Pharmacological agents directed against the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have been reported to inhibit angiogenesis. However, genetic ablations of the genes encoding these integrins fail to block angiogenesis and in some cases even enhance it. This apparent paradox suggests the hypotheses that these integrins are negative regulators of angiogenesis and that the drugs targeting them may be acting as agonists rather than antagonists.

A reevaluation of integrins as regulators of angiogenesis

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Integrins are cell surface receptors mediating adhesion to the extracellular matrix or, in some cases, to adjacent cells[1–3]. Although we know that many integrins and integrin ligands function in angiogenesis[4–6], their exact actions remain unclear. Integrins are accessible to and readily inhibited by antibodies, peptides or peptidomimetics, making them excellent drug targets. The major platelet integrin, $\alpha_{IIb}\beta_3$, is the molecular target for effective antithrombotics, and drugs targeting several leukocyte integrins are in late stages of clinical trials as anti-inflammatory agents. Given this background, integrins have attracted attention as targets for antiangiogenic therapy. My purpose here is to evaluate critically the science underlying this strategy and offer a reevaluation of current and future approaches to this goal.

Starting from an initial observation that the integrin $\alpha_v\beta_3$ is upregulated on certain tumor vasculatures[7], Brooks *et al.* tested inhibitors of this integrin and the closely related $\alpha_v\beta_5$ for antiangiogenic activity in a number of model systems. Brooks and others have reported that the monoclonal antibody, LM609, and various low-molecular-weight reagents based on the tripeptide RGD, which is recognized by these two integrins, block angiogenesis in response to growth factors in tumors and in retinal angiogenesis[8–12]. These results led to the reasonable idea that these two integrins are proangiogenic, and this concept has been extensively reviewed. Based on this model, a humanized version of LM609, known as Vitaxin, has entered early-phase clinical trials[13].

However, a series of papers on genetically altered mice seriously questions the idea that these two integrins are proangiogenic or are even necessary for angiogenesis. The α_v integrin subunit partners selectively with four different β subunits (β_3, β_5, β_6 and β_8) and also with β_1, which in turn can partner with a dozen other α subunits. Genetic ablation of β_1 unsurprisingly causes embryonic lethality[14,15], and further studies show that β_1 integrins are involved in angiogenesis[16], a concept that we will consider later. However, if $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ were essential for angiogenesis, one would predict that ablation of α_v or β_3 and/or β_5 would have severe consequences for angiogenesis. However, that is not so; mice (or people) lacking β_3 are viable and fertile[17], as are mice lacking β_5, β_6, or any pairwise combination of β_3, β_5, and β_6 (refs. 18–20). Furthermore, mice lacking α_v (and thus all α_v integrins) also show extensive angiogenesis[21]. They do show vascular defects selectively in the brain, but the evidence suggests that this arises from defects in the interactions of brain parenchymal cells with the cerebral vasculature, rather than in the vascular cells themselves[22]. β_8-null mice show very similar defects, indicating that the phenotype arises from loss of $\alpha_v\beta_8$ (ref. 23). In contrast, β_3/β_5-double-null mice do not show these defects and are viable and fertile, proving that $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ are not necessary for angiogenesis.

The possibility exists that tumor angiogenesis might be selectively dependent on $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$, but Reynolds *et al.* have also recently shown this not to be true[20]. Indeed, mice lacking one or both of these integrins show enhanced tumor growth and angiogenesis[20]. Rather than supporting the idea that these integrins are proangiogenic, this instead raises the hypothesis that they are antiangiogenic or negative regulators of angiogenesis for at least a significant part of the time. In the rest of this commentary I will explore this alternative hypothesis and evaluate the implications of these results for antiangiogenic therapies targeting integrins.

In the case of α_v integrins and angiogenesis discussed above, there is a major discrepancy between the genetic results and those obtained using antibodies or low-molecular-weight reagents targeting those integrins. It is worth noting that this sort of discrepancy is not typical. Importantly, genetic and pharmacological data are in complete agreement on the role of $\alpha_v\beta_3$ in bone remodeling; both genetic ablation of $\alpha_v\beta_3$ (ref. 24) and antagonists of its role in osteoclast adhesion[25] inhibit bone resorption. Similarly, results on other integrins in angiogenesis are not discrepant; the integrin $\alpha_5\beta_1$ and its ligand, fibronectin, are clearly proangiogenic. Genetic ablation of either one leads to embryonic lethality with major vascular defects[26,27], and antibodies to either, or peptides blocking their interactions, inhibit angiogenesis[28]. Here, all the data are concordant. Less extensive results on two collagen receptor integrins, $\alpha_1\beta_1$ and $\alpha_2\beta_1$, are also in general agreement. Both these integrins are upregulated by angiogenic growth factors (as are $\alpha_5\beta_1$ and fibronectin), and antibodies to $\alpha_1\beta_1$ and $\alpha_2\beta_1$ inhibit tumor-induced angiogenesis[29]. In agreement with these results, α_1-null mice support reduced tumor growth and angiogenesis[30].

