The concept that tumors can be controlled by directly targeting their vascular supply has finally come of age, because clinical trials using a humanized monoclonal antibody that blocks VEGF have demonstrated exciting efficacy in cancer patients, as well as in vascular eye diseases that can lead to blindness. However, data suggest that these current regimens may not provide complete VEGF inhibition and, thus, that the maximum therapeutic potential of VEGF blockade has not yet been achieved. We describe the status of a very potent and high-affinity VEGF blocker, termed the VEGF Trap, that may provide the opportunity to maximize the potential of VEGF blockade in cancer as well as in vascular eye diseases. We also describe use of the VEGF Trap as a research tool, when coupled to high-throughput mouse genetics approaches such as *VelociGene*® that can be exploited in strategies to discover and validate the next generation of angiogenesis targets.

The concept that tumors can be controlled by directly targeting their vascular supply has finally come of age. The first antiangiogenesis approach to be validated in human cancer patients involves blocking vascular endothelial growth factor (VEGF-A). In this regard, the most advanced clinical data have been generated with a humanized monoclonal antibody termed bevacizumab (Avastin) that directly binds and blocks all isoforms of VEGF-A (Ferrara et al. 2004). Despite the promising data achieved to date, dose-response studies suggest that higher doses of bevacizumab may provide even greater benefit (Yang et al. 2003; Yang 2004), implying that current bevacizumab regimens may not provide optimal VEGF inhibition and thus may not have yet demonstrated the maximum potential of VEGF blockade in cancer. In addition to the promise of anti-VEGF approaches in cancer, blocking VEGF-A has also been impressive in maintaining and improving vision in wet age-related macular degeneration (AMD), a disease marked by leaky and proliferating vessels which distort the retina, and these data suggest that VEGF blockade may provide benefit in other eye diseases involving vascular leak and proliferation (Bergsland 2004). Efficacy in wet AMD has most notably been achieved using a modified fragment of the bevacizumab antibody, termed ranibizumab (Lucentis), delivered via monthly intraocular injections (Brown et al. 2006; Heier et al. 2006).

In this paper, we focus on the development and status of a novel VEGF-blocking agent, termed the VEGF Trap, that retains many of the advantages of a blocking antibody but may offer further potential (Holash et al. 2002). The VEGF Trap consists of portions of VEGF receptors that have been fused to the constant region of an antibody, resulting in a fully human biologic with exceedingly high affinity that blocks not only all isoforms of VEGF-A, but also related VEGF family members such as placental growth factor (PlGF). The VEGF Trap also displays ex tended pharmacological half-life, allowing long-term a well as very high affinity blockade. The VEGF Trap ha performed impressively in extensive animal studies c cancer and eye diseases, and initial clinical trials appe promising. The VEGF Trap may provide the opportuni to explore the potential of more complete VEGF bloc ade in cancer, as well as the opportunity for more com plete blockade and even longer-interval dosing regimen in eye diseases. To conclude this paper, we describe ho the VEGF Trap can be used as a research tool in efforts discover and validate the next generation of targets in th field of angiogenesis.

DISCOVERY OF VEGF AND ITS REQUISITE ROLES DURING NORMAL DEVELOPMENT AND IN DISEASE SETTINGS

Initial studies by Dvorak and his colleagues (Senger al. 1986; Dvorak et al. 1999) identified a protein in tum ascites fluid that was capable of inducing vascular leak ar permeability, which they termed vascular permeability fa tor (VPF). Independent efforts by Ferrara and his co leagues to identify secreted factors that could promo tumor angiogenesis led to the discovery of a protein bovine pituitary follicular cell conditioned medium wi mitogenic properties for endothelial cells which the termed vascular endothelial cell growth factor (VEGF (Ferrara and Henzel 1989; Leung et al. 1989). Upon s quencing and further studies, this VEGF protein was une pectedly found to correspond to the VPF previously ide tified by the Dvorak lab. These findings set the stage for concerted effort to define the role of VEGF/VPF (hereo VEGF) in cancer angiogenesis as well as other settings vascular disease, which have led to the realization that bo of its initially realized actions—i.e., promoting vascul

