Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: Implications for cellular surrogate marker analysis of antiangiogenesis

Yuval Shaked,[1] Francesco Bertolini,[2] Shan Man,[1] Michael S. Rogers,[3] Dave Cervi,[1,4] Thomas Foutz,[2] Kimberley Rawn,[1] Daniel Voskas,[1,4] Daniel J. Dumont,[1,4] Yaacov Ben-David,[1,4] Jack Lawler,[5] Jack Henkin,[6] Jim Huber,[7] Daniel J. Hicklin,[7] Robert J. D'Amato,[3] and Robert S. Kerbel[1,4,*]

[1]Department of Molecular and Cellular Biology, Sunnybrook and Women's College Health Sciences Centre, Toronto, Ontario M3N 4M5, Canada
[2]Department of Hematology-Oncology, European Institute of Oncology, Milan 20141, Italy
[3]Vascular Biology Program, Children's Hospital, and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts 02115
[4]Department of Medical Biophysics, University of Toronto, Toronto, Ontario M5G 2M9, Canada
[5]Department of Pathology, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215
[6]Abbott Laboratories, 100 Abbott Park Road, North Chicago, Illinois 60064
[7]ImClone Systems Incorporated, New York, New York 10014
*Correspondence: robert.kerbel@sw.ca

Summary

Development of antiangiogenic therapies would be significantly facilitated by quantitative surrogate pharmacodynamic markers. Circulating peripheral blood endothelial cells (CECs) and/or their putative progenitor subset (CEPs) have been proposed but not yet fully validated for this purpose. Herein, we provide such validation by showing a striking correlation between highly genetically heterogeneous bFGF- or VEGF-induced angiogenesis and intrinsic CEC or CEP levels measured by flow cytometry, among eight different inbred mouse strains. Moreover, studies using genetically altered mice showed that levels of these cells are affected by regulators of angiogenesis, including VEGF, Tie-2, and thrombospondin-1. Finally, treatment with a targeted VEGFR-2 antibody caused a dose-dependent reduction in viable CEPs that precisely paralleled its previously and empirically determined antitumor activity.

Introduction

Antiangiogenic therapy is emerging as a promising treatment strategy, particularly when combined with chemotherapy for the treatment of certain malignancies, e.g., metastatic colorectal carcinoma (Hurwitz et al., 2004). The process that is the target of this therapeutic approach—formation of new blood vessel capillaries in tumors—is thought to be governed primarily by the balance of proangiogenic growth factors, such as vascular endothelial growth factor (VEGF) and endogenous inhibitors of angiogenesis, e.g., thrombospondin-1 (TSP-1) (Hanahan and Folkman, 1996; Bouck et al., 1996; Kerbel and Kamen, 2004). Many of the known proangiogenic regulators act as signaling molecules to induce the division and migration and promote the survival of new differentiated endothelial cells from adjacent mature host vasculature, which together initiate the process of localized "sprouting angiogenesis." In addition, there is thought to be an alternative source for some of the endothelial cells comprising newly forming blood vessel capillaries, namely bone marrow, from which putative VEGF receptor-2-positive endothelial progenitor cells (EPCs) can be mobilized by various cytokines or growth factors, including VEGF (Asahara et al., 1999). These cells enter the peripheral blood circulation to become circulating endothelial progenitors (CEPs) and subsequently incorporate into distal sites of ongoing sprouting angiogenesis, where they can differentiate into mature endothelial cells (Asahara et al., 1997; Rafii et al., 2002; Lyden et al., 2001). This systemic process is referred to as adult or postnatal vasculogenesis, and it appears to variably contribute to the growth and development of new blood vessels, including the neovasculature of tumors (Rafii et al., 2002; Ruzinova et al., 2003; Sikder

SIGNIFICANCE

The early phases of anticancer drug development, especially cytotoxic chemotherapeutic agents, have been largely guided by induction of dose-limiting toxicities and objective responses. In contrast, most antiangiogenic drugs do not cause significant tumor shrinkage and frequently have optimal therapeutic activity below a maximum tolerated dose, when such a dose can be defined. Thus, alternative surrogate pharmacodynamic markers of activity are urgently needed; a blood test would be ideal in this regard. Our results bolster the rationale of using circulating peripheral blood VEGFR-2$^+$ subpopulations as valid cellular surrogates for the highly complex process of new blood vessel formation and its therapeutic inhibition, including determination of optimally efficacious doses for antiangiogenic drugs, or treatment strategies.

et al., 2003; Lyden et al., 2001). CEPs represent a subset of mature VEGF receptor-2-positive circulating endothelial cells (CECs) also found in peripheral blood (Bertolini et al., 2003).

From a cancer treatment perspective, there are a number of reasons for the current interest in circulating peripheral blood cells that contribute to angiogenesis, but two in particular stand out. First, it now appears that various antiangiogenic drugs, or treatment strategies, may work, at least in part, by interfering with the systemic process of CEP mobilization and function, and not necessarily only through suppression of local sprouting angiogenesis (Stoll et al., 2003; Bertolini et al., 2003; Schuch et al., 2003). Second, assessment of levels of these cells, either CEPs or CECs, has the potential to be used as a much-needed surrogate pharmacodynamic marker to monitor antiangiogenic drug activity (Willett et al., 2004); if so, evaluation of these cells might also be used to determine the optimal biologic/therapeutic dose for antiangiogenic drugs.

The problem associated with selecting an efficacious dose, or to be more precise, an optimal biologic dose (OBD), for many, if not most, antiangiogenic drugs or treatments deserves special mention, as it represents one of the more serious challenges to the successful clinical development of such drugs (Cristofanilli et al., 2002; McDonald et al., 2004; Parulekar and Eisenhauer, 2004). The reason is that many such drugs have optimal therapeutic activity below a maximum tolerated dose (MTD), when such a dose can actually be defined; in addition, it is not common to detect rapid "objective tumor responses," i.e., tumor shrinkage of 50% or more in a significant percentage of treated patients when treating advanced stage cancers with an antiangiogenic drug alone (Cristofanilli et al., 2002). Such criteria—MTD/dose-limiting toxicities, and rapid tumor shrinkage—have been used as surrogates for decades to establish the clinical doses used for conventional cytotoxic chemotherapy drugs (Cristofanilli et al., 2002; Park et al., 2004). Thus, alternative surrogate markers are needed to help select the optimal doses for many different antiangiogenic drugs or treatments, in order to minimize the level of empiricism in their clinical development; measuring CECs/CEPs in samples of peripheral blood could clearly represent such a marker for antiangiogenic agents.

However, there is considerable controversy about the nature and function of putative circulating endothelial progenitor cells, and as a consequence, their usefulness as surrogate pharmacodynamic markers of angiogenesis could be called into question. For example, based on transplantation studies in mice using genetically marked donor bone marrow cells transferred to syngeneic, lethally irradiated recipients, estimates of the levels of genetically marked integrated marrow-derived endothelial cells in newly formed blood vessels have been reported to be as high as 50% (Garcia-Barros et al., 2003), while other studies show somewhat lower, but nevertheless substantial levels, e.g., 10%–20% (Asahara et al., 1997; Crosby et al., 2000); in contrast, other studies report much lower levels, e.g., 5% to essentially nondetectable (Ziegelhoeffer et al., 2004; Machein et al., 2003). One recently proposed explanation to account for these variable results raises a potentially significant challenge to the concept of adult vasculogenesis. It is based primarily on the application of more definitive cell detection methods, such as laser-scanning confocal microscopy, that have shown that various types of angiogenesis-promoting bone marrow-derived circulating peripheral blood cells can adhere very closely to the lumen of new blood vessels—but not actually integrate within such vessels to become endothelial cells. Such adherent cells may include monocytes, macrophages, or pericyte precursors (Ziegelhoeffer et al., 2004; Voswinckel et al., 2003; Rehman et al., 2003; Rajantie et al., 2004; Gothert et al., 2004; Heil et al., 2004). Yet another source of controversy comes from some recent studies using spontaneously arising mouse tumors, which, depending on tumor type and stage of disease, appear in some cases to be much less dependent on bone marrow-derived CEPs for tumor growth, in comparison to subcutaneous transplantable tumor models (Ruzinova et al., 2003; Sikder et al., 2003; Li et al., 2004). Thus, it is clear that further studies are required to show that circulating CECs and CEPs can be used as valid biomarkers for angiogenesis and antiangiogenic drug activity. This was the main purpose of our experiments.

