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Article

Proapoptotic Activation of Death Receptor 5 on Tumor Endothelial Cells Disrupts the Vasculature and Reduces Tumor Growth

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SUMMARY

The proapoptotic death receptor DR5 has been studied extensively in cancer cells, but its action in the tumor microenvironment is not well defined. Here, we uncover a role for DR5 signaling in tumor endothelial cells (ECs). We detected DR5 expression in ECs within tumors but not normal tissues. Treatment of tumor-bearing mice with an oligomeric form of the DR5 ligand Apo2L/TRAIL induced apoptosis in tumor ECs, collapsing blood vessels and reducing tumor growth: Vascular disruption and antitumor activity required DR5 expression on tumor ECs but not malignant cells. These results establish a therapeutic paradigm for proapoptotic receptor agonists as selective tumor vascular disruption agents, providing an alternative, perhaps complementary, strategy to their use as activators of apoptosis in malignant cells.

INTRODUCTION

Apoptosis, or type I programmed cell death, is essential for normal development, tissue homeostasis, and tumor suppression in higher organisms (Strasser et al., 2000). The ability of the proapoptotic receptor ligand Apo2L/TRAIL (TNFSF10) to kill malignant cells selectively has prompted an intensive effort to explore its therapeutic potential. Various agonists of the cognate death receptors DR4 and DR5 have been developed, and several have been studied in human cancer clinical trials (Ashkenazi and Herbst, 2008; Johnstone et al., 2008). Referred to as proapoptotic receptor agonists (PARAs) (Ashkenazi, 2008a), these agents include recombinant human Apo2L/TRAIL (dulanermin) and several agonistic antibodies targeting DR4 or DR5. Although certain cancer cells undergo apoptosis in response to PARAs, others exhibit partial or total resistance (Abdulghani and El-Deiry, 2010; Yang et al., 2010). Preclinical studies with these agents have relied mainly on cultured human cancer cells or mouse-xenografted human tumors. However, because most PARAs target human receptors but not their mouse counterparts (Ashkenazi, 2002), the effects of death-receptor engagement on the tumor microenvironment are not well understood.

Some previous studies have used MD5-1, an antibody directed against murine DR5 (or TRAIL-R), the only Apo2L/TRAIL death receptor present in the mouse. MD5-1 is reported to induce apoptosis of cancer cells in vitro; however, its tumoricidal efficacy in vivo requires aspects of innate and adaptive host immunity (Frew et al., 2008; Haynes et al., 2010; Takeda et al., 2004; Uno et al., 2006). Other studies have examined the ability of Apo2L/TRAIL to induce apoptosis in endothelial cells: these were performed mainly by in vitro culture of endothelial cells

Significance

Cancer therapies typically attack the malignant cells of a tumor, but targeting the nonmalignant tumor microenvironment may augment antitumor efficacy. Various types of cancer cells express the proapoptotic death receptor DR5. DR5 agonists induce apoptosis of certain malignant cells and thereby can exert antitumor activity. Here we show that endothelial cells within tumor blood vessels can selectively express DR5. Activation of DR5 on tumor endothelial cells triggers apoptosis: This disrupts the integrity of tumor blood vessels and decreases tumor growth even in the absence of DR5 expression in the malignant cell compartment. Our findings suggest a unique, perhaps complementary, utility for proapoptotic receptor agonists as vascular disruption agents in cancer therapy.

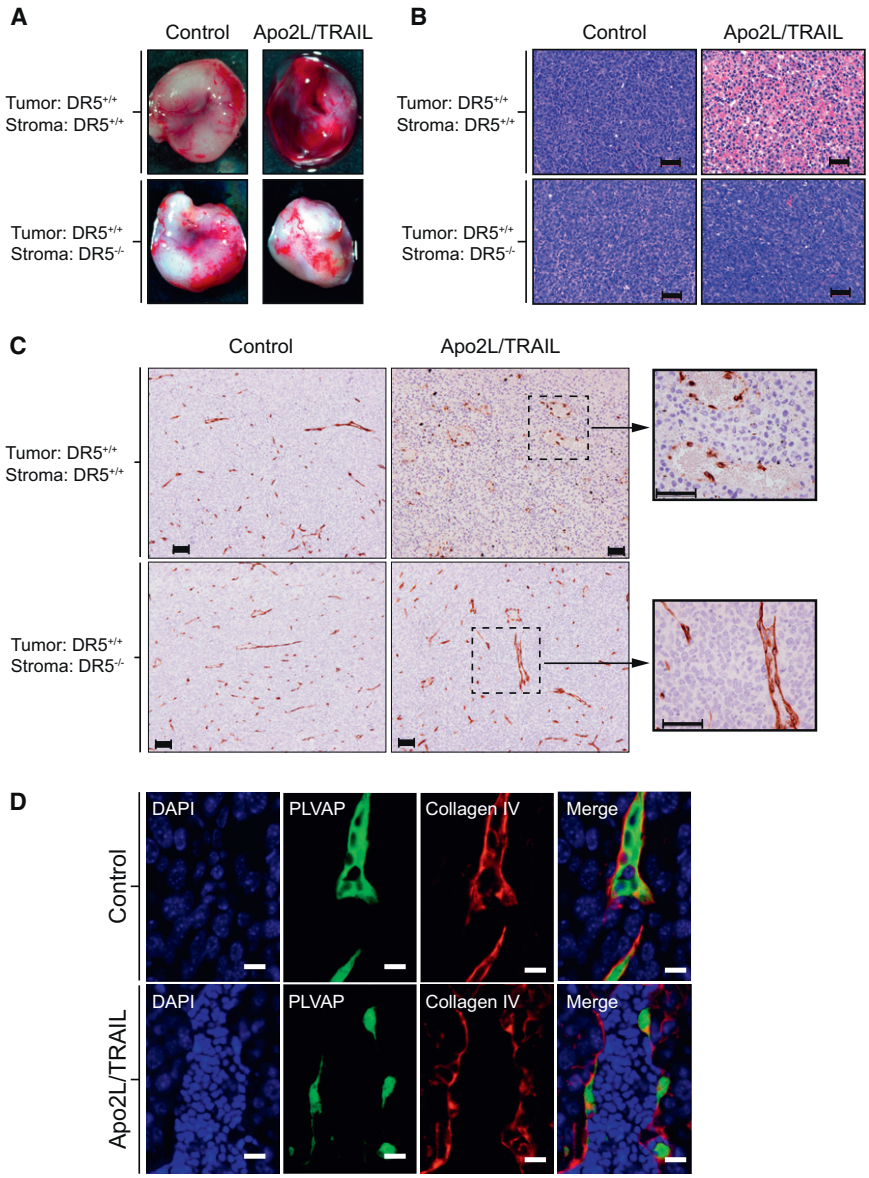


Figure 1. DR5-Dependent Disruption of the Tumor Vasculature by Apo2L/TRAIL

(A) Lewis lung carcinoma (LLC) tumors (>500 mm^3) grown in WT ($DR5^{+/+}$) or DR5-KO ($DR5^{-/-}$) mice were dosed with an intraperitoneal (i.p.) injection of 10 mg/kg of Apo2L/TRAIL (consisting of 10 mg/kg of Flag-tagged Apo2L/TRAIL and 10 mg/kg anti-Flag antibody, given sequentially) or PBS. Tumors were examined macroscopically 24 hr after treatment for the appearance of vascular disruption.

(B) Hematoxylin and eosin (H&E) staining of sections from LLC tumors grown in WT or DR5-KO mice and treated with Apo2L/TRAIL.

(C) Anti-PLVAP staining was used to visualize the tumor endothelium; representative images show disrupted blood vessels (inset = higher magnification image) in tumors from Apo2L/TRAIL-treated WT, but not DR5-KO or untreated (control), mice. Scale bars, 50 μm.

(D) Immunofluorescence colocalization of DAPI (blue), PLVAP (green), and collagen IV (red) on LLC tumor sections from control or Apo2L/TRAIL-treated, WT mice. Single-channel as well as merged images are shown. Scale bar, 10 μm. Data in Figure 1 are representative of two or more independent experiments.

See also Figure S1.

and arrived at conflicting conclusions as to whether this ligand induced apoptosis or proliferation in these cells (Chan et al., 2010; Chen and Easton, 2010; Li et al., 2003). Indeed, the prevailing paradigm for PARAs as anticancer therapeutics remains centered on their ability to induce apoptosis directly in malignant cells.

In the present study, we investigated the biological activity of an oligomeric form of Apo2L/TRAIL capable of activating DR5 in both the malignant and stromal compartments of tumors.