ably, disruption of even a single VEGF allele in developing mice, which decreases VEGF levels by half, also results in embryonic lethality due to severe vascular abnormalities (Carmeliet et al. 1996; Ferrara et al. 1996), demonstrating the need for exquisite regulation of VEGF levels to form normal vessels. Reciprocally, modest increases in VEGF levels during development also lead to vascular disaster and lethality (Miquerol et al. 2000). VEGF continues to be critical during early postnatal growth and development, as evidenced by the lethality and major growth disturbances caused by conditional disruption of the VEGF gene or by administration of VEGF blockers (Ravindranath et al. 1992; Carmeliet et al. 1996; Ferrara et al. 1996, 1998; Gerber et al. 1999a; Ryan et al. 1999; Fraser et al. 2000; Zimmermann et al. 2001; Hazzard et al. 2002; Eremina et al. 2003). However, VEGF blockade in older animals is much less traumatic, affecting only those structures that continue to depend on ongoing vascular remodeling, such as occurs in bone growth plates or during remodeling of the female reproductive organs (Ferrara et al. 1998; Gerber et al. 1999a,b). As discussed in greater detail below, vascular remodeling is absolutely required in a variety of pathological settings, such as during tumor growth, providing major therapeutic opportunities for VEGF blockade in the adult setting in which such blockade can be tolerated.

VEGF ISOFORMS, VEGF FAMILY MEMBERS, AND VEGF RECEPTORS

Further study of the gene encoding human VEGF revealed eight exons separated by seven introns, which results in the generation of four isoforms of increasing size—$VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (subscripts refer to number of amino acids comprising the isoform, with the VEGF isoforms varying in length at their carboxyl termini). The main purpose of these isoforms appears to relate to their bioavailability such that the 121 isoform is diffusible, whereas the higher-molecular-weight isoforms remain bound to the extracellular matrix, requiring cleavage to be released (Houck et al. 1992; Park et al. 1993; Keyt et al. 1996).

Because of the discovery of additional members of the VEGF family, VEGF is now often referred to as VEGF-A. Other members of the VEGF family were

2000). Little is known about VEGF-B, and mice lacki
VEGF-B are overtly healthy and fertile. VEGF-C and
seem to play more critical roles in the lymphatic vas
lature than in the blood vasculature, showing specific
for a VEGF receptor (see below) expressed on this v
culature; administration of both of these factors leads
lymphatic vessel hyperplasia (Joukov et al. 1996; Orla
dini et al. 1996; Olofsson et al. 1999).

Following rapidly on the heels of the discovery
VEGF came the identification of two closely rela
high-affinity receptors for VEGF—FLT1 (FMS-like
rosine kinase) now termed VEGFR1 (de Vries et
1992), and KDR or Flk1, now termed VEGFR2 (Shibu
et al. 1990; Terman et al. 1992; Millauer et al. 199
These high-affinity receptors share features of ma
other growth factor receptors, in that they contain an
tracellular domain which binds and is dimerized by l
and, and a cytoplasmic tyrosine kinase domain that can
regulated upon binding of ligand to the extracellular
main. VEGFR2 seems to be the receptor which media
the major growth and permeability actions of VEG
whereas VEGFR1 may have a negative role, either
acting as a decoy receptor or by suppressing signali
through VEGFR2. Thus, mice engineered to la
VEGFR2 fail to develop a vasculature and have very f
endothelial cells (Shalaby et al. 1995), phenocopyi
mice lacking VEGF, whereas mice lacking VEGF
seem to have excess formation of endothelial cells t
abnormally coalesce into disorganized tubules (Fong
al. 1995). Mice engineered to express only a truncat
form of VEGFR1, lacking its kinase domain, appe
rather normal, consistent with the notion that the prima
role of VEGFR1 may be that of a decoy receptor (Hir
suka et al. 1998), and supporting only a minor role for
cytoplasmic kinase domain. The third member of this
ceptor family, initially called Flt-4 and now term
VEGFR3, does not bind to VEGF-A nor PlGF, and
stead binds to VEGF-C and VEGF-D and seems to me
ate the actions of these latter two factors on the lympha
vasculature (Taipale et al. 1999).