The first approach we took to assess circulating VEGFR-2^+ CECs and putative CEPs as valid peripheral blood cellular markers of angiogenesis was based on the genetic heterogeneity of the angiogenic response in mice. Among the many mechanisms that can affect the ability to respond to anticancer drugs—including antiangiogenic agents—are host pharmacogenomic factors (Rohan et al., 2000). For example, in a seminal study by Rohan et al., a total of 31 different inbred strains of mice, including F_1 hybrids, were analyzed for their relative ability to induce a basic fibroblast growth factor (bFGF)- or VEGF-mediated angiogenic response, using the corneal neovascular micropocket assay (Rohan et al., 2000). Variation of up to 10-fold was observed, with some strains, such as C57BL/6J, being relatively deficient and others, such as 129/SvImJ, showing strong angiogenic responsiveness (Rohan et al., 2000).

Given these aforementioned results, we asked if a similar genetic heterogeneity exists with respect to the potential to mediate vasculogenesis and, if so, whether it parallels angiogenesis. An affirmative answer would strengthen the hypothesis that VEGFR-2^+ peripheral blood circulating cells contribute to the formation of new blood vessels and would provide further compelling rationale for evaluating levels and viability of putative CEPs as a useful pharmacodynamic surrogate marker not only for monitoring the activity of antiangiogenic treatment strategies (Bertolini et al., 2001, 2003; Willett et al., 2004), but also for determining the all important optimal biologic dose. Addressing the latter point constituted a second approach for our studies, in which we undertook retrospective and concurrent dose response studies with an antiangiogenic drug to determine whether a strict correlation exists between optimal antitumor dose and the suppressive effects on VEGFR-2^+ peripheral blood cells.

We therefore tested a large number of different inbred strains of mice for intrinsic levels of nonhematopoietic CEPs, i.e., $CD13^+$/VEGFR-2^+/$CD45^-$/$CD117^+$ in peripheral blood using a four-color flow cytometry assay (Bertolini et al., 2003), which we term the intrinsic "peripheral blood vasculogenic phenotype." In addition, we measured levels of nonhematopoietic CECs, i.e., $CD13^+$/VEGFR-2^+/$CD45^-$, of which CEPs represent a subset. Our results show a pronounced heterogeneity of the intrinsic levels of CEPs or CECs per milliliter of blood, the nature of which is remarkably correlated with the angiogenesis results reported by Rohan et al. (2000), as well as new results we report here. Furthermore, additional results suggest that both CEP and CEC heterogeneity may be explained, at least in part, by the action of well-known stimulators and inhibitors of sprouting angiogenesis, such as VEGF and TSP-1. Finally, we show a

striking retrospective analysis of the utility of measuring CEPs in relation to the problem of establishing optimal biologic antiangiogenic drug dose, using a validated and specific VEGF receptor-2 (*flk-1*)-targeted antiangiogenic agent. Taken together, the results show that measuring VEGFR-2$^+$ peripheral blood cells can be used as a surrogate for the complex process of angiogenesis and its therapeutic inhibition, including establishing the optimal biologic dose for targeted antiangiogenic drugs.

Results

Background to the experimental approach and rationale

In previous studies, some of us (F.B., Y.S., R.S.K.) have used four-color flow cytometry to detect CECs, designated as CD13$^+$/VEGFR-2$^+$/CD45$^-$, and CEPs that have a similar phenotype except for being positive to c-kit (i.e., CD117$^+$), which is present on stem cells and progenitors (Bertolini et al., 2003; Capillo et al., 2003; Monestiroli et al., 2001). As the main purpose of these studies was to assess whether measurement of such cells represents a valid surrogate approach for neovascularization and not to elucidate their precise angiogenic function or identity, we adopted the following strategy: (1) assess whether levels of CECs and/or CEPs are genetically heterogeneous; (2) determine if such heterogeneity retrospectively tracks with previously determined-induced angiogenesis results in the eye or prospectively induced angiogenesis stimulated by bFGF subcutaneously and measured by a perfusion assay, or by VEGF in the eye; (3) evaluate whether several well-known and validated molecular regulators of angiogenesis also regulate intrinsic levels of CECs and CEPs; (4) assess if the previously determined optimal therapeutic (antitumor) dose of a targeted antiangiogenic drug is accurately reflected by measurements of declines in CEPs; and (5) determine if CECs and CEPs are significantly reduced in tumor-bearing mice by the same targeted drug at the optimal dose.

Sprouting angiogenesis among different inbred mouse strains in the corneal neovascular micropocket assay induced with VEGF

Measurement of sprouting angiogenesis in numerous inbred mouse strains was previously assessed by Rohan et. al. using a corneal neovascular micropocket assay with bFGF as the stimulus (Rohan et al., 2000). We conducted a similar experiment in which we examined eight inbred mouse strains and assessed sprouting angiogenesis using the same method, i.e., corneal neovascular micropocket assay, but in this case we used 180 ng VEGF as the stimulus, instead of 10 ng bFGF (as described in the Experimental Procedures). The results in Table 1 show that the sprouting vessel area on the cornea induced by 180 ng VEGF pellets is comparable to the results that were reported previously (Rohan et al., 2000). Both VEGF and bFGF pellets have shown a range of up to ~10-fold differences in the ability to induce angiogenesis. Thus, a strain such as 129/SvImJ, which was previously shown to have strong angiogenic ability using the bFGF as the angiogenic stimulus, was found to have strong angiogenic ability when VEGF was used as the stimulus. Similarly, strains such as C57BL/6J, C3H/HeJ, and CD-1, previously shown to express low levels of bFGF-induced angiogenic responsiveness, had a similar limited ability to induce angiogenesis when VEGF was used as the stimulus.

Table 1. Corneal neovascular area induced by VEGF in different mouse strains

Strain	Vessel area induced by 180 ng VEGF
129/SvImJ	2.2 ± 0.4**
DBA/2J	1.4 ± 0.3**
BALB/cJ	1.0 ± 0.2**
FVB/NJ	0.9 ± 0.1*
CBA/J	0.8 ± 0.1
C57BL/6J	0.8 ± 0.2
C3H/HeJ	0.8 ± 0.1
CD-1	0.8 ± 0.2

The average vessel area (mean ± SD) in mm^2 in response to pellets of 180 ng VEGF is listed for various strains of mice ($n > 9$ eyes/strain). The strains are ranked (highest to lowest). *Significantly different ($p < 0.05$) from C57BL/6J; **significantly different ($p < 0.05$) from all other strains.

Assessment of CECs/CEPs in the peripheral blood of different inbred mouse strains

We next undertook an analysis of levels of CECs/CEPs among eight different inbred mouse strains, selecting strains representative of "high," "medium," and "low" angiogenic responsiveness, as assessed by the corneal micropocket angiogenesis assay. For this purpose, a group of 9- to 12-week-old males from different mouse strains, as summarized in Figure 1, were examined for levels of CECs (Figure 1A) and CEPs (Figure 1B), by using flow cytometry (as described in the Experimental Pro-

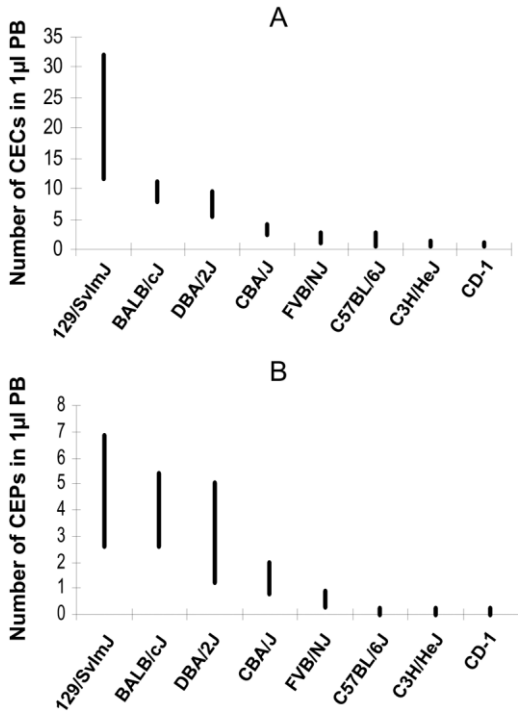

Figure 1. The evaluation of CECs/CEPs in different mouse strains

Four to six males per group, 9–12 weeks of age, from different mouse strains, as described in the figure, were bled by cardiac puncture and evaluated for the number of CECs (**A**) and CEPs (**B**) in 1 μl peripheral blood (PB) using a four-color flow cytometry assay.