RESULTS

DR5-Dependent Disruption of the Tumor Vasculature by Crosslinked Apo2L/TRAIL

Although murine cancer cells often express DR5, they do not respond to dulanermin, the trimeric recombinant soluble version of human Apo2L/TRAIL that has been studied in clinical trials (Ashkenazi et al., 1999; Herbst et al., 2010) (Figure S1A available online; data not shown). However, we found that crosslinking of a Flag epitope-tagged version of Apo2L/TRAIL with an anti-Flag antibody into higher order oligomers enabled the ligand to induce apoptosis in a range of mouse cancer cell lines: These included Renca331 cells (Figure S1B), which are particularly sensitive to membrane-bound Apo2L/TRAIL (Seki et al., 2003), as well as Lewis lung carcinoma (LLC) cells (Figure S1C). To determine the efficacy of this oligomeric DR5-ligand in vivo, we implanted LLC cells into C57BL/6 wild-type recipient mice and treated the animals with a single dose of crosslinked Apo2L/TRAIL (Apo2L/TRAIL hereafter). Surprisingly, we observed a striking hemorrhagic appearance in tumors within 24 hr of treatment (Figure 1A). Histological examination confirmed extensive tumor hemorrhage, as well as widespread tumor-cell death (Figure 1B). Such effects were not induced by dulanermin (Figure S1D), consistent with its lack of activity toward mouse DR5. Immunohistochemical staining with the Meca-32 antibody (Hallmann et al., 1995), which recognizes the plasmalemma vesicle-associated protein (PLVAP; also PV-1)—an endothelial marker—revealed severe disruption of the tumor vasculature in response to Apo2L/TRAIL. Tumor blood vessels in treated mice showed evidence of congestion, discontinuity in ECs lining the vessel, and blood-cell leakage (Figure 1C). To evaluate this further, we performed immunofluorescence staining for PLVAP in conjunction with collagen IV, a marker for the basement membrane. In tumors

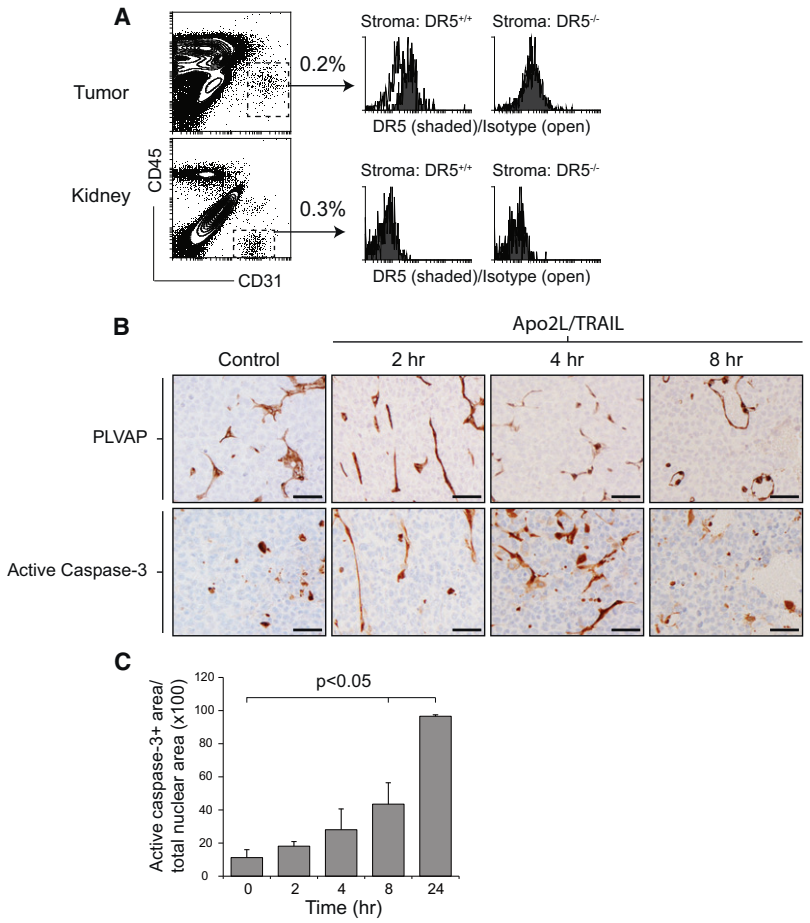


Figure 2. DR5-Mediated Apoptosis in Tumor-Associated Endothelial Cells

(A) Top, analysis of DR5 expression by CD45^{low}CD31^{high} expressing tumor endothelial cells (ECs) in LLC tumors grown in WT (DR5$^{+/+}$) or DR5-KO (DR5$^{-/-}$) mice. DR5 expression (shaded) versus isotype control (open) on cell lines are shown from a pooled cell fraction generated from WT (n = 4) or DR5-KO LLC tumors. Bottom, analysis of DR5 expression by CD45^{low}CD31^{high} expressing, "normal" kidney endothelial cells isolated from WT or DR5-KO mice. Pooled kidney cell fractions were generated from the same mice that were analyzed above.

(B) Anti-PLVAP and activated capsase-3 (CC3) staining in serial LLC tumor sections collected from WT or DR5-KO mice treated for the indicated times with Apo2L/TRAIL or PBS (control).

(C) Quantitation of cleaved caspase-3 immunohistochemical staining on LLC tumor sections from a time course of Apo2L/TRAIL treatment. The average of n = 5 tumors for each time point is plotted; error bars indicate the SEM. Student's t test was used to calculate statistical significance. Data in Figure 2 are representative of two or more independent experiments.

See also Figure S2.

from untreated mice, blood vessels were characterized by a contiguous layer of PLVAP-positive cells within the collagen IV-stained basement membrane (Figure 1D); in contrast, tumors from mice treated with Apo2L/TRAIL for 24 hr displayed congested blood vessels, with only occasional PLVAP-positive cells and with large areas of "naked" basement membrane. These observations suggest that Apo2L/TRAIL treatment leads to ablation and detachment of ECs lining blood vessels within tumors, thereby perturbing the integrity of these vessels.

DR5-Dependent Apoptosis Activation in Tumor Endothelial Cells

Remarkably, implantation of LLC tumor cells in DR5-deficient mice completely abrogated the effects of Apo2L/TRAIL on the tumor vasculature (Figures 1A–1C). We therefore hypothesized that the proapoptotic ligand might be exerting a direct biological effect on the tumor-stromal compartment. To evaluate this possibility, we dissociated LLC tumors grown in wild-type (WT) or DR5-knockout (KO) recipients and stained the isolated cells for flow cytometric analysis with antibodies to three markers: DR5; the leukocyte common antigen, CD45; and the endothelial cell-associated antigen, CD31 (Tang et al., 1993). We used differential CD45 and CD31 expression to broadly define tumor-associated leukocytes (CD45^{high}), a tumor epithelial-cell enriched fraction (CD45^{low}CD31^{low}), and tumor ECs (CD45^{low}CD31^{high}). We detected DR5 protein expression on CD45^{low}CD31^{low} malignant epithelial cells from tumors grown in WT or DR5-KO mice, but not on CD45^{high} leukocytes from tumors grown in mice with either DR5 genotype (Figure S2A) (Tang et al., 1993). Importantly, we also observed DR5 expression on CD45^{low}CD31^{high} endothelial cells from tumors grown in WT but not DR5-KO mice (Figure 2A). By contrast, we did not detect significant DR5 expression on CD45^{low}CD31^{high} endothelial cells isolated from the kidneys of normal WT mice (Figure 2A). Immunohistochemistry confirmed DR5 expression on ECs within the tumor stroma of WT mice (Figure S2B).