Thus, the discordant results on α_v integrins and angiogenesis are the exception, suggesting that these receptors have a somewhat different role than do the fibronectin ($\alpha_5\beta_1$) and collagen ($\alpha_1\beta_1$ and $\alpha_2\beta_1$) receptors. It is often suggested that the genetic results could underestimate the importance of α_v integrins because of overlapping functions or some sort of compensation. The genetic data show that $\alpha_v\beta_5$ cannot be compensating for $\alpha_v\beta_3$, and there is no evidence for upregulation of any other integrins[20]. So, there is no evidence for overlapping functions or compensation among the integrins, but the possibility of some unknown form of compensation cannot be eliminated. Future experiments, employing cell type–specific and/or regulated gene ablation may provide additional information on this point. Nonetheless, what is already clear from the genetics is that α_v integrins are not necessary for angiogenesis, whereas $\alpha_5\beta_1$ and fibronectin are.

What then should one say about the antibody and peptide inhibition of angiogenesis? Assuming that the reagents are specific for $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$, we need to consider how they could be antiangiogenic. Initially investigators characterized these reagents as antagonists of cell adhesion and migration mediated by α_v integrins. But are they antagonists of all functions of these integrins? We know that integrins are signaling receptors, activating many intracellular signal transduction pathways[1–3,31]. Soluble reagents (antibodies, small molecules) that block integrin interactions with a solid-phase substrate (as in adhesion and migration)

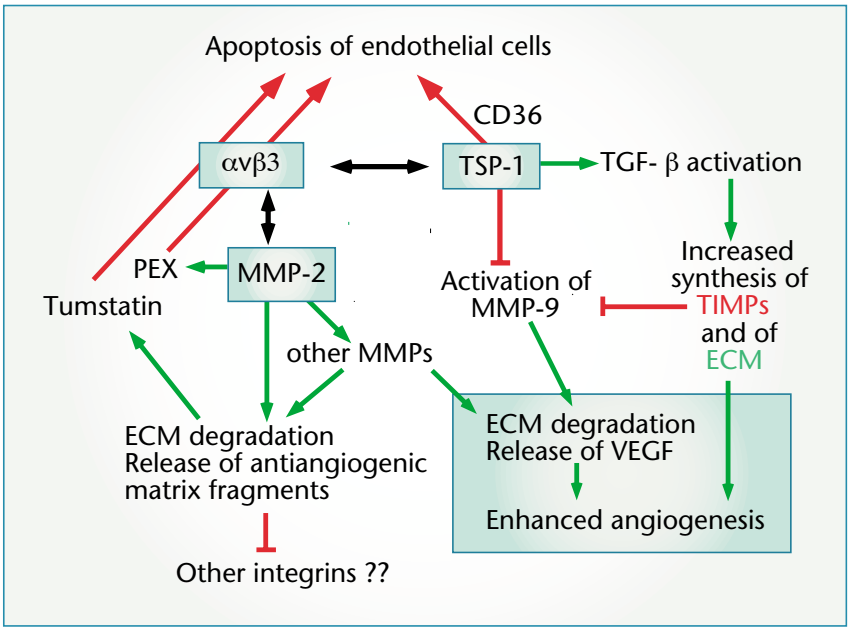

Fig. 1 $\alpha_v\beta_3$ as a mediator of antiangiogenic regulators. Thrombospondin (TSP-1) is a known antiangiogenic protein acting in several ways, three of which are shown (see text)[36–39]. TSP-1 is a ligand for $\alpha_v\beta_3$, which could therefore localize its antiangiogenic effects. Another alternative negative regulatory pathway invokes $\alpha_v\beta_3$ binding and activation of MMP-2 (refs. 46,47), leading to matrix degradation and release of antiangiogenic matrix fragments, at least one of which (tumstatin) can act to inhibit angiogenesis via $\alpha_v\beta_3$ (ref. 45), or other integrins. MMP-2 can also cleave itself to yield an antiangiogenic fragment called PEX, which can act in a negative feedback loop[47]. Note that both TSP-1 and MMPs could also have proangiogenic effects (shown in box at lower right). It seems likely that $\alpha_v\beta_3$ could play roles as a balancer of both proangiogenic and antiangiogenic effects.

could nonetheless be agonists of signal transduction by the same integrins. There is abundant evidence that RGD-based reagents can activate integrins[32–35].