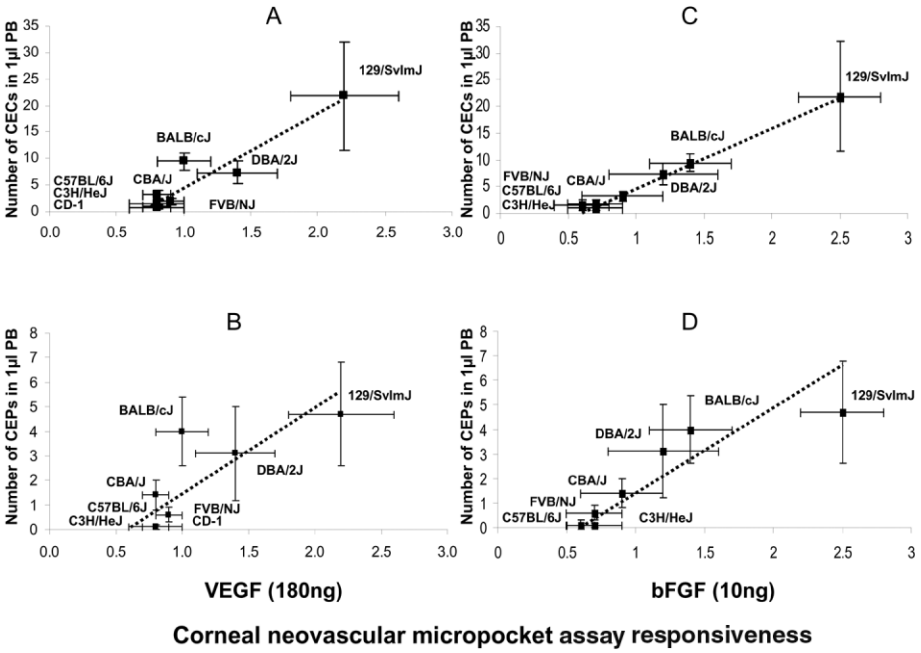

Figure 2. The correlation between angiogenic responses evaluated either by VEGF or bFGF with the number of peripheral blood CECs/CEPs

Correlation with angiogenic responsiveness was conducted by plotting the results obtained from the corneal neovascular micropocket assay and the levels of CEC/CEP. x axis values represent the average (mean ± SD) of neovascularization vessel area in mm^2 stimulated by either 180 ng VEGF (results from Table 1) or 10 ng bFGF, as previously reported (Rohan et al., 2000). y axis values represent the baseline number (mean ± SD) levels of cells/µl peripheral blood of either CECs (from Figure 1A) or CEPs (from Figure 1B) of the same strains.

cedures). The results in Figure 1 demonstrate a striking correlation between the angiogenic ability assessed by the corneal neovascular micropocket assay and the absolute number of CECs. Thus, the 129/SvImJ mouse strain, shown to have the strongest angiogenic ability among the strains tested (Table 1 and Rohan et al., 2000), also expressed the highest numbers of CECs. In contrast, mice of C57BL/6J, C3H/HeJ, and CD-1 strains, all of which were shown to have significantly less angiogenic ability (Table 1 and Rohan et al., 2000), were found to have much lower numbers of CECs (Figure 1A). A range of approximately 20-fold was found among the various strains we tested. Moreover, we also observed a correlation between the number of CECs and CEPs. Thus, 129/SvImJ that presents with high levels of CECs (11–32 cells/µl peripheral blood) also had high levels of CEPs (3–7 cells/µl in the peripheral blood). In contrast, strains that were shown to have mid or low levels of CECs were found to have the lowest levels of CEPs. The nature of this correlation suggests that both angiogenesis and the vasculogenic phenotype are likely regulated by the same genetic heterogeneity among different mouse strains. Similar results were obtained when female mice were used (data not shown).

A comparison was undertaken between both CECs or CEPs with the corneal neovascular micropocket assay results induced by 180 ng VEGF, this time in a prospective analysis (Table 1); the results again revealed a striking correlation, which was confirmed by using the nonparametrical Spearman test ($r = 0.85$, $p = 0.01$ for CECs; $r = 0.87$, $p = 0.007$ for CEPs). The same correlation was found among strains having mid and low angiogenic ability, as shown in Table 1, and as previously reported (Rohan et al., 2000). This linear correlation is shown in Figures 2A and 2B, where we plotted the "angiogenic responsiveness" results from Table 1 versus the number of CECs or CEPs per milliliter of blood. In addition, an even stronger and striking correlation is shown in Figures 2C and 2D, where we plotted the previous reported angiogenic responsiveness results obtained by the corneal neovascular micropocket assay induced with 10 ng bFGF (Rohan et al., 2000) and our prospective evaluation of the number of CECs/CEPs ($r = 0.93$, $p = 0.006$ for CECs; $r = 0.97$, $p = 0.002$ for CEPs).

Relative angiogenic ability evaluated by a Matrigel plug perfusion assay

In order to show that the angiogenesis results are not restricted to this particular assay and the ophthalmic environment, we used a different method, i.e., the Matrigel (subcutaneous) plug perfusion assay (as described in the Experimental Procedures) to measure the angiogenic ability in some of the mouse strains that we studied for levels of CECs/CEPs. For this approach, three mouse strains, 129/SvImJ, BALB/cJ, and C57BL/6J, which demonstrated high, mid, and low levels of CECs/CEPs and angiogenic ability using the eye assay, respectively, were tested using the Matrigel plug perfusion assay.

Figure 3 shows the ratio between Matrigel plug fluorescence and plasma fluorescence. This ratio was the highest (0.0036) in the 129/SvImJ strain, which also has shown the highest levels of both CECs/CEPs and the strongest ability to induce angiogenesis in the corneal neovascular micropocket assay (Figure 1 and Table 1, respectively). In contrast, the C57BL/6J mouse strain, which demonstrated a low angiogenesis/vasculogenic phenotype (Figure 1), was also found to have a low ratio between Matrigel plug fluorescence and plasma fluorescence (0.0014). The results from the Matrigel plug perfusion assay showed a statistically significant difference between 129/SvImJ mice and C57BL/6J mice ($p = 0.002$). Consistent with this trend, BALB/cJ mice demonstrated a mid range of angiogenic ability, with respect to intrinsic levels of CECs/CEPs and the Matrigel plug perfusion assay (0.0024). These results confirm our observations, shown in Figure 1 and Table 1, and suggest that the correlation is not restricted to angiogenic responses induced in an ophthalmic microenvironment, or to the assay used.

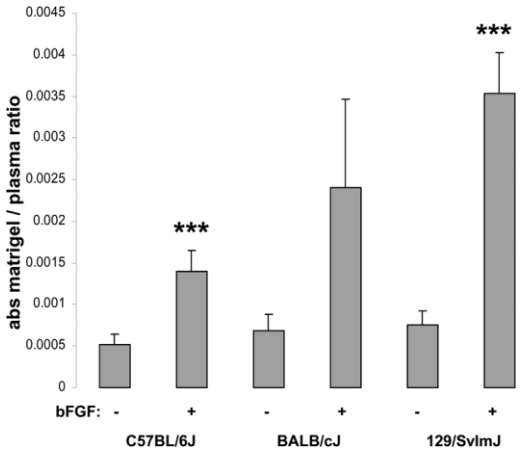

Figure 3. The assessment of angiogenic ability by a Matrigel plug perfusion assay

Six males per group (C57BL/6J, BALB/cJ, and 129/SvImJ), age 9–10 weeks, were assessed for angiogenic ability by a Matrigel plug perfusion assay (as described in the Experimental Procedures). Mice were divided into two groups: Matrigel alone, i.e., bFGF (−), or in the presence of bFGF (+). The results presented are a ratio between Matrigel plug fluorescence and systemic plasma fluorescence (***p = 0.002).

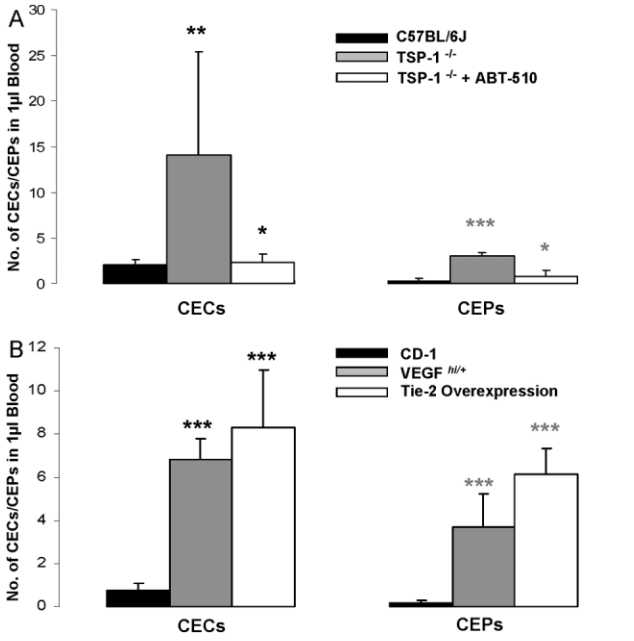

Figure 4. Assessment of CEC and CEP levels in TSP-1 null C57BL/6 mice, VEGF hypermorphic, and Tie-2-overexpressing mice

A: Ten 10-week-old *TSP-1*$^{-/-}$ C57BL/6J mice were divided into two groups; five mice were injected with the vehicle only, and five were injected with ABT-510 TSP-1 peptide mimetic, and another five age-matched wild-type controls were all bled by cardiac puncture and assessed for CECs/CEPs by the four-color flow cytometry assay, as described in the Experimental Procedures.