ECs in various tissues are phenotypically and functionally diverse, with differential surface-marker expression and distinct adherens and tight junctional complexes (Dejana, 2004; Jain, 2005; Pober and Sessa, 2007). Consistent with the lack of DR5 expression by ECs in normal tissues, we did not observe any evidence of vascular disruption or hemorrhage outside of the tumor microenvironment in Apo2L/TRAIL-treated mice. The apparent selectivity of DR5 expression on tumor ECs as compared to normal-tissue ECs may reflect local stressful conditions within the tumor such as hypoxia, which has been shown to increase DR5 expression in cancer cells (Mahajan et al., 2008). To assess proapoptotic signaling in tumor ECs, we monitored the appearance of apoptotic markers over time in LLC tumors from mice treated with Apo2L/TRAIL. Serial sections of tumor tissue were stained for PLVAP to localize tumor ECs, or with an antibody specific to active (cleaved) caspase-3 as an indicator of proapoptotic signaling. Some areas of active caspase-3 staining appeared in tumor epithelial cells regardless of treatment (Figure 2B), suggesting spontaneous focal apoptosis as is commonly seen in mouse tumors. However, we detected rapid generation of active caspase-3 in tumor ECs, within 2 hr after

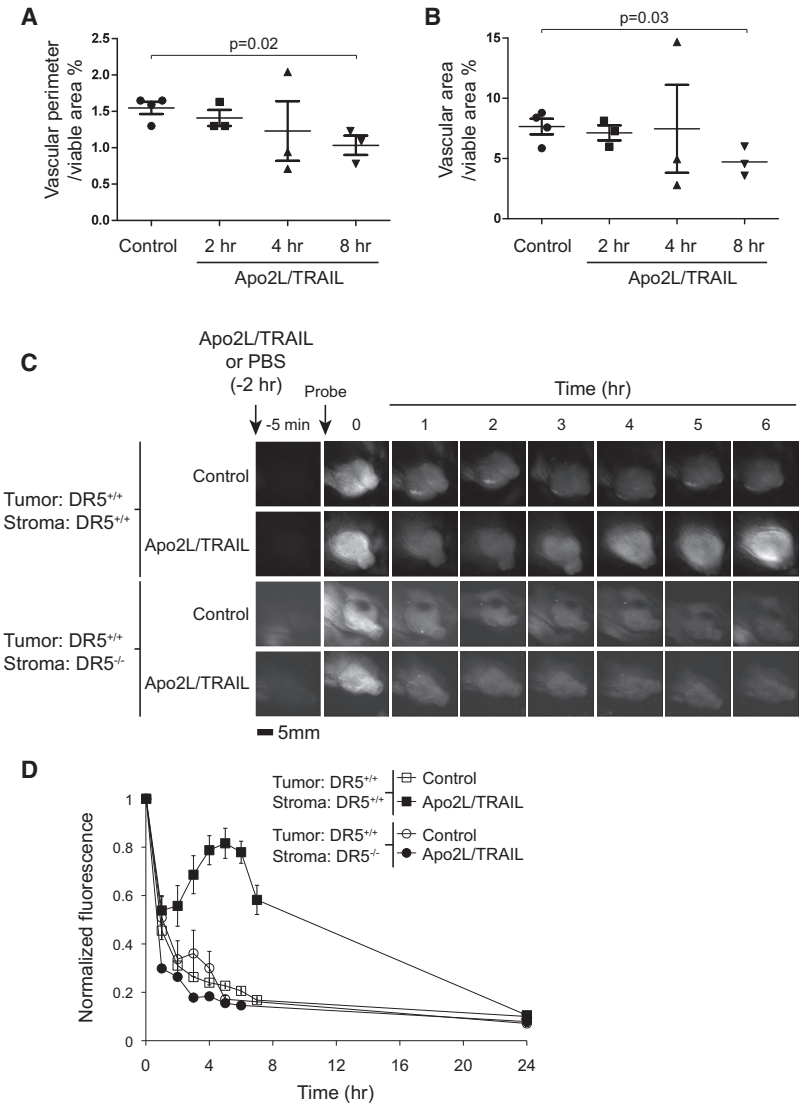


Figure 3. Temporal Analysis of Tumor-Vascular Changes Induced by Apo2L/TRAIL

Quantitation of anti-PLVAP immunostaining was performed on tumor sections from mice treated with Apo2L/TRAIL for the indicated time period.

(A) Vascular perimeter (presented as vessel perimeter in μm, divided by viable area in μm^2 multiplied by 100). Horizontal bars indicate the mean ± SEM. Statistical significance was calculated using a two-tailed, independent samples t test.

(B) Vascular area (presented as vessel area in μm^2, divided by viable area in μm^2 multiplied by 100). Vascular perimeter and area were calculated as described in Supplemental Experimental Procedures. Horizontal bars indicate the mean ± SEM. Statistical significance was calculated using a two-tailed, independent samples t test.

(C) LLC tumor-bearing WT or DR5-KO mice (n = 3–5/group) were treated with PBS or Apo2L/TRAIL. Two hr after treatment, mice were injected intravenously with the fluorescent blood pool probe AngioSense680IVM. Distribution of the fluorescent probe in the tumor was monitored at the indicated times on anesthetized mice. Shown are representative images from a time course following injection of the probe.

(D) Quantification from (C). Error bars indicate the SEM. See also Figure S3.

Apo2L/TRAIL treatment. By 8 hr, there was evidence of vessel congestion and hemorrhage (indicated by the presence of blood cells outside of PLVAP-marked vascular spaces). Distinct changes in EC morphology were also apparent, with cell bodies rounded and protruding into the lumen (Figure 2B). By 24 hr after Apo2L/TRAIL treatment, extensive active caspase-3 staining could be seen throughout the tumor (Figure 2C; Figure S2C). Remarkably, at early time points, little caspase-3 activity was present within malignant epithelial cells (Figures 2B and 2C), suggesting that Apo2L/TRAIL-induced apoptosis in tumor ECs preceded, and was independent of, apoptosis in the malignant-cell compartment. Apo2L/TRAIL did not induce EC apoptosis in LLC tumors grown in DR5-deficient mice (Figure S2C), confirming direct DR5-dependent signaling in tumor ECs.

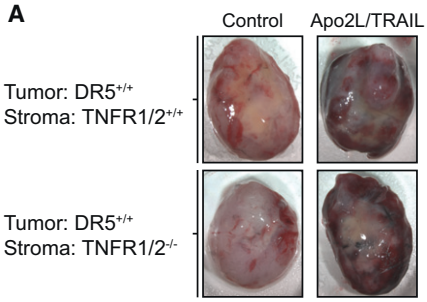
In contrast to Apo2L/TRAIL, the MD5-1 antibody, which targets mouse DR5, did not induce discernible effects on the tumor vasculature. LLC tumors from mice treated with MD5-1 showed no evidence of hemorrhage (Figure S2D), and activation of caspase-3 in these tumors by MD5-1 was significantly weaker than stimulation by Apo2L/TRAIL (Figure S2E). However, MD5-1 did inhibit tumor growth in vivo (Figure S2F). These results suggest that the enhanced potency achieved by crosslinking of Apo2L/TRAIL, perhaps better mimicking the transmembrane form of this ligand, is important for activation of DR5-mediated apoptosis in tumor ECs. Nevertheless, additional mechanisms supported by MD5-1, such as innate or adaptive immune-cell functions (Frew et al., 2008; Haynes et al., 2010; Takeda et al., 2004; Uno et al., 2006), may mediate generation of an apoptotic signal by this anti-DR5 antibody in tumor cells.

Temporal Analysis of DR5-Mediated Tumor-Vascular Disruption

We next asked whether the apoptotic effects of Apo2L/TRAIL on ECs were associated with changes in tumor-vascular density. Using a method previously described (Brey et al., 2003), we quantitated PLVAP immunostaining in control and Apo2L/TRAIL-treated tumor specimens over time. By 8 hr after treatment with Apo2L/TRAIL, both the vascular perimeter and area significantly decreased (Figures 3A and 3B), indicating diminished tumor-vascular density. This decrease was consistent with the changes in vessel morphology seen at the same timepoint (Figure 2B), suggesting a cumulative effect of EC apoptosis over time.

To examine the kinetics of tumor-vascular disruption more closely, we used noninvasive, near-infrared imaging of a fluorescent macromolecular blood-pool probe. Intravenous (i.v.)

A



B

Recipient	Treatment	Tumor Hemorrhage	(n)
Wildtype	Control	0%	14
Wildtype	Apo2L/TRAIL	100%	12
DR5-deficient	Control	0%	15
DR5-deficient	Apo2L/TRAIL	0%	14
TNFR1/2-deficient	Control	0%*	10
TNFR1/2-deficient	Apo2L/TRAIL	100%	10

* Untreated tumors in TNFR1/2-deficient exhibited small areas of hemorrhage

C

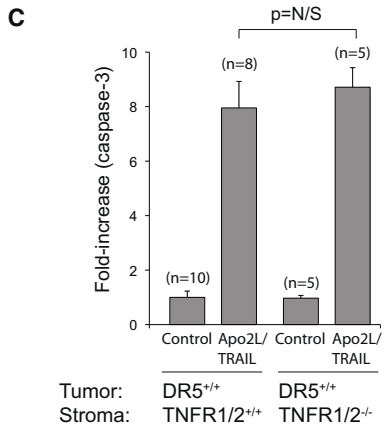


Figure 4. DR5-Mediated Vascular Disruption Is Independent of TNFR Signaling

(A) LLC tumors (>500 mm^3) grown in WT or TNFR1/2-double-KO (TNFR1/$2^{-/-}$) mice were dosed intraperitoneally with 10 mg/kg of Apo2L/TRAIL or PBS. Tumors were examined macroscopically 24 hr after treatment for the appearance of vascular disruption.