If we adopt the working hypothesis that the blocking reagents are acting as agonists of a negative regulatory function of the α_v integrins, then the data are all in concordance. Removal of these integrins should lead to enhanced angiogenesis as seen in tumors and some other angiogenic responses[20], and engagement of the α_v integrins by agonists should activate their negative regulatory functions and suppress angiogenesis. This negative regulatory model for α_v integrins actually does a better job of explaining the data than the proangiogenic model and is fully in agreement with the genetic data.

How might α_v integrins act as negative regulators? We can consider several models, each well founded on known properties of these integrins. One group of negative regulatory models for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins invoke them as receptors for known negative regulatory molecules (Fig. 1). Thrombospondin (TSP-1) is a well-established negative regulator of angiogenesis[36–39]. It can act as an activator of transforming growth factor-β (TGF-β) and as a negative regulator of matrix metalloproteinase-9 (MMP-9) activation. TGF-β can, in turn, upregulate tissue inhibitors of metalloproteinases (TIMPs), and MMP-9 is a known activator of angiogenesis by its ability to release vascular endothelial growth factor (VEGF) from sequestered stores (presumably in the extracellular matrix, ECM) so that it can bind and activate its receptor on endothelial cells[40]. TSP-1 can also bind the CD36 receptor on endothelial cells and activate apoptotic pathways[39]. Whether or not these mechanisms explain, in whole or in part, the antiangiogenic effects of thrombospondin, several studies have shown clearly that both TSP-1 and TSP-2 suppress angiogenesis[36–39]. Because $\alpha_v\beta_3$ can act as a receptor for TSP-1 and TSP-2, it could function by localizing them and thus mediate their antiangiogenic effects.

Several groups have also implicated $\alpha_v\beta_3$ as a receptor for various proteolytic fragments of ECM proteins that can act as antiangiogenic factors[41–44]. Maeshima *et al.* have shown this most clearly for tumstatin, a fragment of type IV collagen α3 chain[45]. Tumstatin acts through $\alpha_v\beta_3$, phosphatidylinositol-3 (PI3) kinase, protein kinase B (PKB/Akt), and mammalian target of rapamycin (mTOR) to inhibit protein synthesis in endothelial cells, inducing apoptosis and inhibiting angiogenesis[45]. If indeed tumstatin or other ECM fragments do act as endogenous negative regulators of angiogenesis, a possibility that is not yet clear, they may act through $\alpha_v\beta_3$ or other integrins (Fig. 1). As shown in Figure 1, $\alpha_v\beta_3$ could also participate in the generation of these ECM fragments by its activation of MMP-2, and MMP-2 can cleave itself to generate an antiangiogenic fragment, PEX, also acting through $\alpha_v\beta_3$ (refs. 46,47).

Another class of model involves crosstalk between integrins and growth factor receptors. For example, Reynolds *et al.* have shown that $\alpha_v\beta_3$ downregulates the VEGF receptor, flk-1, also known as VEGFR2 or KDR, in endothelial cells[20]. High levels of $\alpha_v\beta_3$ thus reduce responses to VEGF, and this could be a normal mechanism for negative feedback regulation of angiogenesis. In this model, absence of this negative feedback in β_3-deficient mice would allow upregulation of VEGFR2 and increased responsive-