B: Nine- to twelve-week-old VEGF hypermorphic or Tie-2-overexpressing and CD-1 strain background control male mice (n = 4–6) were analyzed for CEC/CEP levels by four-color flow cytometry assay, similar to the analysis conducted for *TSP-1*$^{-/-}$ mice. Black asterisks: ***p $< 5.7 \times 10^{-5}$; **p = 0.024; * p = 0.61. Gray asterisks: ***p < 0.0011; *p = 0.29 from background control.

TSP-1$^{-/-}$ C57BL/6J mice demonstrate enhanced mobilization of CECs/CEPs

We next addressed the question of whether the basis of the genetic heterogeneity in levels of CECs and CEPs is, at least in part, regulated by known and validated molecular regulators of sprouting angiogenesis, either stimulators such as VEGF or inhibitors such as TSP-1. To do so, we used primarily an in vivo genetic approach and assessed levels of the cells in various mutant (knockout) or transgenic mouse models. First, we evaluated a mutant of the C57BL/6J mouse strain (which normally expresses low angiogenic ability relative to most other evaluated strains [Rohan et al., 2000]) that has a targeted disruption in the *tsp-1* gene (Lawler et al., 1998). TSP-1 is a well-known endogenous inhibitor of angiogenesis (Bouck et al., 1996), but its effect on CEPs and CECs has not yet been determined. The results shown in Figure 4A indicate that the numbers of both CECs and CEPs are approximately 5-fold higher in the *TSP-1* null C57BL/6J mice (p = 0.024, p = 0.0005, respectively), relative to the C57BL/6J control mice. In addition, to confirm that TSP-1 has a direct effect on levels of CECs/CEPs, we undertook a pharmacologic "knockout" approach in which *TSP-1* null mice were treated on a daily basis with the antiangiogenic drug ABT-510, a peptide mimetic derived from the type I (properdine-like) repeats of TSP-1 that mimics the interaction between TSP-1 and CD36 receptors (Reiher et al., 2002; Viloria-Petit et al., 2003). After 2 weeks of daily administration with either ABT-510 peptide or the vehicle (5% dextrose in water), mice were evaluated for CECs/CEPs. Figure 4A shows that the treatment of *TSP-1* null mice with ABT-510 results in a marked reduction of the intrinsically high CEC/CEP levels. The daily dose selected for this experiment (60 mg/kg/day) was known to be in the range of an optimal biologic dose (J.H., unpublished data).

VEGF-A$^{hi/+}$ as well as CD-1 *Tie-2* transgenic mice have increased mobilization and viability of CECs/CEPs

In order to extend the aforementioned findings, we next evaluated two different stimulatory regulators of angiogenesis, namely VEGF or Tie-2, on intrinsic levels of CECs/CEPs. To do so, we analyzed peripheral blood either from VEGF hypermorphic (*VEGF-A*$^{hi/+}$) (Miquerol et al., 2000) or Tie-2-overexpressing mice (Sarao and Dumont, 1998; Maisonpierre et al., 1993). *VEGF-A*$^{hi/+}$ mice (kindly provided by Dr. Andras Nagy, University of Toronto, Canada) have a 2- to 3-fold increase in circulating VEGF levels during embryonic development (Miquerol et al., 2000) as well as in adulthood (Y.S. and D.C., unpublished data). In addition, angiopoietin-1 and -2 binding Tie-2 is a highly endothelial cell-specific receptor tyrosine kinase that is essential for embryonic vascular development (Sato et al., 1995; Maisonpierre et al., 1993). Embryos lacking Tie-2 signaling pathways result in lethality due to impaired cardiac function and vascular hemorrhaging (Sarao and Dumont, 1998). The background of both of these mutant mouse strains is CD-1, which was previously shown to have low angiogenic responsiveness (Rohan et al., 2000) and, by us, to have low levels of CECs/CEPs. Male mice, 9–12 weeks old, from either *VEGF-A*$^{hi/+}$, *Tie-2* transgenic, or CD-1 background groups were anesthetized, bled by cardiac puncture, and blood prepared for the evaluation of CECs/CEPs. The results in Figure 4B show that *VEGF-A*$^{hi/+}$ as well as mice overexpressing Tie-2 receptor exhibit a 7- to 8-fold increase in CEC levels in comparison to CD-1 wild-type controls ($p = 7.8 \times 10^{-5}$, p = 0.001, respectively); similarly, there was a marked elevation levels of CEPs (p = 0.005, $p = 5.8 \times 10^{-5}$, respec-

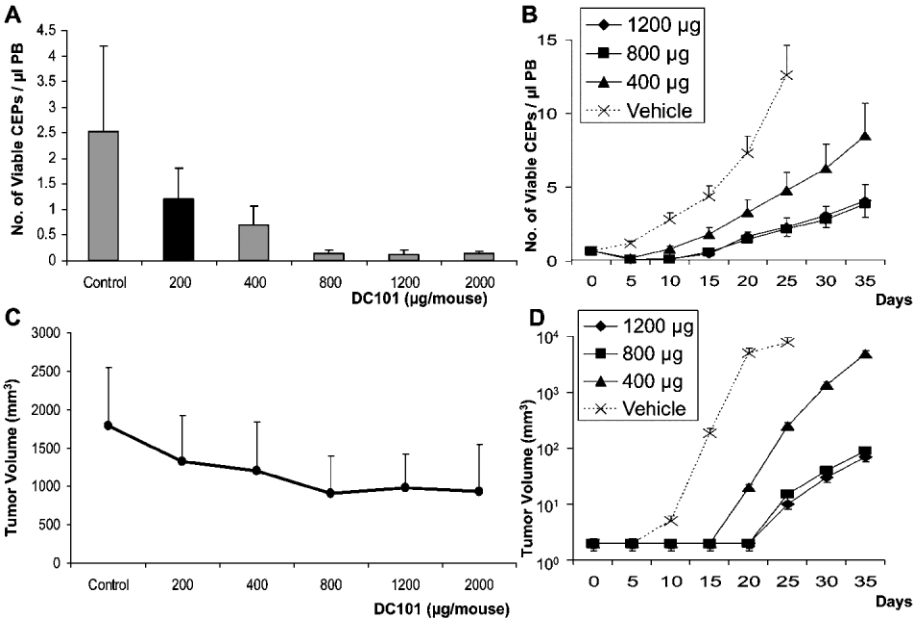

Figure 5. The assessment of viable CEPs analyzed in a dose response of DC101 in Lewis lung carcinoma (LL/2) C57BL/6J and Namalwa lymphoma CB-17 NOD/SCID mice

LL/2 C57BL/6J (n = 5/group) or Namalwa CB-17 NOD/SICD (n = 6/group) treatments were initiated with escalating doses (0–2000 μg) of DC101 as indicated in the figure. The LL/2-bearing mice were sacrificed after a week of DC101 administration, and blood was drawn by cardiac puncture for the assessment of viable CEPs (**A**). Tumor volumes were measured in all groups (**C**). The Namalwa-bearing mice were bled from the orbital sinus for the evaluation of CEPs (**B**) and measured for their tumor growth (**D**) every 5 days up to 5 weeks of treatment. PB, peripheral blood.

tively). Interestingly, in the Tie-2-overexpressing mice, a very marked increase of approximately 60-fold in the levels of CEPs (from 0.1 cells/μl in the wild-type to 6 cells/μl in mice overexpressing Tie-2) was observed. The percentage of the CEP subpopulation (approximately 75%), from the general CEC population, was found to be the highest among all genetically altered mice we have tested thus far. Moreover, these results further support a role for Tie-2 in adult vasculogenesis primarily by priming endothelial cell progenitor mobilization, as shown by increased levels of the CEPs, and are in line with recent reports showing that Tie-2 is expressed by putative CEPs (Shaw et al., 2004).