(B) Table summarizing the incidence of hemorrhage in tumors grown in recipient mice with the indicated genotypes.

(C) LLC tumor cells were implanted in C57BL/6 WT or TNFR1/2-double-KO mice. After a 24 hr treatment with Apo2L/TRAIL or PBS (control), tumors were harvested and flow cytometry was used to measure cleaved caspase-3 in tumor cells. Caspase-3 activation is represented as fold over control (PBS). Error bars indicate the SEM. Student's t test was used to calculate statistical significance. N/S, not significant. Data in Figure 4 are representative of two or more independent experiments.

administration of macromolecular probes enables longitudinal assessment of vascular permeability, because probe distribution within tumors is governed by endothelial permeability and the differential rates of clearance between the vascular and interstitial compartments (for further discussion see Figure S3). In untreated tumor-bearing mice, fluorescence intensity within tumors decreased exponentially following i.v. probe injection (Figures 3C and 3D), consistent with the expected systemic half-life of the probe. By contrast, in animals treated with ApoL2/TRAIL 2 hr before probe injection, tumor-associated fluorescence decreased initially, comparable to control, but then increased between 2 and 6 hr. Both the timing and characteristics of the phenotype were consistent with an acute change in vascular permeability (Figure S3C), rather than vascular normalization due to an antiangiogenic effect (Jain, 2005; McKeage and Baguley, 2010). Apo2L/TRAIL treatment of DR5-KO tumor-bearing mice did not result in abnormal probe clearance, confirming that the disruption of tumor-vascular integrity required stromal DR5. Together, these data suggest that Apo2L/TRAIL induces DR5-dependent changes in the tumor vasculature within 4 hr of treatment.

DR5-Mediated Tumor-Vascular Disruption Is Independent of TNFR Signaling

In addition to proapoptotic signaling, engagement of DR5 under certain circumstances may activate nonapoptotic pathways, such as the nuclear factor-κB (NF-κB) cascade, which promotes cytokine and chemokine production, among other cellular effects (Wilson et al., 2009). Tumor necrosis factor alpha (TNFα), often induced by NF-κB stimulation, has been reported to trigger dramatic tumor-vascular effects (Corti and Ponzoni, 2004; ten Hagen et al., 2008). This raised the possibility that the impact of DR5 activation on the tumor vasculature might be exerted indirectly, via TNFα. To examine this notion, we implanted TNF receptor (TNFR) 1 and 2 double-KO mice with LLC tumors and treated them with Apo2L/TRAIL. The appearance and incidence of tumor-vascular disruption induced by Apo2L/TRAIL in TNFR1/2-KO mice were indistinguishable from those in WT mice, yet were absent in DR5-KO recipients (Figures 4A and 4B). In accordance, TNFR1/2 deficiency in the stromal compartment had no impact on Apo2L/TRAIL-induced caspase-3 activation in tumor epithelial cells (Figure 4C). These results indicate that Apo2L/TRAIL exerts its tumor-vascular effects independently of TNFR signaling.

Tumor-Vascular Disruption Is Independent of DR5 Expression in Malignant Cells

Our findings suggested that DR5 ligation on tumor ECs might directly induce apoptotic death of these cells; alternatively, proapoptotic signaling in DR5-expressing malignant cells might indirectly mediate the observed apoptotic outcome in tumor ECs. To interrogate this, we generated methylcholanthrene (MCA)-induced fibrosarcomas in WT and DR5-KO mice and established cell lines from the resulting tumors. We confirmed significant DR5 expression or its absence by flow cytometric analysis of the corresponding tumor cell lines (Figure 5A). We then generated WT or DR5-KO MCA tumors by implanting these fibrosarcoma cells in WT or DR5-KO recipient mice. Induction of tumor hemorrhage by Apo2L/TRAIL required DR5 expression in the stroma, but not the malignant tumor-cell compartment (Figure 5B; Figure S4). Immunostaining with anti-PLVAP and anti-active caspase-3 antibodies confirmed proapoptotic signaling in tumor ECs, regardless of DR5 expression status in the malignant cells (Figures 5C and 5D). Hence, disruption of the tumor vasculature by Apo2L/TRAIL occurs independently of DR5 engagement in cancer cells.

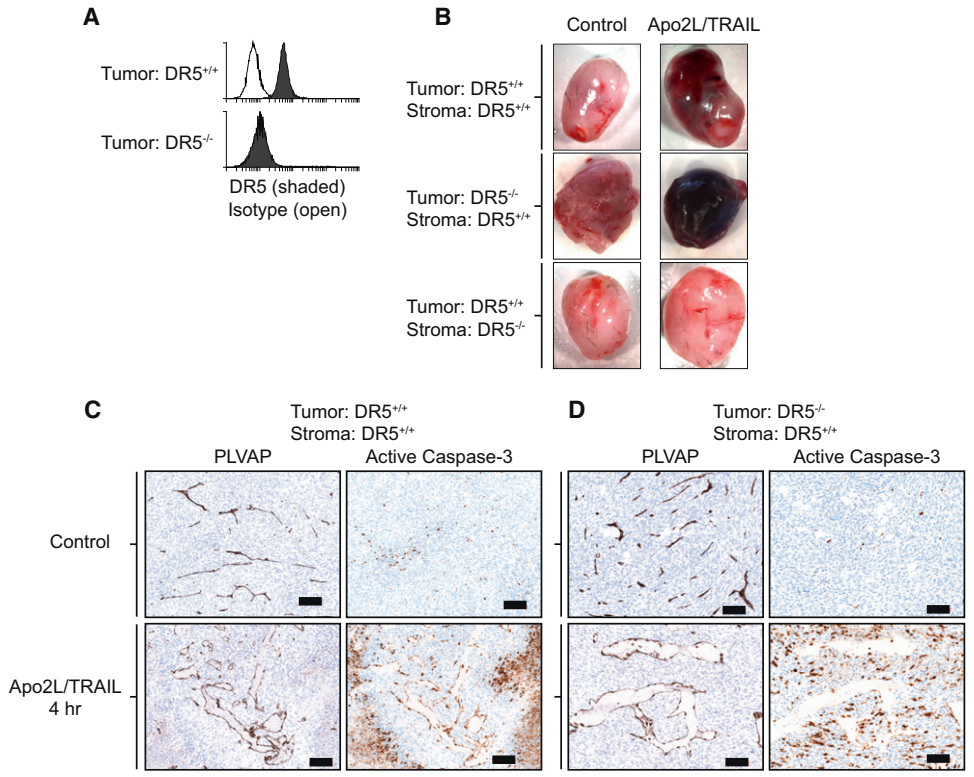


Figure 5. Effect of Apo2L/TRAIL on the Tumor Vasculature Is Independent of DR5 Expression in Malignant Cells

(A) Methylcholanthrene (MCA)-induced fibrosarcoma cell lines were derived from WT ($DR5^{+/+}$) or DR5-KO ($DR5^{-/-}$) C57BL/6 mice and assayed for DR5 expression by flow cytometry.

(B) Images are shown of $DR5^{+/+}$ or $DR5^{-/-}$ fibrosarcoma tumors, grown in C57BL/6 $DR5^{+/+}Rag2^{-/-}$ (top and middle panels), or C57BL/6 $DR5^{-/-}$ (bottom panels), recipients. Tumors were harvested at 24 hr post-treatment with Apo2L/TRAIL and compared with PBS-treated controls.

(C and D) Apoptosis in tumor vasculature of MCA-induced tumors. $DR5^{+/+}$ (C) or $DR5^{-/-}$ (D) MCA-induced fibrosarcoma cells were implanted in C57BL/6 $DR5^{+/+}Rag2^{-/-}$ recipients and treated with Apo2L/TRAIL (10 mg/kg) for 4 hr. Serial sections from tumors were stained with antibodies specific for PLVAP or cleaved casapse-3 to localize endothelial and apoptotic cells, respectively. Scale bars, 100 μm. Data in Figure 5 are representative of two or more independent experiments.

See also Figure S4.