In addition to these primary receptors, a number of p
tential accessory receptors for the VEGFs have be
identified, although the requisite roles of these recept
in mediating VEGF responses have not been clearly e
cidated. These potential accessory receptors include
neuropilins (Soker et al. 1998).

out of these studies came the interesting finding that one tumor type, renal cell carcinoma, had particularly high VEGF expression which correlated with inactivation of the von Hippel Lindau locus, resulting in loss of control of the tumor's oxygen sensor, hypoxia-inducible factor (HIF) (Iliopoulos et al. 1996; Lonser et al. 2003). The up-regulation of VEGF in an attempt to reoxygenate the tumor through revascularization led to the belief that this tumor may either be highly sensitive to anti-VEGF therapy or highly refractory. Fortunately, the former seems to be the case (Yang et al. 2003).

Concomitant with the analysis of human tumors for VEGF expression came the development of animal models of cancer where the hypothesis that VEGF was required for tumor vasculature, and thus tumor growth, could be tested. In 1993, 4 years after their discovery of VEGF, Ferrara and colleagues demonstrated that a mouse monoclonal antibody to human VEGF (A.4.6.1) could inhibit the growth of several human tumor types in nude mice with inhibition ranging from 70% to more than 90% (Kim et al. 1993). Subsequent to this observation, a number of laboratories using different strategies to inhibit VEGF signaling have shown to a greater or lesser extent that inhibition of VEGF can have a major impact on tumor growth in mice. In addition to numerous studies using the VEGF-blocking antibody, other strategies to block VEGF in tumor models included blocking antibodies targeting VEGFR2 (Prewett et al. 1999), soluble VEGF receptors acting as circulating decoys to capture VEGF and preventing it from binding cell-surface receptors (Ferrara et al. 1998; Gerber et al. 1999a,b; Liang et al. 2006), dominant-negative VEGF receptors expressed at high levels on tumor surfaces, small-molecule inhibitors of VEGF receptor kinases and other kinases (Smith et al. 2004), antisense oligonucleotides targeting VEGF, and VEGF siRNA (Grunweller and Hartmann 2005; Lu et al. 2005).

As the number of studies increased comparing the different modes of inhibiting VEGF, it became apparent that blocking tumor-derived VEGF without blocking stromal VEGF was not as efficacious, implicating stromal VEGF as a crucial player in tumor growth and angiogenesis. Thus, antibodies such as A.4.6.1 which only block human VEGF did not fare as well in blocking human tumor growth in immunocompromised mice as reagents blocking both tumor and host stroma-derived VEGF (Gerber et al. 2000; Liang et al. 2006).

tumor experiments this VEGFR1-Fc reagent was effic
cious at approximately 500-fold lower concentratio
than a similar VEGFR2-Fc construct (Kuo et al. 2001
Despite its high affinity, the VEGFR1-Fc was not a fe
sible clinical candidate because of its poor pharmac
kinetic profile; in rodent studies, this protein had to b
administered frequently and at very high doses
achieve efficacious levels. In addition, this agent a
peared to have nonspecific toxicity effects that did n
seem to be accounted for by its blocking of VEGF (Ku
et al. 2001). We decided to exploit our Trap technolog
platform (Economides et al. 2003), which involv
defining and fusing minimal binding units from diffe
ent receptor components to generate chimeric fusio
proteins that act as high-affinity soluble blockers, in a
attempt to create a potent and well-behaved Trap f
VEGF. The result was a chimeric fusion protein co
taining a modified domain 2 of VEGFR1 and the thi
Ig domain of VEGFR2 fused to the Fc region of huma
IgG1, resulting in a fully human protein that we ter
VEGF Trap (Holash et al. 2002). This reagent has th
advantage of being fully human and thus potential
non-immunogenic, as well as being substantial
smaller than previous fusion proteins and antibodie
raising the possibility that it might allow improved ti
sue and tumor penetration. In addition, this VEGF Tra
had greatly improved pharmacological bioavailabili
as compared to the initial VEGFR1-Fc reagent, exhibi
ing about a 300-fold increase in the maximum concen
tration achieved in the circulation (i.e., C_{max}), as well a
about a 1000-fold increase in total circulation exposu
(i.e., AUC) (Holash et al. 2002). Importantly, the affi
ity of VEGF Trap binding to both mouse and huma
VEGF isoforms (0.58 pM, 0.46 pM) was superior to th
of the parental VEGFR1-Fc (~ 5–20 pM) (Holash et a
2002). In addition, the VEGF Trap also bound PlG
with high affinity (1.8 pM).