Furthermore, since the absolute number of CECs/CEPs is calculated using the WBC count multiplied by the percentage of CECs or its CEP subset, we asked whether any correlation exists between the WBC count and potential angiogenic responsiveness in different strains or transgenic mice we studied (Supplemental Table S1 at http://www.cancercell.org/cgi/content/full/7/1/101/DC1/). We found that there was no significant correlation between WBC counts and the absolute number of CECs/CEPs.

Strict correlation of antiangiogenic drug dose response with respect to antitumor therapeutic activity and effects on peripheral blood VEGFR-2$^+$ CEPs

There are several reports showing suppressive effects of various antiangiogenic drugs on the CECs/CEPs, e.g., reduction in the viability and/or mobilization of CEPs, both preclinically (Capillo et al., 2003; Bertolini et al., 2003; Schuch et al., 2003) and clinically (Willett et al., 2004). In this regard, empirical dose response preclinical studies with DC101, a rat monoclonal blocking antibody specific for the mouse VEGFR-2 (*flk-1*) receptor (Prewett et al., 1999; Bocci et al., 2004), have shown that the optimal antitumor therapeutic dose of DC101 is in the range of 800 μg–1 mg/mouse, administered every 3 days. In two such studies, the dose response analysis was undertaken using the Lewis lung carcinoma grown in syngeneic C57BL/6J mice (Prewett et al., 1999) and, more recently, using the HT29 human colon xenograft model in nude mice (Bocci et al., 2004). We therefore undertook a retrospective analysis in order to test whether the evaluation of CECs/CEPs can, in principle, help establish an optimal dose of such an antiangiogenic drug. To do so, we first evaluated the viability of CEPs in two different tumor models. We tested the effect of DC101 in escalating doses (0–2000 μg/mouse/dose) on a syngeneic Lewis lung carcinoma in C57BL/6J mice, and on a human lymphoma (Namalwa) xenograft in CB-17 NOD/SCID mice.

Since the effect of DC101 is to block VEGFR-2, we first tested whether a possible interference might exist between our flow cytometry *flk-1* (VEGFR-2)-directed antibody (purchased from BD Biosciences) and the DC101 (anti-VEGFR-2 blocking antibody). Blood pooled from five mice was incubated with 20 ng–2 mg of DC101 for 1 hr (as recommended by the manufacturer), and then samples were analyzed for CECs/CEPs according to the Experimental Procedures. No blocking was detected between the different samples, since an equal number of events was observed in the CD13$^+$/CD45$^-$ and VEGFR-2$^+$/CD45$^-$ gates at all DC101 concentrations tested (data not shown).

Next, we evaluated levels of viable CEPs after DC101 treatment in both LL/2 tumor-bearing C57BL/6J and Namalwa xenograft CB-17 NOD/SCID mice. To do so, either 10-week-old C57BL/6J mice transplanted with LL/2 tumor cells or 6- to 8-week-old CB-17 NOD/SCID mice transplanted with Namalwa lymphoma cells were assessed for tumor volumes according to the Experimental Procedures. When the tumor volume reached approximately 200 mm^3, mice (n = 5–6/group) were treated with DC101 in escalating doses, i.e., 0, 200, 400, 800, 1200, and 2000 μg/mouse either for 1 week (i.e., three injections) in the LL/2 model or for 5 weeks (injected every 3 days) in the Namalwa model. LL/2 C57BL/6J mice were sacrificed 24 hr after the last injection, blood was drawn by cardiac puncture for the assessment of viable CEPs, and tumor volumes were measured. In the case of Namalwa CB-17 NOD/SCID mice, blood was

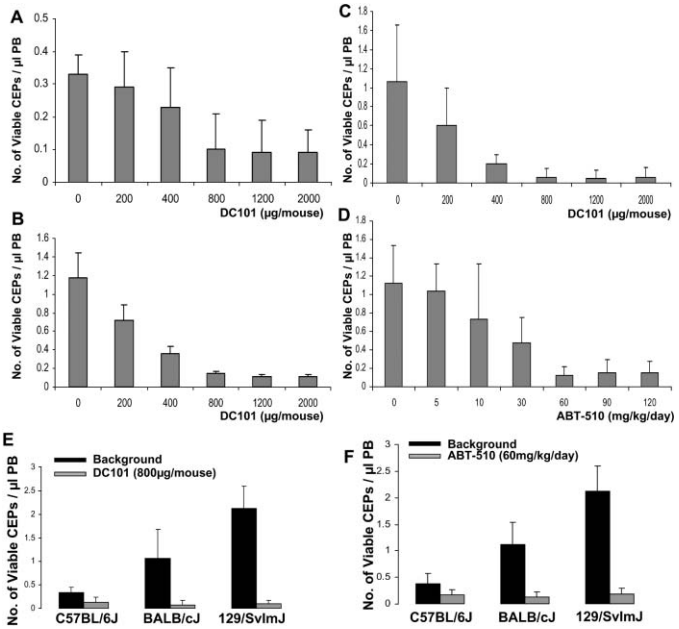

Figure 6. Evaluation of viable CEPs in different strains (non-tumor-bearing) treated with either DC101 or ABT-510

C57BL/6J (**A**), CB-17 NOD/SCID (**B**), and BALB/cJ (**C and D**) mice (n = 5–6/group) were treated with either DC101 in doses of 0–2000 μg/mouse or ABT-510 in doses of 0–120 mg/kg/day, as indicated in the figure. In addition, C57BL/6J and 129/SvImJ strains were treated in the optimal dose with either DC101 in 800 μg/mouse (**E**) or ABT-510 in 60 mg/kg/day (**F**). In all cases, after a week of treatment, mice were sacrificed and analyzed for the CEC/CEP levels by four-color flow cytometry assay, similar to the analysis conducted in Figures 4 and 5. PB, peripheral blood.

drawn from the orbital sinus every 5 days for the assessment of viable CEPs and measured for tumor volumes as well. The results show that 800 μg/mouse of DC101 is the optimal biological dose in both LL/2 C57BL/6J model (Figure 5A) and Namalwa lymphoma CB-17 NOD/SCID model (Figure 5B), since it induced the lowest level of viable CEPs and greatest decrease in tumor volume (Figures 5C and 5D, respectively). These results were not altered by further escalating the DC101 dose up to 2000 μg/mouse.

Since the CEP levels are elevated by tumor growth as can be seen in Figure 5B, this raises the possibility that the reduction in the viable CEPs is due to the tumor volume changes, as a secondary effect and not to the optimal biological dose of the drug. Therefore, we evaluated the viable CEPs in a correlation study in non-tumor-bearing mice. First, we performed a DC101 dose response study in both tumor-free background mice that we have tested above (i.e., C57BL/6J and CB-17 NOD/SCID). In both cases, DC101 in escalating doses has shown that the optimal dose is 800 μg/mouse, since it induced the greatest reduction in the viable CEPs (Figures 6A and 6B, respectively) that was not altered using higher doses. Second, we tested the viable CEPs in other strains and also with another drug—ABT-510, a TSP-1 peptide mimetic. For this approach, 10-week-old tumor-free BALB/cJ mice (n = 5/group) were injected either three times (once every 3 days) with DC101 in escalating doses (similar to tumor-bearing mice, i.e., 0, 200, 400, 800, 1200, and 2000 μg/mouse), or for a week (once a day) with ABT-510 at 0, 5, 10, 30, 60, 90, and 120 mg/kg/day. Twenty-four hours after the last injection, mice were sacrificed, and blood was drawn by cardiac puncture for the assessment of the viable CEPs in a manner similar to tumor-bearing mice that were treated with DC101. The results in Figures 6C and 6D show that either 800 μg/mouse of DC101 or 60 mg/kg/day of ABT-510 appear to be optimal biological doses, respectively, since they induced the lowest level of viable CEPs. This is similar to the reduction in the viable CEPs that we observed in LL/2 C57BL/6J mice and Namalwa CB-17 NOD/SCID mice and is also consistent with the optimal dose of ABT-510 (J.H., unpublished data).

In addition, we tested the optimal biologic dose of either DC101 or ABT-510 in different strains to determine whether similar effects are detected in "high angiogenic strains" such as 129/SvImJ or "low-angiogenic" strains such as C57BL/6J, i.e., regardless of the genetic background. Both DC101 and ABT-510 (Figures 6E and 6F, respectively), caused a similar marked reduction in the viable CEPs in all three strains, declining to basal levels of approximately 0.1 viable CEPs/μl peripheral blood.