DR5-Mediated Vascular Disruption Reduces Tumor Growth

The induction of DR5-mediated apoptosis in tumor ECs suggested that it might be possible to harness this activity for therapeutic gain. To assess this, we compared the ability of Apo2L/TRAIL to inhibit tumor growth in WT mice bearing either DR5-expressing or DR5-deficient tumors. In vitro analysis of caspase-8 and caspase-3/7 activation and loss of cell viability confirmed a lack of proapoptotic signaling in DR5-KO MCA tumor cells (Figure 6A). Consistent with the immunohistochemical evidence (Figures 5C and 5D), both DR5-expressing and DR5-KO fibrosarcomas showed significant caspase-3 activation in vivo in response to Apo2L/TRAIL (Figure 6B). This result suggested that the death of malignant cells occurred as an indirect consequence of tumor-vascular disruption. Supporting this hypothesis, the growth of pre-established fibrosarcomas possessing either DR5 genotype was significantly attenuated by Apo2L/TRAIL (Figures 6C and 6D). Moreover, in both cases, extensive hemorrhagic tumor necrosis occurred (Figure S4). Thus, DR5-mediated apoptosis in tumor ECs can contribute to antitumor efficacy in a manner that is distinct and separable from DR5-dependent malignant-cell apoptosis. We also evaluated vascular density and morphology at an "end of study" time point (i.e., when control and Apo2L/TRAIL-treated tumors had grown to similar size). Although overall vascular density was significantly reduced in tumors exposed to Apo2L/TRAIL as compared with controls, vascular morphology was indistinguishable (Figures S5A and S5B). Hence, although repeat dosing of Apo2L/TRAIL exerts a long-term effect on tumor-vascular density, treatment cessation allows the tumor vasculature to reform.

Consistent with its modest proapoptotic activity toward the fibrosarcoma and LLC cell lines in vitro (Figure 6A; Figure S1C), Apo2L/TRAIL did not induce significant proapoptotic signaling in the malignant-cell compartment of corresponding tumors in DR5-KO mice (Figure 6E; Figure S5C). Although DR5 status in the recipient mice did not affect tumor initiation or growth (data not shown), antitumor activity of Apo2L/TRAIL required DR5 expression in the stromal compartment (Figures S5D and S5E). Therefore, in these particular models, DR5 engagement on tumor ECs may be the primary mechanism mediating the inhibition of tumor growth by Apo2L/TRAIL. The

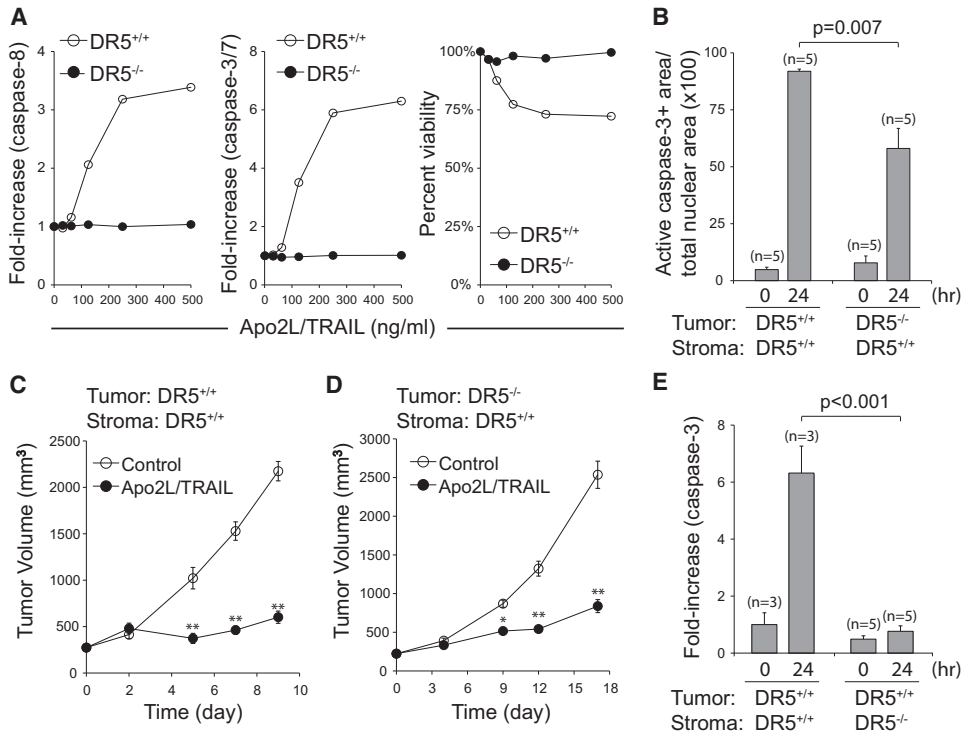


Figure 6. Vascular Disruption by Apo2L/TRAIL Contributes to Antitumor Effects In Vivo

(A) DR5$^{+/+}$ or DR5$^{-/-}$ fibrosarcoma cell lines were treated in vitro with a dose titration of Apo2L/TRAIL. Caspase-8 and caspase 3/7 activity was quantified 4 hr after treatment using luminescent substrate assays. Cell viability was determined 24 hr after Apo2L/TRAIL treatment using an ATP-based Cell Titer Glo assay.

(B) DR5$^{+/+}$ or DR5$^{-/-}$ fibrosarcoma cell lines were grown in DR5$^{+/+}$Rag2$^{-/-}$ recipient mice, treated with a single dose (10 mg/kg) of Apo2L/TRAIL, and harvested for immunohistochemical (IHC) staining with antibodies against cleaved caspase-3. Graph shows quantitation of cleaved caspase-3 IHC staining on tumor sections from control (0 hr) or Apo2L/TRAIL-treated (24 hr) mice. The mean of n = 5 tumors for each group is plotted; error bars indicate the SEM. Student's t test was used to calculate statistical significance.

(C and D) C57BL/6 DR5$^{+/+}$Rag2$^{-/-}$ mice bearing wild-type (C) or DR5$^{-/-}$ (D) MCA-induced tumors were treated with Apo2L/TRAIL five times per week for two weeks, and tumor growth was compared with untreated controls. Error bars indicate the SEM (n = 8–10 mice/group). p values were calculated using Student's t test; asterisk indicates $p < 0.01$; double asterisks indicate $p < 0.001$.

(E) DR5$^{+/+}$ MCA-induced fibrosarcoma cells were grown in DR5$^{+/+}$ or DR5$^{-/-}$ C57BL/6 mice. Tumors were harvested 24 hr after treatment with Apo2L/TRAIL, and flow cytometry was used to measure cleaved caspase-3 in tumor cells. Caspase-3 activation is shown as fold increase over control (0 hr). Error bars indicate the SEM (n = 3–5 mice/group). Data in Figure 6 are representative of two or more independent experiments.

See also Figure S5.

lack of malignant-cell apoptosis in the absence of stromal DR5 could be attributed alternatively to a nonoptimal distribution of the crosslinked ligand into the tumor in this context. To address this, we used H2122 human lung carcinoma cells, which exhibit strong sensitivity to Apo2L/TRAIL (Wagner et al., 2007). We established H2122 tumor xenografts in Rag2-deficient mice with DR5-WT or DR5-KO stromal compartments. In the presence of stromal DR5, but not in its absence, Apo2L/TRAIL induced H2122 tumor hemorrhage similar to that seen in the fibrosarcoma and LLC models (Figure S5F). Nonetheless, activation of caspase-3 in H2122 tumor cells was comparable in both DR5 genotypic backgrounds (Figure S5G). Hence, antibody-crosslinked Apo2L/TRAIL can access the malignant tumor cells to induce apoptosis independently of stromal DR5 expression or effects on the tumor vasculature. These data suggest that DR5-mediated vascular disruption represents an alternative, possibly complementary, mechanism of antitumor efficacy of Apo2L/TRAIL.

Apo2L/TRAIL Disrupts the Tumor Vasculature in a Genetic Model of Pancreatic Cancer

Our results demonstrated that DR5 engagement could disrupt the tumor vasculature in murine syngeneic tumor models (Figures 2, 3, 4, and 5) as well as in mouse xenograft models of human lung and breast cancer (Figure S5F; data not shown). Genetically engineered mouse models of cancer have been increasingly recognized for their ability to recapitulate many aspects of human cancer, including pathogenesis and response to therapy. Therefore, to assess more rigorously the ability of Apo2L/TRAIL to disrupt tumor vessels, we turned to a previously established, genetically engineered mouse model of pancreatic ductal adenocarcinoma (Singh et al., 2010). Seven out of eight (87.5%) tumors from *KrasLSL−G12D; p16/p19$^{fl/fl}$; Pdx1-Cre* mice treated with a single dose of Apo2L/TRAIL showed extensive hemorrhage (Figures 7A and 7B), similar to that seen in syngeneic or xenografted tumor models. Anti-PLVAP staining confirmed tumor-vessel disruption in Apo2L/TRAIL-treated

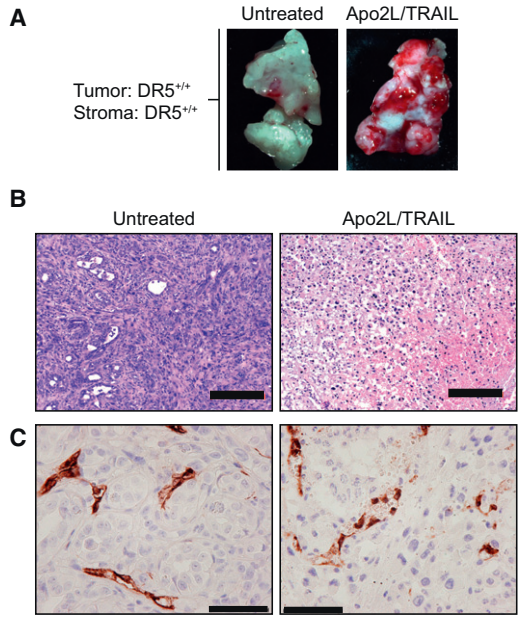


Figure 7. Vascular Disruption by Apo2L/TRAIL in a Mouse Genetic Model of Human Pancreatic Cancer

(A) Tumor-bearing *KrasLSL-G12D; p16/19$^{fl/fl}$; Pdx1-Cre* mice were dosed intraperitoneally with 10 mg/kg of Apo2L/TRAIL or PBS. Tumors were examined macroscopically 24 hr after treatment for the appearance of vascular disruption.