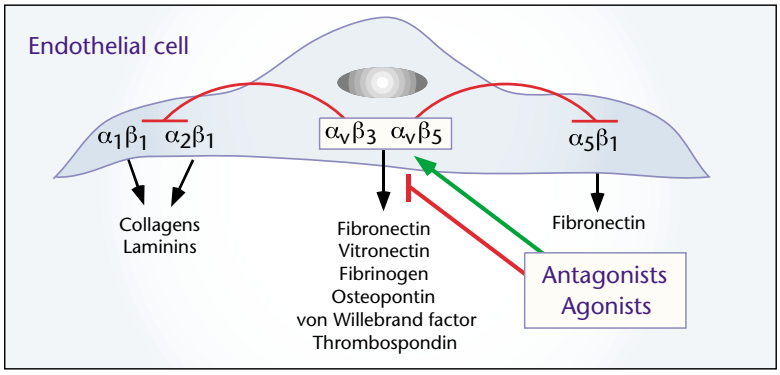

Fig. 2 *Trans*-dominant inhibition of proangiogenic integrins. $\alpha_5\beta_1$–fibronectin interactions are clearly proangiogenic, and ablation or blocking their interaction blocks angiogenesis[26–28]. The same seems to be true for the collagen receptors, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (refs. 29, 30). We also know that specific interference with β_3 integrins can indirectly inhibit the functions of $\alpha_5\beta_1$ and $\alpha_2\beta_1$ in the same cell[33,48–50]. A plausible model is that reagents specific for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ could affect angiogenesis by such *trans* inhibition of $\alpha_5\beta_1$ and the other integrins (red arrows), The mechanism could involve signal transduction and/or competition for a shared component[33,48–52]. Such *trans*-dominant inhibition works best when the targeted integrin is at high levels, as are $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on active endothelium.

ness to VEGF that may contribute to the enhanced angiogenesis seen in these mice.

Another very plausible class of model for negative regulation by $\alpha_v\beta_3$ (and $\alpha_v\beta_5$) involves *trans*-dominant inhibition of other, proangiogenic integrins ($\alpha_5\beta_1$, $\alpha_1\beta_1$, $\alpha_2\beta_1$) in endothelial cells (Fig. 2). Diaz-Gonzalez *et al.* have clearly demonstrated *trans*-dominant negative regulation of $\alpha_5\beta_1$ and $\alpha_2\beta_1$ by $\alpha_{IIb}\beta_3$, a close relative of $\alpha_v\beta_3$ (ref. 33). This group showed that reagents specifically targeting $\alpha_{IIb}\beta_3$ inhibit (indirectly) the functions of $\alpha_5\beta_1$ and $\alpha_2\beta_1$ in the same cells. There is also good evidence that antibodies to $\alpha_v\beta_3$ can inhibit the role of $\alpha_5\beta_1$ in phagocytosis[48] or cell migration[49,50]. Crosstalk among integrins, both positive and negative, is well established[48–52]. The mechanisms are not entirely clear, but are believed to include signal transduction and possibly also competition for limiting components. Be that as it may, it is completely clear that integrins can affect each other's functions. Thus, it is entirely plausible that reagents directed at $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ may be having their effects, not by inhibiting directly some proangiogenic role of those integrins, but rather by inhibiting indirectly the well-established proangiogenic roles of some other integrin such as $\alpha_5\beta_1$ linking to fibronectin (Fig. 2).

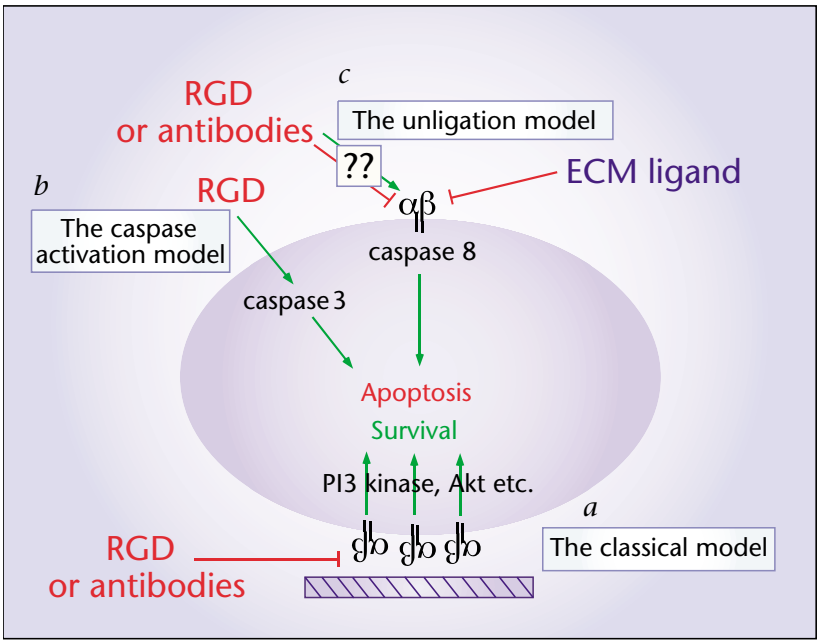

Fig. 3 Three models for endothelial apoptosis. ***a***, The classical model, in which integrin engagement by ligand is necessary to provide survival signals. Inhibitors block ligand binding and thus the survival signals[8,53,54]. ***b***, The caspase activation model, in which RGD peptides directly activate caspases and trigger apoptosis without any involvement of integrins[55,56]. ***c***, The unligation model, or "integrin-mediated cell death"[57,58], in which unligated integrins directly bind and activate caspase-8. ECM ligands block this, but RGD peptides and antibodies binding to the same integrins are not proposed to do so[57,58], even though they are known to activate integrins[32–35].