Both DC101 and ABT-510 reduce the levels of viable CEPs in different and diverse models of tumor-bearing mice

In addition to retrospectively showing the optimal antiangiogenic dose of either DC101 or ABT-510 in BALB/cJ mice and the impact of both drugs at the optimal dose on the reduction of viable CEPs in different strains representing low, mid, and high angiogenic ability, we asked whether either 800 μg/mouse of DC101 or 60 mg/kg/day of ABT-510 would have a significant effect on the CECs/CEPs in different mouse tumor models similar to what has been previously observed for certain other antiangiogenic drugs or treatments (Schuch et al., 2003; Bertolini et al., 2003; Capillo et al., 2003). We tested a spontaneous erythroleukemia model in BALB/cJ (Figure 7A), an orthotopic human breast cancer (MDA-MB-231) xenograft model in CB-17 SCID mice (Figure 7B), and a subcutaneously implanted, syngeneic Lewis lung carcinoma (LL/2) model in C57BL/6J mice (Figures 7C and 7D). In each case, mice were divided into two groups (n = 5/group). One group was injected for a week with either DC101 antibody (every 3 days, except in the erythroleukemia model where mice were treated for 3 weeks, after which an antitumor effect was observed), or ABT-510 peptide (once a day), while the other (control) group was injected with the respective vehicle only (PBS for DC101 and 5% dextrose in water for ABT-510) as an untreated control arm. In all tumor models, an increase in the levels of both CECs and CEPs was found in the tumor-bearing mice in comparison to the background normal control mice (data not shown). In addition, a significant increase, i.e., 10-fold for erythroleukemia model (Figure 7A; $p = 0.017$), 20-fold for MDA-MB-231 human xenograft model (Figure 7B; $p = 0.047$), and approximately 7- to 10-fold for Lewis lung carcinoma model (Figures 7C and 7D; $p < 0.02$), was in each case detected with respect to the viability and mobilization of CEPs. Both DC101 and ABT-510 treatments caused a reduction in the number of CECs/CEPs (data not shown) as well as in the viable CEPs, and indeed, the levels were not significantly different from values detected in normal controls, as shown in Figure 7 ($p > 0.16$), with the exception of ABT-510 treatment in LL/2 mice, which showed a reduction in viable CEP levels that was significantly different from the

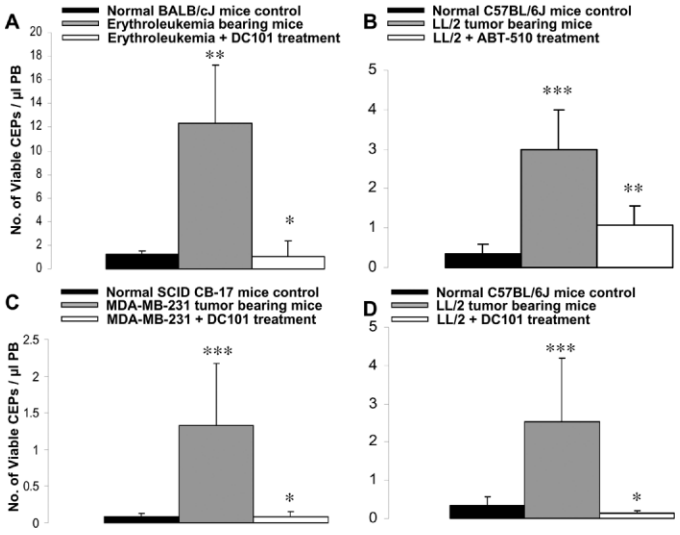

Figure 7. The viability of CEPs in various tumor-bearing mouse models treated with either DC101 or ABT-510 in their optimal doses

Mice induced with erythroleukemia (**A**), MDA-MB-231 human breast cancer (**C**), or Lewis lung carcinoma (**B and D**) were divided into two groups (n = 5/group); one group was treated with either DC101 or ABT-510 in the optimal dose (i.e., 800 μg/mouse for DC101 and 60 mg/kg/day for ABT-510), whereas the other group was injected with the vehicle (PBS for DC101 and 5% dextrose in water for ABT-510), and another group age-matched wild type controls. Mice were bled by cardiac puncture and assessed for viable CEPs according to the Experimental Procedures. ***$p < 0.01$; **$0.05 < p < 0.01$; *$p > 0.05$, from background control. PB, peripheral blood.

background control but nevertheless still significantly different from the untreated tumor-bearing control mice ($p = 0.009$). Thus, in all of these tumor models—which involved spontaneous and transplanted tumors, both solid and leukemic, and in different mouse strains—we found marked increases of CECs and CEPs in tumor-bearing mice, which were reduced to background levels using the optimal dose range of two very different antiangiogenic drugs, one an antibody and the other a peptide, having distinctly different endothelial cell molecular targets.

Discussion

Our results regarding measurements of $CD13^+$/VEGFR-2^+/$CD45^-$/$CD117^+$ peripheral blood circulating cells, historically considered to be bone marrow-derived CEPs and $CD13^+$/VEGFR-2^+/$CD45^-$ cells, designated as mature CECs, are of considerable importance with respect to the question of whether such cell types (or perhaps more importantly, whether circulating VEGFR-2^+ peripheral blood cells in general) in all probability functionally contribute to angiogenesis and can be used reliably as valid quantitative surrogate markers of angiogenesis and antiangiogenic drug activity. Thus, we report here five results relevant to providing an affirmative answer to this question. First, intrinsic levels of the aforementioned two circulating VEGFR-2^+ cell types vary significantly, up to 20-fold, between different mouse strains, i.e., they are highly genetically heterogeneous. Second, this genetic heterogeneity correlates to a remarkable degree with the ability of these same strains of mice to respond to an angiogenic stimulus whether induced by bFGF or VEGF, both in the ophthalmic or subcutaneous environment, and measured by two different assays. Third, two of the best characterized stimulators of sprouting angiogenesis, i.e., VEGF and Tie-2, as well as a well-known endogenous angiogenesis inhibitor (TSP-1), appear to also regulate the intrinsic levels of the two peripheral blood circulating cell types we measured in our experiments. Fourth, a dose-dependent decrease in these cell types was induced in three different mouse strains by a well-characterized, validated, and highly specific targeted antiangiogenic drug—the DC101 monoclonal antibody to mouse VEGFR-2—in a manner that strikingly correlated with its dose-dependent biologic activity, including its known optimal biologic dose; moreover, this was observed in either normal or tumor-bearing mice using several different strains. In addition, a dose response with TSP-1 peptide mimetic antiangiogenic drug (ABT-510) showed a marked reduction in the range of a known optimal dose, i.e., 60 mg/kg/day. Fifth, the drugs were also shown to be similarly efficacious in their suppressive effects on both CECs and CEPs when tested at the optimal dose in high, mid, and low angiogenic strains (e.g., 129/SvImJ, BALB/cJ, and C57BL/6J, respectively). Finally, we also show that both DC101 and ABT-510, at an optimal dose, have potent suppressive activity on these two circulating cell types in four different tumor mouse models, which included a spontaneous (nontransplanted) leukemia, a syngeneic lung tumor, a transplanted human breast cancer xenograft, and a human Burkitt's lymphoma; in all cases, a marked reduction in the viable CEPs was observed, down to baseline, control levels in most cases.

Our results do not directly address the current controversy surrounding the validity of the concept of circulating bone marrow-derived endothelial "progenitor" cells, which have been hypothesized to integrate into newly forming blood vessels and differentiate into mature endothelial cells, thus contributing to new blood vessel formation, i.e., "systemic vasculogenesis" (Stoll et al., 2003; Jain and Duda, 2003). As cited in the Introduction, several recent reports, some based on confocal microscopy techniques, have questioned the authenticity or relative importance of this concept and have in some cases instead implicated circulating bone marrow-derived cells, which stimulate neovascularization after homing to sites of angiogenesis and adhering closely to the endothelial cells of the newly forming vessels (Ziegelhoeffer et al., 2004; Voswinckel et al., 2003; Rehman et al., 2003; Rajantie et al., 2004; Gothert et al., 2004; Heil et al., 2004). In this regard, our results, taken together, strongly support the hypothesis that there are measurable, defined circulating peripheral blood VEGFR-2^+ cells that in all likelihood functionally contribute to angiogenesis, whether such cells are true endothelial progenitors or vessel adherent "support" cells such as monocytes, macrophages, or precursor pericytes. Regardless of their quantitative contribution to angiogenesis, the results are strongly supportive of assessing their levels as valid surrogate markers of angiogenesis, including assessing the effects of antiangiogenic agents or treatments. As such, the conclusion reached in several previous studies—that an antiangiogenic effect was achieved based on declines of the types of circulating cell we have measured, after treatment of tumor-bearing mice with such drugs as endostatin (Schuch et al., 2003; Capillo et al., 2003) or prolonged low-dose metronomic chemotherapy (Bertolini et al., 2003) or treatment of rectal carcinoma patients with bevacizumab, the humanized anti-VEGF antibody (Willett et al., 2004)—is supported strongly by our results. This is a critical point, because measuring and documenting antiangio-

genic drug activity using a blood-based assay, and establishing the optimal biologic/therapeutic dose of such drugs, would greatly aid in their clinical development and application.