(B) H&E staining of sections from pancreatic ductal adenocarcinomas resected from Apo2L/TRAIL or untreated mice. Representative images show widespread hemorrhaging observed in 7 of 8 tumors from mice treated with Apo2L/TRAIL. Scale bars, 200 μm.

(C) Anti-PLVAP staining was used to visualize the tumor endothelium; representative images show disrupted blood vessels in tumors from Apo2L/TRAIL-treated but not in control mice. Scale bars, 50 μm.

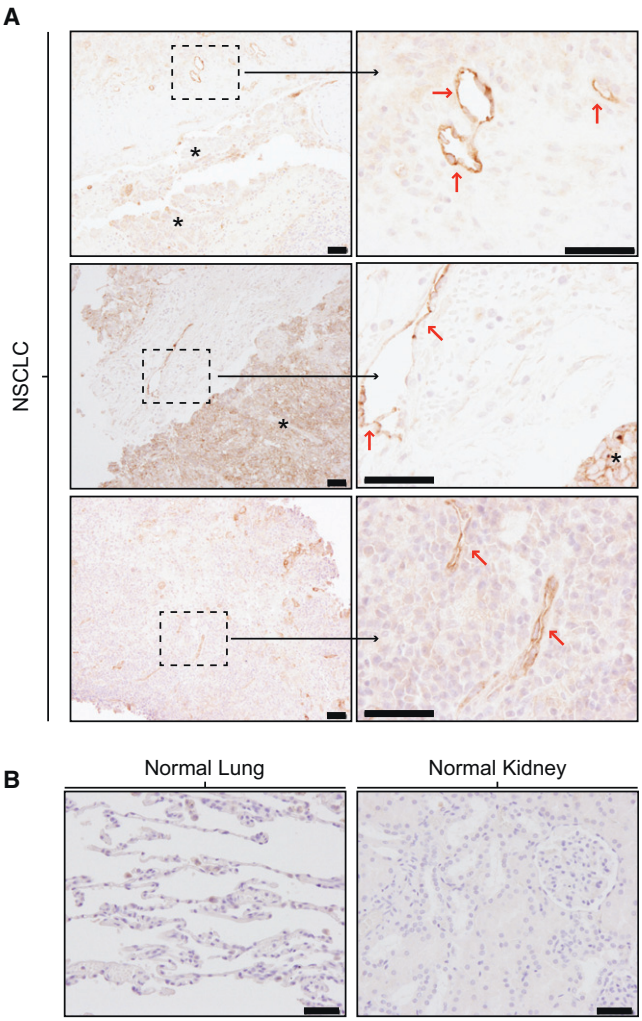


Figure 8. Analysis of DR5 Expression in Human Tumor Endothelial Cells

(A) Representative sections from three separate human nonsmall cell lung cancer (NSCLC) specimens with DR5 expression in the tumor endothelium. Dashed boxes indicate areas shown at higher magnification (right panels). Red arrows indicate focal DR5-positive tumor endothelial cells.

(B) Representative sections from normal human lung and kidney, respectively. Scale bars, 50 μm.

See also Figure S6.

mice (Figure 7C). The ability of Apo2L/TRAIL to trigger vascular disruption in a range of tumor types and contexts supports the potential relevance of this activity for treating human cancer.

Evidence for DR5 Expression in Human Tumor Endothelial Cells

To evaluate the frequency of DR5 expression in ECs within human tumors, we analyzed a panel of surgical tissue specimens from nonsmall cell lung cancer (NSCLC) patients by DR5 immunohistochemistry. Of 43 samples, four (~10%) showed focal regions of DR5 expression in the tumor endothelium (Figure 8A). We also surveyed a panel of normal tissues from human and mouse: With the exception of some staining in human stomach, DR5 expression was not detected in the endothelium or in other cell types of normal tissues (Figure 8; Figure S6). Consistent with the lack of DR5 expression in nontumor vasculature, repeated dosing of naive C57BL/6 mice with Apo2L/TRAIL did not significantly affect body weight or survival, despite some small, transient elevations in liver enzyme activity, detected in serum from some of the animals (Figures S6C–S6E and data not shown). These findings suggest that endothelial expression of DR5 is present in a subset of human NSCLC tumors. In contrast, expression in normal tissues is rarely detected, permitting repeated administration of crosslinked Apo2L/TRAIL in mice without significant adverse effects.

DISCUSSION

The current experimental paradigm for PARAs as cancer-therapeutic agents is based on their ability to induce direct cancer-cell apoptosis via DR5 and/or DR4 (Ashkenazi, 2002, 2008b; Johnstone et al., 2008). Yet, clinical studies with PARAs to date have suggested that malignant cells are relatively refractory to death-receptor engagement, suggesting that various mechanisms of apoptosis evasion may limit the efficacy of such agents in patients with cancer (Yang et al., 2010). In the

present study, we show that an oligomeric form of Apo2L/TRAIL, which displays more potent agonistic activity toward DR5 and is capable of engaging the murine receptor, can achieve antitumor effects by directly perturbing the tumor vasculature. Oligomeric Apo2L/TRAIL induced vascular disruption in several tumor settings and models.

Our data suggest that DR5 activation in tumor ECs induces apoptotic death of these cells, thereby compromising vascular integrity and causing vessel congestion, intratumoral hemorrhage, decreased vascular density, and diminished tumor growth. Several lines of evidence indicate that Apo2L/TRAIL can act directly on the tumor stroma: First, its effects on the vasculature and on tumor growth in the fibrosarcoma model persisted even when DR5 was genetically ablated from the malignant cell compartment. Conversely, the effects on the tumor vasculature were abolished upon genetic ablation of DR5 the stroma, diminishing antitumor efficacy in both the fibrosarcoma and LLC models. Second, Apo2L/TRAIL induced significant levels of caspase-3 activation in tumor ECs prior to engaging apoptosis in the malignant cell compartment. Considering that other stromal cells (e.g., immune infiltrates) did not show appreciable DR5 expression, our data demonstrate the ability of Apo2L/TRAIL to induce direct, DR5-dependent apoptosis in tumor ECs.

Importantly, DR5-mediated vascular disruption exerted antitumor activity even in the absence of DR5 in malignant cells, thus highlighting the potential for attacking tumors that otherwise might be expected to resist PARA therapy. Although vascular disruption appears to be the primary mechanism for antitumor effects in the fibrosarcoma and LLC models studied here, this may not apply to all tumors. Indeed, direct effects of PARAs on cancer cells are well established and further supported by the activity of Apo2L/TRAIL against H2122 human xenografts in the absence of stromal DR5 expression and tumor-vascular disruption. Thus, DR5-mediated perturbation of the tumor vasculature may constitute an alternative, and perhaps complementary, antitumor strategy to the current PARA-based approach of directly inducing tumor-cell apoptosis.