Finally, we must consider the potential role of integrins as regulators of apoptosis; there are several very distinct versions of this class of models (Fig. 3). In the classical view (Fig. 3*a*), for which there is a great deal of evidence, integrin ligation provides a survival signal (through the PI3 kinase–Akt pathway), and cells are dependent for survival on anchorage[53]. In this view, when cells lose their integrin-mediated adhesion they undergo apoptosis, sometimes called anoikis[54]. Indeed, in the early experiments that showed LM609 or RGD-based peptides to inhibit angiogenesis, they induced apoptosis of endothelial cells and this was interpreted as anoikis[8]. Later work showed that RGD-based peptides could induce apoptosis by directly activating caspases, without any involvement (positive or negative) of integrins (Fig. 3*b*)[55,56]. Whether or not such integrin-independent effects contribute to the apoptosis seen in any of the angiogenesis inhibition experiments remains unclear, but should be evaluated. Most recently, Stupack *et al.* have proposed a third model suggesting that unligated integrins promote apoptosis by directly recruiting caspase-8 (ref. 57) (Fig. 3*c*). Cheresh and Stupack have suggested that this "integrin-mediated death" might explain the enhanced angiogenesis in β_3-deficient mice[58]. These workers suggest that, in β_3^+ mice, or when inhibitors block $\alpha_v\beta_3$, unoccupied $\alpha_v\beta_3$ triggers apoptosis of endothelial cells[57,58]. However, since RGD peptides and mimetics are known to act as agonists[32–35], it is difficult to see how this model explains the inhibition data. It is also not easy to see how it could explain the difference between wild-type and $\alpha_v\beta_3$-deficient mice. Because $\alpha_v\beta_3$ is the most promiscuous integrin known, it seems highly unlikely that it is ever unligated in a wild-type mouse. It seems much more likely that apoptosis could be induced by negative regulators acting through $\alpha_v\beta_3$, such as tumstatin, or organized by $\alpha_v\beta_3$, for example thrombospondin (see Fig. 1). Perhaps the blocking agents interfere with positive stimuli (such as vitronectin) and allow negative stimuli (such as tumstatin) to impinge on the α_v integrins.

In presenting these models for negative regulation by α_v integrins, I do not wish to suggest that these integrins never have positive effects, just that there is good reason to believe that they can themselves be antiangiogenic. It is quite conceivable that these integrins could play both positive and negative roles in different phases of angiogenesis and/or could act as balancers or integrators of positive and negative signals. However, the genetic data suggest that, on balance, their role is as negative regulators.

What bearing do these considerations have on the potential and design of antiangiogenic drugs targeting α_v integrins? It might appear logical to target some unambiguously proangiogenic integrin such as $\alpha_5\beta_1$, and certainly that would be a good idea. However, α_v integrins are frequently expressed at high levels on angiogenic blood vessels, and there are data suggesting that angiogenesis can somehow be inhibited by targeting them. The point of the present discussion is not that such drugs will not work, but that we do not understand how they work and that we need to do so if we are to design the most effective drugs. The original idea that antagonists of α_v integrins should be antiangiogenic requires reexamination. Although such reagents were originally isolated as antagonists of cell adhesion, I argue here that they may be acting also and more importantly as agonists of some normal negative regulatory role of α_v integrins. One is driven to consider such models by the genetic data, and I have outlined several plausible possibilities. We need to find out which of these or other mechanisms actually function during angiogenesis. We need then to screen for drugs that act as agonists of negative regulatory functions or as antagonists of any

positive roles. This could easily be done by examining potential drugs for their effects on signal transduction (activation or inhibition of FAK, PI3 kinase, Akt, mTOR, VEGFR2, caspases, etc.) or on activation of α_v integrins or other integrins in the same cells. Integrins may yet turn out to be good targets for antiangiogenic drugs, but the route to effective drug design necessarily includes critical consideration of the mechanistic bases for their involvement.

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