Because we did not evaluate directly how the two circulating VEGFR-2$^+$ cell types contribute to angiogenesis, or whether they integrate into newly formed vasculature, consideration might be given to terminology such as circulating peripheral blood "neovasculature progenitor cells" or "vasculogenic promoting cells," as these terms could encompass perivascular adherent/support cells as well as endothelial progenitor cells. Recent clinical results showing similar robust declines in both VEGFR-2$^+$ CECs and CEPs in a small number of evaluated cancer patients as a result of treatment with bevacizumab are especially encouraging in this regard (Schuch et al., 2003), since they suggest the potential of evaluating such cells in peripheral blood samples to monitor clinical antiangiogenic drug activity. Our results show that this might now be extended to help determine optimal antiangiogenic drug dosing and as such may be a more tractable and much less expensive approach of doing so compared to such methods as measuring tumor blood flow and vascular permeability by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) (Morgan et al., 2003). It is also noteworthy that molecular surrogates of circulating VEGFR-2$^+$ cells such as VE-cadherin (for CECs) measured by RT-PCR of whole RNA in blood or similar molecular-based approaches should make evaluation of such cell types more feasible in the near future (Rabascio et al., 2004).

In summary, lack of a validated in vivo assay to measure angiogenesis has been a major impediment to the clinical development of many antiangiogenic drugs. Our results reported herein highlight the possibility of a peripheral blood-based cellular assay to both measure and monitor angiogenesis, as well as to monitor antiangiogenic drug activity, the latter of which can be exploited to help establish the optimal biologic dose of such drugs.

Experimental procedures

Animal models and strains

The analyses of all strains and transgenic mice were performed on groups of four to six males, 9–12 weeks old, unless indicated otherwise (for detailed information, see the Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/7/1/101/DC1/).

Corneal neovascular micropocket assay

The corneal neovascular micropocket assay was performed as described (Kenyon et al., 1996); for detailed information, see the Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/7/1/101/DC1/.

In vivo angiogenesis assessment by Matrigel plug perfusion assay

The Matrigel plug perfusion (angiogenesis) assay was performed as previously described (Klement et al., 2000; Bocci et al., 2003) with some minor modifications (for detailed information, see the Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/7/1/101/DC1/).

CEC and CEP measurements by flow cytometry

Evaluation of CECs and CEPs was carried out on blood collected by cardiac puncture, followed by enumeration using four-color flow cytometry as described previously (Bertolini et al., 2003); for detailed information, see the Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/7/1/101/DC1/.

In vivo tumor induction and measurement

The spontaneous Friend erythroleukemia model has been described previously (Shibuya and Mak, 1983). Syngeneic Lewis lung carcinoma (LL/2) model was described previously (Prewett et al., 1999; Bocci et al., 2003). Orthotopic human breast cancer model (MDA-MB-231) was described previously (Bocci et al., 2004). Human Burkitt's lymphoma (Namalwa) model was described previously (Bertolini et al., 2003). For detailed information on tumor models and measurements, see the Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/7/1/101/DC1/.

Pharmacological and biological reagents and dose schedule

A rat anti-mouse neutralizing monoclonal antibody directed to mouse VEGFR-2 (flk-1) called DC101 was generated by ImClone Systems Inc. (New York, NY). This antibody was reconstituted in sterile PBS and injected intraperitoneally into mice twice a week, as described in the text and elsewhere (Prewett et al., 1999; Klement et al., 2000). Control mice were injected with the vehicle alone (PBS).

The nonapeptide Ac-Sar-Gly-Val-D-allo-Ile-Thr-Nva-Ile-Arg-Pro-ethylamide, which contains a fragment of the second type-I TSP-1 repeat (ABT-510 or DI-TSPa), was generated by Abbott Laboratories (Reiher et al., 2002). The peptide was dissolved in 5% dextrose and injected intraperitoneally in a dose of 60 mg/kg/day into *TSP-1* null mice. Control mice were injected with the vehicle only (5% dextrose in water).

Statistical analysis

Results are reported as the mean ± SD. Statistical significance of differences was assessed by Student-Newman-Keuls test, using the Microsoft Office 2000 (Microsoft Office-Excel software). The level of significance was set at $p < 0.05$. In addition, results from the correlation between corneal neovascular micropocket assay and the numbers of CEC/CEP reported are significantly correlated with the nonparametrical Spearman test using the GraphPad Prism v. 4.0 (GraphPad Software). The level of significance was set at Spearman $r \geq 0.85$ and $p < 0.05$.

Acknowledgments

We thank Cassandra Cheng for her excellent secretarial work. We are grateful to numerous colleagues for their comments and suggestions regarding the studies summarized in this manuscript and Dr. Douglas Hanahan for critical reading of the manuscript. This work was supported primarily by grants to R.S.K. from the National Cancer Institute of Canada, and also from the National Institutes of Health (NIH) (CA-41233) and the Canadian Institutes of Health Research (CIHR). Support was also received from Associazione Italiana per la Ricerca sul Cancro (AIRC), Instituto Superiore di Sanita (ISS), and the 6th EU framework program "Angiotargeting" to F.B. and from HL68003 and CA92644 (NIH) to J.L. R.S.K. is a Canada Research Chair in Molecular Medicine. Y.S. is a recipient of a postdoctoral fellowship award from the CIHR. D.C. is supported by a studentship award from the CIHR. D.V. is supported by a studentship award from the Heart and Stroke Foundation of Canada (HSFC). D.J.D. is supported by a grant from NIH (NHLBI-R01 HL63224-01A1). Y.B.-D. is supported by grants from NCIC and CIHR. R.J.D. is supported by grants from the NIH (R01-EY12726).

Received: August 17, 2004
Revised: October 25, 2004
Accepted: November 18, 2004
Published: January 17, 2005

References

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J.M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964–967.

Asahara, T., Takahashi, T., Masuda, H., Kalka, C., Chen, D., Iwaguro, H., Inai, Y., Silver, M., and Isner, J.M. (1999). VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J. *18*, 3964–3972.

Bertolini, F., Mingrone, W., Alietti, A., Ferrucci, P.F., Cocorocchio, E., Peccatori, F., Cineri, S., Mancuso, P., Corsini, C., Burlini, A., et al. (2001). Thalidomide in multiple myeloma, myelodysplastic syndromes and histiocytosis. Analysis of clinical results and of surrogate angiogenesis markers. Ann. Oncol. *12*, 987–990.

Bertolini, F., Paul, S., Mancuso, P., Monestiroli, S., Gobbi, A., Shaked, Y., and Kerbel, R.S. (2003). Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. Cancer Res. *63*, 4342–4346.

Bocci, G., Francia, G., Man, S., Lawler, J., and Kerbel, R.S. (2003). Thrombospondin-1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. Proc. Natl. Acad. Sci. USA *100*, 12917–12922.

Bocci, G., Man, S.M., Green, S.K., Francia, G., Ebos, J.M.L., du Manoir, J.M., Emmenegger, U., Ma, L., Thorpe, P., Davidoff, A.M., et al. (2004). Increased plasma VEGF as a surrogate marker for optimal therapeutic dosing of VEGFR-2 monoclonal antibodies. Cancer Res. *64*, 6616–6625.

Bouck, N., Stellmach, V., and Hsu, S.C. (1996). How tumors become angiogenic. Adv. Cancer Res. *69*, 135–174.

Capillo, M., Mancuso, P., Gobbi, A., Monestiroli, S., Pruneri, G., Dell'Agnola, C., Martinelli, G., Shultz, L., and Bertolini, F. (2003). Continuous infusion of endostatin inhibits differentiation, mobilization, and clonogenic potential of endothelial cell progenitors. Clin. Cancer Res. *9*, 377–382.

Cristofanilli, M., Charnsangavej, C., and Hortobagyi, G.N. (2002). Angiogenesis modulation in cancer research: novel clinical approaches. Nat. Rev. Drug Discov. *1*, 415–426.

Crosby, J.R., Kaminski, W.E., Schatteman, G., Martin, P.J., Raines, E.W., Seifert, R.A., and Bowen-Pope, D.F. (2000). Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ. Res. *87*, 728–730.

Garcia-Barros, M., Paris, F., Cordon-Cardo, C., Lyden, D., Rafii, S., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. (2003). Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science *300*, 1155–1159.