Several vascular disrupting agents (VDAs) are in clinical development for cancer treatment. However, there is concern that the therapeutic utility of these agents might be limited by adverse events, such as on-target effects against normal vasculature, as well as a lack of efficacy (Heath and Bicknell, 2009; Lara et al., 2011; Lorusso et al., 2011; McKeage and Baguley, 2010). Certain effects of Apo2L/TRAIL on the tumor vasculature—namely, the rapid disruption of existing vessels and ensuing intratumoral hemorrhage—are similar to those of other VDAs. However, despite these similarities, there are key differences in the mechanisms of action of Apo2L/TRAIL versus known VDAs. One class of VDAs, including combretastatin and its analogs, acts primarily by inhibiting tubulin polymerization and inducing changes in EC shape (McKeage and Baguley, 2010; Siemann et al., 2009). Although its specific molecular target is unknown, another clinically tested VDA, the flavonoid ASA404, induces vascular permeabilization and EC apoptosis via TNFα-dependent mechanisms (Baguley, 2011). In contrast to these agents, Apo2L/TRAIL disrupts the tumor vasculature strictly through direct DR5-mediated apoptosis in tumor ECs. This mechanistic difference may have important implications for both safety and efficacy outcomes in patients. Nevertheless, additional studies will be necessary to understand more fully the impact of DR5 activation in tumor ECs and the longer-term effects on vascular function.

An additional consideration is that specific PARAs exhibit differences in potency that may affect their biological activity and potential toxicity. The enhanced activity of oligomeric Apo2L/TRAIL toward mouse DR5 suggests that this form of the protein may better mimic the endogenous transmembrane ligand. It is possible that the noncrosslinked trimeric Apo2L/TRAIL molecule represented by dulanermin, as well as DR5 agonistic antibodies, although active against malignant cells, do not achieve the signaling threshold required to induce significant apoptosis in tumor ECs. Thus, variants of PARAs with greater potency might display stronger vascular disrupting activity. Although more potent variants may not be as well tolerated as dulanermin (Lawrence et al., 2001), the lack of systemic toxicity in mice with the oligomeric Apo2L/TRAIL used here is encouraging, and suggests that a significant therapeutic window may be achievable. This is further supported by the apparent absence of DR5 expression in most normal vasculature and tissues. However, more focused studies on the nontumor vasculature, quiescent or inflamed, may reveal additional examples of DR5 modulation besides the tumor stroma. Regardless, any clinical development of more potent PARAs would necessitate extensive testing in animals, including nonhuman primates, to exclude potential side effects on normal tissues including the vasculature.

Only a minority (~10%) of human NSCLC specimens showed endothelial DR5 expression. However, given the very high prevalence of this lethal malignancy, our data suggest significant impact for potential clinical translation and provide a compelling rationale for assessing endothelial DR5 expression in other human cancers. Furthermore, it will be interesting to determine what specific features of the tumor microenvironment drive endothelial DR5 expression and whether DR5 levels can be manipulated by certain combinatorial treatment regimes. To date, clinical efficacy with PARAs has not been observed in unselected patients, with the exception of some individual subjects (Ashkenazi and Herbst, 2008; Yang et al., 2010). In future clinical studies, it will be important to assess more specifically whether DR5 expression in tumor ECs correlates with responsiveness to PARAs, and whether efficacy in individual patients is associated with tumor-vascular disruption.

In conclusion, our studies uncover DR5-mediated proapoptotic signaling in tumor ECs. We propose that PARAs may act as a unique class of tumor-selective vascular disruption agents, even for tumors in which the malignant cell compartment is resistant to direct apoptosis induction via DR5.

EXPERIMENTAL PROCEDURES

Mouse Models

C57BL/6 (wild-type) mice were obtained from the Jackson Laboratory, and C57BL/6.Rag2$^{-/-}$ mice were obtained from Taconic, Inc. C57BL/6.DR5$^{-/-}$ (Diehl et al., 2004), C57BL/6.TNFR1$^{-/-}$TNFR2$^{-/-}$, Rag2$^{-/-}$;DR5$^{-/-}$ and *KrasLSL−G12D; p16/p19fl/fl; Pdx1-Cre* (Singh et al., 2010) mice were bred and maintained at Genentech, Inc. under specific pathogen-free conditions. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Genentech, Inc.

Human Tissue Samples

Archival formalin fixed paraffin embedded (FFPE) tissue specimens of nonsmall cell lung cancer (NSCLC) were collected and used in this study in accordance with the protocol (APM4074 g) approved by the Institutional Review Board of Kaiser Permanente Medical Center, (Vallejo CA and Portland, OR), Ohio State University (Columbus, OH), Medical College of Wisconsin (Milwaukee WI), and M. D. Anderson Cancer Center (Houston TX), and written informed consent was obtained from patients in all cases at time of enrollment. Normal tissue specimens were purchased from Biomax Inc., (Rockville, MD). In all analyses, a slide was H&E stained and verified by a board-certified pathologist.

Fibrosarcoma Tumor Initiation

C57BL/6 (wild-type) or C57BL/6.DR5$^{-/-}$ mice were inoculated subcutaneously in the hind flank with 200 μg of methylcholanthrene (MCA) (Sigma-Aldrich) in 0.1 ml of corn oil, as previously described (Koebel et al., 2007). Mice were assessed weekly for tumor development from 90 days after MCA treatment.

In Vivo Dosing

Dulanermin (trimeric Apo2L/TRAIL) and recombinant soluble Flag-tagged human Apo2L/TRAIL was prepared according to a published method (Ashkenazi et al., 1999; Kischkel et al., 2000). Tumor-bearing mice were dosed intraperitoneally with 60 mg/kg of dulanermin, or 10 mg/kg of Apo2L/TRAIL followed by 10 mg/kg of the anti-Flag antibody (M2) (Sigma). For tumor growth studies and the end-of-study experiment in Figure S5, mice were dosed with 1 or 2 cycles of Apo2L/TRAIL (10 mg/kg) as indicated. Each cycle consisted of 5 days of consecutive treatment, with 2 days of no treatment in between cycles.

Cell Lines and Tumor Transplant Models

Lewis lung carcinoma, fibrosarcoma, and H2122 cells were maintained in RPMI medium supplemented with L-glutamine and 10% fetal bovine serum (FBS) under conditions of 5% CO_2 at 37°C. Mice were injected subcutaneously with 5×10^6 cancer cells. Tumors were measured in two dimensions using a caliper. Tumor volume was calculated using the formula: $V = 0.5a \times b^2$, where a and b are the long and the short diameters of the tumor, respectively. Additional details are available in Supplemental Experimental Procedures.

Cell Viability and Caspase-3 Assays

Cell viability following Apo2L/TRAIL treatment was determined in vitro using the Cell-titer Glo cell viability assay (Promega). Caspase-3/7 or 8 activity was measured in vitro using the Caspase-Glo 3/7 or Caspase-Glo 8 assay (Promega), according to the manufacturer's instructions. For in vitro viability and caspase assays, Apo2L and M2 were combined sequentially at a 1:1 molar ratio. Ex vivo caspase-3 processing in tumor cells was monitored by flow cytometry using the cleaved caspase-3-specific antibody (clone C92-605, BD PharMingen). Caspase-3 activation is represented as a fold-increase relative to control treated mice.

Immunohistochemistry for Mouse Specimens

Primary antibodies were used at 10 μg/ml for mouse DR5 (clone MD5-1, BD Biosciences; Franklin Lakes, NJ), 2 μg/ml for PLVAP (clone MECA-32, BD Biosciences, NJ), and 0.06 μg/ml for cleaved caspase 3 (Asp175) (Cell Signaling Technologies; Danvers, MA). Quantitation of caspase-3 and PLVAP immunostaining and additional details are described in Supplemental Experimental Procedures.

In Vivo Near Infrared Fluorescence Imaging

Two hr after treatment with Apo2L/TRAIL or PBS, mice (n = 3–5/treatment group) were injected intravenously with the fluorescent blood pool marker AngioSense680IVM (PerkinElmer). The temporal distribution of AngioSense680IVM within tumors and neighboring tissue was measured by visualizing fluorescence (650 nm excitation/700 nm emission) with a Kodak 4000 FX Pro imaging system (CareStream Health) and quantifying fluorescence intensities within regions of interest placed over tumor or adjoining tissue normalized to time = 0, $(I_{ROIt=x} - I_{BG}) / (I_{ROIt=0} - I_{BG})$. At each indicated time point, animals were anesthetized under isoflurane, with body temperature maintained at 37°C, and were imaged.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.05.014.

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REFERENCES

Abdulghani, J., and El-Deiry, W.S. (2010). TRAIL receptor signaling and therapeutics. Expert Opin. Ther. Targets *14*, 1091–1108.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat. Rev. Cancer *2*, 420–430.

Ashkenazi, A. (2008a). Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. Nat. Rev. Drug Discov. *7*, 1001–1012.

Ashkenazi, A. (2008b). Targeting the extrinsic apoptosis pathway in cancer. Cytokine Growth Factor Rev. *19*, 325–331.

Ashkenazi, A., and Herbst, R.S. (2008). To kill a tumor cell: the potential of proapoptotic receptor agonists. J. Clin. Invest. *118*, 1979–1990.