To determine whether the improved pharmacologic
bioavailabity and high-affinity binding of VEGF Tra
translated into superior performance in vivo, we fir
used a short-term and quantitative in vivo model
VEGF activity in which a single dose of VEGF induc
a stereotypic reduction in blood pressure. In this acu
assay model, we found that equivalent doses of VEG
Trap were indeed far superior to that of the parent
VEGFR1-Fc (Holash et al. 2002).

almost every case. In addition to its activity in multiple subcutaneous models of melanoma, glioma, and rhabdomyosarcoma tumors (Holash et al. 2002), the VEGF Trap has been shown to work in multiple pancreatic cancer models (Fukasawa and Korc 2004), Wilms' tumor (Huang et al. 2003), Ewing's sarcoma (Dalal et al. 2005), glioblastoma (Wachsberger et al. 2005), and models of ovarian cancer as well as associated malignant ascites (Hu et al. 2005).

In addition to the above published studies, recent unpublished temporal studies indicate that vascular regression can be seen in most tumors within hours of VEGF Trap treatment, resulting in marked and widespread hypoxia within the tumors. In addition, transcription profiling studies during these temporal studies have revealed a set of endothelial-specific genes that are rapidly and profoundly regulated in response to VEGF Trap treatment. Further studies on some of these genes have led to their identification as potential targets for new antiangiogenesis therapies (see below).

In summary, animal tumor studies have indicated that treatment with VEGF Trap effectively inhibited tumor growth of a wide variety of murine, rat, and human tumor cell lines implanted either subcutaneously or orthotopically in mice. VEGF Trap treatment inhibited the growth of tumors representing a variety of tumor types, including melanoma, glioma, rhabdomyosarcoma, ovarian, pancreatic, renal, and mammary tumor tissue, with a broad therapeutic index. Growth of small established tumors was also inhibited. Histological analysis indicated that treatment with VEGF Trap resulted in the formation of largely avascular and necrotic tumors, demonstrating that tumor-induced angiogenesis was blocked. VEGF Trap was also active in blocking tumor growth in similar animal tumor models in combination with paclitaxel, docetaxel, or radiation, and was synergistic with 5-fluorouracil. VEGF Trap as a single agent and in combination with paclitaxel also prevented the formation of ascites in mouse tumor models (Byrne et al. 2003; Hu et al. 2005).

VEGF TRAP IN CLINICAL TRIALS FOR CANCER

The above results in animal tumor models supported the exploration of the VEGF Trap in human studies. Initial clinical studies are promising (Dupont et al. 2005;

EFFICACY IN PRECLINICAL MODELS OF VASCULAR EYE DISEASES

In addition to the role for VEGF in tumor angiogenes a variety of studies have indicated that VEGF may pla key pathological role in vascular eye diseases, in parti lar in diabetic edema and retinopathy settings, and in a related macular degeneration (AMD), which are lead causes of vision loss and blindness. In these diseases, cess VEGF is thought to result in vascular leak that c tributes to abnormal swelling of the retina and result vision impairment, as well as in the abnormal growth choroidal and retinal vessels that can destroy normal r nal architecture. Consistent with these possibilities, VEGF Trap has demonstrated impressive efficacy in assortment of animal models of these eye diseases.