Gothert, J.R., Gustin, S.E., Van Eekelen, A.M., Schmidt, U., Hall, M.A., Jane, S.M., Green, A.R., Gottgens, B., Izon, D.J., and Begley, C.G. (2004). Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. Blood *104*, 1769–1777.

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell *86*, 353–364.

Heil, M., Ziegelhoeffer, T., Mees, B., and Schaper, W. (2004). A different outlook on the role of bone marrow stem cells in vascular growth: bone marrow delivers software not hardware. Circ. Res. *94*, 573–574.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, H., Helm, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., et al. (2004). Addition of bevacizumab (rhuMab VEGF) to bolus IFL in the first line treatment of patients with metastatic colorectal cancer: results of a randomized phase III trial. N. Engl. J. Med. *350*, 2335–2342.

Jain, R.K., and Duda, D.G. (2003). Role of bone marrow-derived cells in tumor angiogenesis and treatment. Cancer Cell *3*, 515–516.

Kenyon, B.M., Voest, E.E., Chen, C.C., Flynn, E., Folkman, J., and D'Amato, R.J. (1996). A model of angiogenesis in the mouse cornea. Invest. Ophthalmol. Vis. Sci. *37*, 1625–1632.

Kerbel, R.S., and Kamen, B.A. (2004). Antiangiogenic basis of low-dose metronomic chemotherapy. Nat. Rev. Cancer *4*, 423–436.

Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D., Bohlen, P., and Kerbel, R.S. (2000). Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J. Clin. Invest. *105*, R15–R24.

Lawler, J., Sunday, M., Thibert, V., Duquette, M., George, E.L., Rayburn, H., and Hynes, R.O. (1998). Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J. Clin. Invest. *101*, 982–992.

Li, H., Gerald, W.L., and Benezra, R. (2004). Utilization of bone marrow-derived endothelial cell precursors in spontaneous prostate tumors varies with tumor grade. Cancer Res. *64*, 6137–6143.

Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., Chadburn, A., Heissig, B., Marks, W., Witte, L., et al. (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat. Med. *7*, 1194–1201.

Machein, M.R., Renninger, S., Lima-Hahn, E., and Plate, K.H. (2003). Minor contribution of bone marrow-derived endothelial progenitors to the vascularization of murine gliomas. Brain Pathol. *13*, 582–597.

Maisonpierre, P.C., Goldfarb, M., Yancopoulos, G.D., and Gao, G. (1993). Distinct rat genes with related profiles of expression define a TIE receptor tyrosine kinase family. Oncogene *8*, 1631–1637.

McDonald, D.M., Teicher, B.A., Stetler-Stevenson, W., Ng, S.S., Figg, W.D., Folkman, J., Hanahan, D., Auerbach, R., O'Reilly, M., Herbst, R., et al. (2004). Report from the society for biological therapy and vascular biology faculty of the NCI workshop on angiogenesis monitoring. J. Immunother. *27*, 161–175.

Miquerol, L., Langille, B.L., and Nagy, A. (2000). Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. Development *127*, 3941–3946.

Monestiroli, S., Mancuso, P., Burlini, A., Pruneri, G., Dell'Agnola, C., Gobbi, A., Martinelli, G., and Bertolini, F. (2001). Kinetics and viability of circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. Cancer Res. *61*, 4341–4344.

Morgan, B., Thomas, A.L., Drevs, J., Hennig, J., Buchert, M., Jivan, A., Horsfield, M.A., Mross, K., Ball, H.A., Lee, L., et al. (2003). Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies. J. Clin. Oncol. *21*, 3955–3964.

Park, J.W., Kerbel, R.S., Kelloff, G.J., Barrett, J.C., Chabner, B.A., Parkinson, D.R., Peck, J., Ruddon, R.W., Sigman, C.C., and Slamon, D.J. (2004). Rationale for biomarkers and surrogate end points in mechanism-driven oncology drug development. Clin. Cancer Res. *10*, 3885–3896.

Parulekar, W.R., and Eisenhauer, E.A. (2004). Phase I trial design for solid tumor studies of targeted, non-cytotoxic agents: theory and practice. J. Natl. Cancer Inst. *96*, 990–997.

Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L., et al. (1999). Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res. *59*, 5209–5218.

Rabascio, C., Muratori, E., Mancuso, P., Calleri, A., Raia, V., Foutz, T., Cinieri, S., Veronesi, G., Pruneri, G., Lampertico, P., et al. (2004). Assessing tumor angiogenesis: increased circulating VE-cadherin RNA in patients with cancer indicates viability of circulating endothelial cells. Cancer Res. *64*, 4373–4377.

Rafii, S., Lyden, D., Benezra, R., Hattori, K., and Heissig, B. (2002). Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat. Rev. Cancer *2*, 826–835.

Rajantie, I., Ilmonen, M., Alminaite, A., Ozerdem, U., Alitalo, K., and Salven, P. (2004). Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. Blood *104*, 2084–2086 Published online June 10, 2004. 10.1182/blood-2004-01-0336.

Rehman, J., Li, J., Orschell, C.M., and March, K.L. (2003). Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation *107*, 1164–1169.

Reiher, F.K., Volpert, O.V., Jimenez, B., Crawford, S.E., Dinney, C.P., Henkin, J., Haviv, F., Bouck, N.P., and Campbell, S.C. (2002). Inhibition of tumor growth by systemic treatment with thrombospondin-1 peptide mimetics. Int. J. Cancer *98*, 682–689.

Rohan, R.M., Fernandez, A., Udagawa, T., Yuan, J., and D'Amato, R.J. (2000). Genetic heterogeneity of angiogenesis in mice. FASEB J. *14*, 871–876.

Ruzinova, M.B., Schoer, R.A., Gerald, W., Egan, J.E., Pandolfi, P.P., Rafii,

S., Manova, K., Mittal, V., and Benezra, R. (2003). Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. Cancer Cell *4*, 277–289.

Sarao, R., and Dumont, D.J. (1998). Conditional transgene expression in endothelial cells. Transgenic Res. *7*, 421–427.

Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature *376*, 70–74.

Schuch, G., Heymach, J.V., Nomi, M., Machluf, M., Force, J., Atala, A., Eder, J.P., Jr., Folkman, J., and Soker, S. (2003). Endostatin inhibits the vascular endothelial growth factor-induced mobilization of endothelial progenitor cells. Cancer Res. *63*, 8345–8350.

Shaw, J.P., Basch, R., and Shamamian, P. (2004). Hematopoietic stem cells and endothelial cell precursors express Tie-2, CD31 and CD45. Blood Cells Mol. Dis. *32*, 168–175.

Shibuya, T., and Mak, T.W. (1983). Isolation and induction of erythroleukemic cell lines with properties of erythroid progenitor burst-forming cell (BFU-E) and erythroid precursor cell (CFU-E). Proc. Natl. Acad. Sci. USA *80*, 3721–3725.

Sikder, H., Huso, D.L., Zhang, H., Wang, B., Ryu, B., Hwang, S.T., Powell, J.D., and Alani, R.M. (2003). Disruption of Id1 reveals major differences in angiogenesis between transplanted and autochthonous tumors. Cancer Cell *4*, 291–299.

Stoll, B.R., Migliorini, C., Kadambi, A., Munn, L.L., and Jain, R.K. (2003). A mathematical model of the contribution of endothelial progenitor cells to angiogenesis in tumors: implications for anti-angiogenic therapy. Blood *102*, 2555–2561.

Viloria-Petit, A.M., Miguerol, L., Yu, J.L., Gertenstein, M., Sheehan, C., May, L., Henkin, J., Lobe, C., Nagy, A., Kerbel, R.S., and Rak, J. (2003). Contrasting effects of VEGF gene disruption in embryonic stem cell-derived versus oncogene-induced tumors. EMBO J. *22*, 4091–4102.

Voswinckel, R., Ziegelhoeffer, T., Heil, M., Kostin, S., Breier, G., Mehling, T., Haberberger, R., Clauss, M., Gaumann, A., Schaper, W., and Seeger, W. (2003). Circulating vascular progenitor cells do not contribute to compensatory lung growth. Circ. Res. *93*, 372–379.

Willett, C.G., Boucher, Y., di Tomaso, E., Duda, D.G., Munn, L.L., Tong, R.T., Chung, D.C., Sahani, D.V., Kalva, S.P., Kozin, S.V., et al. (2004). Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat. Med. *10*, 145–147.

Ziegelhoeffer, T., Fernandez, B., Kostin, S., Heil, M., Voswinckel, R., Helisch, A., and Schaper, W. (2004). Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ. Res. *94*, 230–238.