Ashkenazi, A., Pai, R.C., Fong, S., Leung, S., Lawrence, D.A., Marsters, S.A., Blackie, C., Chang, L., McMurtrey, A.E., Hebert, A., et al. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Invest. *104*, 155–162.

Baguley, B.C. (2011). Preclinical efficacy of vascular disrupting agents in non-small-cell lung cancer. Clin. Lung Cancer *12*, 81–86.

Brey, E.M., Lalani, Z., Johnston, C., Wong, M., McIntire, L.V., Duke, P.J., and Patrick, C.W., Jr. (2003). Automated selection of DAB-labeled tissue for immunohistochemical quantification. J. Histochem. Cytochem. *51*, 575–584.

Chan, J., Prado-Lourenco, L., Khachigian, L.M., Bennett, M.R., Di Bartolo, B.A., and Kavurma, M.M. (2010). TRAIL promotes VSMC proliferation and neointima formation in a FGF-2-, Sp1 phosphorylation-, and NFkappaB-dependent manner. Circ. Res. *106*, 1061–1071.

Chen, P.L., and Easton, A.S. (2010). Evidence that tumor necrosis factor-related apoptosis inducing ligand (TRAIL) inhibits angiogenesis by inducing vascular endothelial cell apoptosis. Biochem. Biophys. Res. Commun. *391*, 936–941.

Corti, A., and Ponzoni, M. (2004). Tumor vascular targeting with tumor necrosis factor alpha and chemotherapeutic drugs. Ann. N Y Acad. Sci. *1028*, 104–112.

Dejana, E. (2004). Endothelial cell-cell junctions: happy together. Nat. Rev. Mol. Cell Biol. *5*, 261–270.

Diehl, G.E., Yue, H.H., Hsieh, K., Kuang, A.A., Ho, M., Morici, L.A., Lenz, L.L., Cado, D., Riley, L.W., and Winoto, A. (2004). TRAIL-R as a negative regulator of innate immune cell responses. Immunity *21*, 877–889.

Frew, A.J., Lindemann, R.K., Martin, B.P., Clarke, C.J., Sharkey, J., Anthony, D.A., Banks, K.M., Haynes, N.M., Gangatirkar, P., Stanley, K., et al. (2008).

Combination therapy of established cancer using a histone deacetylase inhibitor and a TRAIL receptor agonist. Proc. Natl. Acad. Sci. USA *105*, 11317–11322.

Hallmann, R., Mayer, D.N., Berg, E.L., Broermann, R., and Butcher, E.C. (1995). Novel mouse endothelial cell surface marker is suppressed during differentiation of the blood brain barrier. Dev. Dyn. *202*, 325–332.

Haynes, N.M., Hawkins, E.D., Li, M., McLaughlin, N.M., Hämmerling, G.J., Schwendener, R., Winoto, A., Wensky, A., Yagita, H., Takeda, K., et al. (2010). CD11c+ dendritic cells and B cells contribute to the tumoricidal activity of anti-DR5 antibody therapy in established tumors. J. Immunol. *185*, 532–541.

Heath, V.L., and Bicknell, R. (2009). Anticancer strategies involving the vasculature. Nat. Rev. Clin. Oncol. *6*, 395–404.

Herbst, R.S., Eckhardt, S.G., Kurzrock, R., Ebbinghaus, S., O'Dwyer, P.J., Gordon, M.S., Novotny, W., Goldwasser, M.A., Tohnya, T.M., Lum, B.L., et al. (2010). Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer. J. Clin. Oncol. *28*, 2839–2846.

Jain, R.K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science *307*, 58–62.

Johnstone, R.W., Frew, A.J., and Smyth, M.J. (2008). The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat. Rev. Cancer *8*, 782–798.

Kischkel, F.C., Lawrence, D.A., Chuntharapai, A., Schow, P., Kim, K.J., and Ashkenazi, A. (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity *12*, 611–620.

Koebel, C.M., Vermi, W., Swann, J.B., Zerafa, N., Rodig, S.J., Old, L.J., Smyth, M.J., and Schreiber, R.D. (2007). Adaptive immunity maintains occult cancer in an equilibrium state. Nature *450*, 903–907.

Lara, P.N., Jr., Douillard, J.Y., Nakagawa, K., von Pawel, J., McKeage, M.J., Albert, I., Losonczy, G., Reck, M., Heo, D.S., Fan, X., et al. (2011). Randomized phase III placebo-controlled trial of carboplatin and paclitaxel with or without the vascular disrupting agent vadimezan (ASA404) in advanced non-small-cell lung cancer. J. Clin. Oncol. *29*, 2965–2971.

Lawrence, D., Shahrokh, Z., Marsters, S., Achilles, K., Shih, D., Mounho, B., Hillan, K., Totpal, K., DeForge, L., Schow, P., et al. (2001). Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat. Med. *7*, 383–385.

Li, J.H., Kirkiles-Smith, N.C., McNiff, J.M., and Pober, J.S. (2003). TRAIL induces apoptosis and inflammatory gene expression in human endothelial cells. J. Immunol. *171*, 1526–1533.

Lorusso, P.M., Boerner, S.A., and Hunsberger, S. (2011). Clinical development of vascular disrupting agents: what lessons can we learn from ASA404? J. Clin. Oncol. *29*, 2952–2955.

Mahajan, S., Dammai, V., Hsu, T., and Kraft, A.S. (2008). Hypoxia-inducible factor-2alpha regulates the expression of TRAIL receptor DR5 in renal cancer cells. Carcinogenesis *29*, 1734–1741.

McKeage, M.J., and Baguley, B.C. (2010). Disrupting established tumor blood vessels: an emerging therapeutic strategy for cancer. Cancer *116*, 1859–1871.

Pober, J.S., and Sessa, W.C. (2007). Evolving functions of endothelial cells in inflammation. Nat. Rev. Immunol. *7*, 803–815.

Seki, N., Hayakawa, Y., Brooks, A.D., Wine, J., Wiltrout, R.H., Yagita, H., Tanner, J.E., Smyth, M.J., and Sayers, T.J. (2003). Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis is an important endogenous mechanism for resistance to liver metastases in murine renal cancer. Cancer Res. *63*, 207–213.

Siemann, D.W., Chaplin, D.J., and Walicke, P.A. (2009). A review and update of the current status of the vasculature-disabling agent combretastatin-A4 phosphate (CA4P). Expert Opin. Investig. Drugs *18*, 189–197.

Singh, M., Lima, A., Molina, R., Hamilton, P., Clermont, A.C., Devasthali, V., Thompson, J.D., Cheng, J.H., Bou Reslan, H., Ho, C.C., et al. (2010). Assessing therapeutic responses in Kras mutant cancers using genetically engineered mouse models. Nat. Biotechnol. *28*, 585–593.

Strasser, A., O'Connor, L., and Dixit, V.M. (2000). Apoptosis signaling. Annu. Rev. Biochem. *69*, 217–245.

Takeda, K., Yamaguchi, N., Akiba, H., Kojima, Y., Hayakawa, Y., Tanner, J.E., Sayers, T.J., Seki, N., Okumura, K., Yagita, H., and Smyth, M.J. (2004). Induction of tumor-specific T cell immunity by anti-DR5 antibody therapy. J. Exp. Med. *199*, 437–448.

Tang, D.G., Chen, Y.Q., Newman, P.J., Shi, L., Gao, X., Diglio, C.A., and Honn, K.V. (1993). Identification of PECAM-1 in solid tumor cells and its potential involvement in tumor cell adhesion to endothelium. J. Biol. Chem. *268*, 22883–22894.

ten Hagen, T.L., Seynhaeve, A.L., and Eggermont, A.M. (2008). Tumor necrosis factor-mediated interactions between inflammatory response and tumor vascular bed. Immunol. Rev. *222*, 299–315.

Uno, T., Takeda, K., Kojima, Y., Yoshizawa, H., Akiba, H., Mittler, R.S., Gejyo, F., Okumura, K., Yagita, H., and Smyth, M.J. (2006). Eradication of established tumors in mice by a combination antibody-based therapy. Nat. Med. *12*, 693–698.

Wagner, K.W., Punnoose, E.A., Januario, T., Lawrence, D.A., Pitti, R.M., Lancaster, K., Lee, D., von Goetz, M., Yee, S.F., Totpal, K., et al. (2007). Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat. Med. *13*, 1070–1077.

Wilson, N.S., Dixit, V., and Ashkenazi, A. (2009). Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat. Immunol. *10*, 348–355.

Yang, A., Wilson, N.S., and Ashkenazi, A. (2010). Proapoptotic DR4 and DR5 signaling in cancer cells: toward clinical translation. Curr. Opin. Cell Biol. *22*, 837–844.