Preclinical studies in rodents have shown that VE Trap can inhibit choroidal (Saishin et al. 2003) a corneal (Wiegand et al. 2003) neovascularization, as w as suppress vascular leak into the retina (Qaum et 2001), and that the VEGF Trap can also promote the s vival of corneal transplants by inhibiting associated n vascularization (Cursiefen et al. 2004). In addition, i primate model of AMD, in which choroidal neovascu lesions and vascular leak are induced by using a laser create small lesions in the retinas of adult cynomolg macaques, both systemically and intravitreally deliver VEGF Trap not only prevented development of vascu leak and neovascular membranes when administer prior to laser lesion, but also induced regression when a ministered after lesions had developed (Wiegand et 2005). These preclinical results support a role for VE blockade, and in particular for local delivery of the VE Trap, in multiple vascular eye diseases ranging fr AMD and diabetic eye diseases to corneal injury a transplantation.

VEGF TRAP IN CLINICAL TRIALS FOR VASCULAR EYE DISEASES

The above results in animal models have supported exploration of the VEGF Trap in human studies of vas lar eye diseases. Initial clinical studies in human patie suffering from both AMD and diabetic edema a retinopathy appear quite promising, with evidence early trials that the VEGF Trap can rapidly and impr sively decrease retinal swelling, and that these chan

other tumors can continue to grow even in the face of anti-VEGF treatments. The realization that some tumors can be relatively resistant to anti-VEGF approaches raises the need for additional antiangiogenesis approaches that might be useful in such settings. Toward this end, as noted above, we performed transcriptional profiling screens to identify endothelial-specific targets that are markedly regulated either by VEGF blockade or by excess VEGF activity, reasoning that such targets might prove interesting as new antiangiogenesis targets. Confirming the potential of such a screen, one target that was "rediscovered" via such screens was Angiopoietin-2. We had previously independently identified the Angiopoietins as key new angiogenic regulators that seemed to work in tandem with the VEGFs (Davis et al. 1996; Suri et al. 1996; Maisonpierre et al. 1997; Valenzuela et al. 1999; Yancopoulos et al. 2000; Gale et al. 2002), and moreover, obtained substantial data that Angiopoietin-2 in particular was specifically induced in tumor vasculature and that it was important for tumor angiogenesis (Holash et al. 1999); a recent study employing Angiopoietin-2-blocking antibodies confirmed notable antitumor effects (Oliner et al. 2004). On the basis of the confidence in these transcriptional profiling screens engendered by the reidentification of Angiopoietin-2, we explored additional potential targets identified by the screens. Among these targets we have reported the identification of Delta-like ligand 4 (Dll4) (a ligand for the Notch family of receptors) as a gene that is markedly and specifically induced in tumor vasculature (Gale et al. 2004). Moreover, Dll4 is strikingly up-regulated in VEGF-overexpressing tumors and down-regulated in tumors by VEGF blockade. Using VelociGene® technology, which provides a high-throughput approach to create mouse mutants for genes of interest (Valenzuela et al. 2003), we found that mice lacking Dll4 exhibit profound vascular defects early in development (Gale et al. 2004). Remarkably, and as previously seen only for VEGF (see above), deletion of even just one of the two Dll4 alleles in developing embryos resulted in embryonic lethality due to vascular defects (Gale et al. 2004). All this evidence for a critical role for Dll4 in normal as well as tumor angiogenesis provided a rationale to develop blockers for Dll4. Recent testing in tumor models indicates that Dll4 may indeed prove to be an important new antiangiogenesis target, either alone or in combination with the VEGF Trap, or in settings of relative resistance to anti-VEGF therapies.

sion in some settings. In other preclinical cancer model
we have found that combination of VEGF Trap with a cy
totoxic agent can result in potency far greater than that
either single agent. Furthermore, the VEGF Trap is als
very effective in animal models of vascular eye disease
The impressive efficacy in preclinical models of canc
and eye diseases provided a rationale for advancement
the VEGF Trap into clinical trials, where it is producin
promising initial results in both cancer and eye diseases

In addition to its potential therapeutic value in canc
and vascular eye diseases, the VEGF Trap is also an i
valuable research tool. Transcription profiling screens u
ing VEGF Trap have allowed a number of strategies d
signed to identify new antiangiogenesis targets. It
hoped that these strategies are helping to identify the ne
generation of antiangiogenesis targets, which may wo
either alone or in combination with the VEGF Trap, or
settings of relative resistance to anti-VEGF therapies.

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