium; and (c) ROS generated by tumor cells as a byproduct of metabolism or as a result of exposure to hypoxia followed by reoxygenation. In this regard, we have demonstrated that inflammatory

cytokines, acidity, thrombin, hypoxia/reoxygenation, and ROS all induce PS exposure on EC *in vitro* [14,15]. Chemotherapy, radiation and androgen deprivation therapy increase stresses within tumors, with repercussions on the vasculature that elevate PS exposure.

The bavituximab family of anti-PS antibodies

Bavituximab binds to complexes of the PS-binding plasma protein beta2-glycoprotein 1 (β2GP1) and anionic phospholipids. The antibody binds to PS-expressing membranes by crosslinking two molecules of β2GP1bound to PS on the membrane (Fig. 1) [16]. The Bavituximab-β2GP1-PS complex is only stably formed on PS surfaces. Bavituximab recognizes domain II in β2GP1, which has not been implicated in anti-phospholipid syndromes. Bavituximab was developed from 3G4, the original mouse IgG3 monoclonal antibody [15]. A recombinant mouse IgG2a anti-PS antibody was constructed, designated 2aG4, which contains the 3G4 Fv region fused to a murine IgG2a heavy chain constant region. 2aG4 has higher affinity interaction with mouse FcγR receptors on murine host immune cells. Bavituximab was developed for human clinical use. Bavituximab is a human-mouse chimeric antibody having the 3G4 Fv regions joined to human IgG1κ constant regions. The affinity constant (K_D) for the binding of all these antibodies to human β2GP1 complexed with PS is 4×10^{-10} M. All the antibodies recognize human and rat β2GP1 but not mouse β2GP1 [16]. Experiments in the mouse require supplementation with a source of reactive β2GP1.

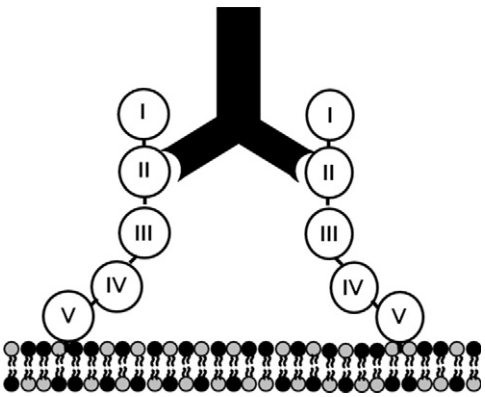

Fig. 1. Binding of bavituximab to cell membrane surfaces with exposed anionic phospholipids (gray) is mediated through β2GP1.

In vivo tumor localization of antibody

Externalized PS is one of the most specific markers of tumor vasculature yet identified. After injection into tumor-bearing mice or rats, bavituximab and related antibodies localize specifically to endothelial cells in tumor vessels but not to those in normal tissues [1]. Tumor vessel targeting has been confirmed immunohistochemically in numerous tumor types and visualized using positron emission tomography of ^{74}As-labeled anti-PS antibodies in rats. Biodistribution studies have confirmed the excellent specificity of bavituximab for tumor vasculature [17].

Anti-tumor effects in rodents

Treatment of rodents with 2aG4 or 3G4 as single agents retards the growth of multiple different tumors, including human and rodent tumors, orthotopic, ectopic, spontaneous and metastatic tumors [18–22]. The antibodies have improved anti-tumor activity when used in combination with chemotherapeutic drugs, androgen-deprivation therapy and irradiation. 2aG4 has enhanced anti-tumor activity when given together with docetaxel in a human breast cancer model (Fig. 2) [19]. It significantly reduces

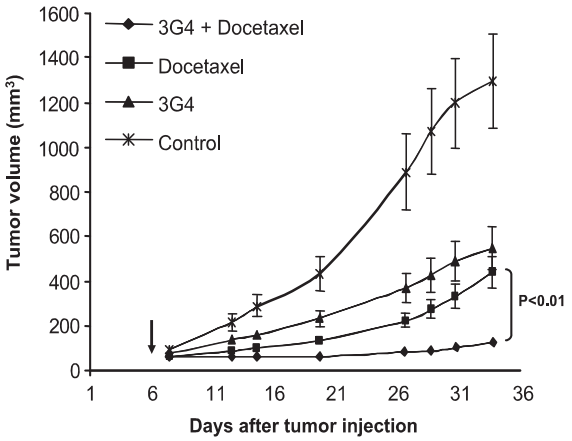

Fig. 2. Combination of 3G4 with docetaxel in SCID mice bearing human MDA-MB-43:5 breast tumors. 3G4 antibody (100 μg/dose), docetaxel (10 mg/kg) or both were administered 3 times a week for 3 weeks starting 6 days after tumor implantation.

tumor burden and metastatic lesions when co-administered with gemcitabine in an orthotopic model of pancreatic cancer [20]. It acts in combination with androgen-deprivation therapy to cause regressions of large prostate tumors in mice [21]. It acts with cisplatin to prolong the growth of cisplatin-resistant tumors, as expected for a therapy that targets tumor vasculature. The anti-tumor effects of the antibodies are enhanced by focal irradiation in non-small lung cell carcinoma [22] and orthotopic glioma [18] models. Radiation resistant tumors become responsive to the therapy because it targets the radiosensitive vascular endothelium. The enhanced therapeutic effects appear to be due to the ability of the chemotherapeutic drugs, androgen-deprivation therapy and irradiation to generate reactive oxygen species (ROS), which increase exposure of PS on tumor blood vessels and amplify the target for 3G4 or 2aG4. In all situations, coadministration of antibody improves efficacy without contributing any toxicity.

Mechanism of action

(i) Anti-vascular activity: 3G4 and 2aG4 have a strong vascular targeting action. Histologic examination of tumors from treated mice reveals a marked reduction in the vascular density and plasma content of tumors (Fig. 3). Time course studies show

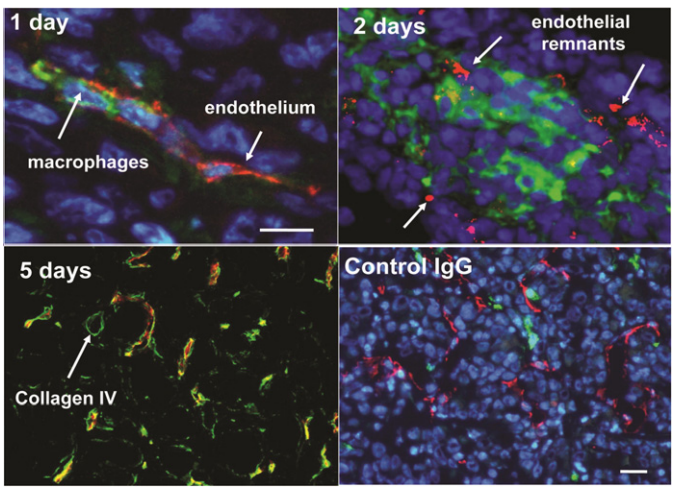

Fig. 3. Time course of events leading to tumor vessel destruction. Intravenously administered 3G4 or 2aG4 localize rapidly (<1 hour) to PS-expressing tumor blood vessels in mice and rats. By 1 day after injection, monocytes/macrophages are seen adhering to the antibody-coated tumor endothelium. By 2 days, vascular destruction is evident. By 5 days, de-endothelialized 'ghosts' of former blood vessels are visible by immunohistochemical detection of basement membrane collagen IV. None of these effects is seen in animals treated with isotype-matched negative control IgG.