Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity

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Summary
Slit is a secreted protein known to function through the Roundabout (Robo) receptor as a chemorepellent in axon guidance and neuronal migration, and as an inhibitor in leukocyte chemotaxis. Here we show Slit2 expression in a large number of solid tumors and Robo1 expression in vascular endothelial cells. Recombinant Slit2 protein attracted endothelial cells and promoted tube formation in a Robo1- and phosphatidylinositol kinase-dependent manner. Neutralization of Robo1 reduced the microvessel density and the tumor mass of human malignant melanoma A375 cells in vivo. These findings demonstrate the angiogenic function of Slit-Robo signaling, reveal a mechanism in mediating the crosstalk between cancer cells and endothelial cells, and indicate the effectiveness of blocking this signaling pathway in treating cancers.

Introduction
Angiogenesis is a cellular process of capillary sprouting and configuring of neovasculature. It is of crucial importance in a variety of physiological and pathological conditions, including ischemia and hypoxia, wound healing, diabetic retinopathy, macular degeneration, neovascular glaucoma, psoriasis, rheumatoid arthritis, and cancer growth and metastasis (Hanahan and Folkman, 1996; Risau, 1997). The obligatory requirement of Slit for the growth and metastasis of cancers is now well recognized. For instance, the growth of solid tumors requires concomitant expansion of vascular networks to maintain the blood supply of oxygen and nutrients; an insufficient supply of blood (to tissues located more than 100 to 200 μm away from blood vessels) can lead to cancer necrosis. Although vascular endothelial cell growth factors (VEGFs), fibroblast growth factors (FGFs), and several other angiogenic molecules are indispensable for vessel formation (Gale and Yancopoulos, 1999; Yancopoulos et al., 2000; Folkman, 2001, 2002; Kerbel and Folkman, 2002), the molecular and cellular mechanisms regulating tumor angiogenesis are still not well understood.

Several families of extracellular proteins including the neotrans, the semaphorins, the ephrins, and the Slits function as guidance cues to attract or repel projecting axons and migrating neurons during the development of the nervous system (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). Slit is a new family of secreted repellents in axon guidance (Wang et al., 1999; Brose et al., 1999; Kidd et al., 1999; Li et al., 1999) and neuronal migration (Wu et al., 2001). The receptor for Slit is the transmembrane protein Roundabout (Robo) (Kidd et al., 1998, 1999; Brose et al., 1999; Li et al., 1999). Slit has recently been shown to be an endogenously available inhibitor of leukocyte chemotaxis (Wu et al., 2001). There are three Slits and four Robos in mammals (for a recent review, see Wong et al., 2002). Some of the Slits and Robos are expressed in adult tissues and outside the nervous system (Holmes et al., 1998; Wu et al., 2001). For example, SLIT2 mRNA in endothelial cells and ROBO1 mRNA in leukocytes have been reported (Wu et al., 2001). However, it has not been determined whether human cancer cells can express these genes, either at the mRNA or protein levels. Potential roles of Slit-Robo signaling in pathological conditions have been suggested (Wu et al., 2001), but not established. We
have now investigated whether Slit and Robo could play roles in tumor angiogenesis in vitro and in vivo. Results from our current studies have established that Slit-Robo signaling attracts vascular endothelial cells, which significantly extends the previous conclusion of a fundamentally conserved mechanism for guiding somatic cell migration (Wu et al., 2001; Rao et al., 2002). Furthermore, our findings of Slit-Robo signaling in promoting tumor-induced angiogenesis indicate that Slit and Robo play significant roles in pathological conditions. The effectiveness of Robo blockade in limiting tumor growth demonstrates a novel target as well as molecular tools for cancer therapy.

Results

Expression of Slit2 on cancer cells and Robo1 in endothelial cells

Using reverse-transcription coupled to the polymerase chain reaction (RT-PCR), we found that A375 cells, a cell line derived from a human malignant melanoma, expressed SLIT2 mRNA and human umbilical vein endothelial cells (HUVECs) expressed ROBO1 mRNA, respectively (Figures 1A and 1D). The expression of SLIT2 mRNA in A375 cells and ROBO1 mRNA in HUVECs was confirmed by Northern blotting with 32P-labeled SLIT2 and ROBO1 cDNA fragments (Figures 1B and 1E). The G3PDH mRNA, a house-keeping gene, was probed as the loading control of total input RNA. We generated antibodies to Slit2 and ROBO1 to detect the expression of Slit2 protein in A375 cells (Figure 1C) and Robo1 protein in HUVECs (Figure 1F). Slit2/293 cells and Robo1/293 cells were used as the positive controls, and V/293 cells were used as the negative controls (Li et al., 1999; Wu et al., 1999; Wu et al., 2001). The expression of Slit2 in A375 cell derived solid tumors was detected by immunohistochemical staining with the anti-Slit2 antibody, but not with the preimmune IgG (Figure 1G). The expression of Robo1, which co-localized with vWF (von Willbrand factor) expression, in tumor endothelial cells was visualized with the anti-Robo1 antibody (Figure 1H), but not with the preimmune IgG (data not shown). Interestingly, there appeared to be a gradient of Slit2 protein with a higher concentration near the center of the tumor and a lower concentration in the periphery (Figure 1G). The expression of Slit2 in malignant melanoma and Robo1 in tumor endothelial cells suggests a possible paracrine Slit2/Robo1 interaction in the pathogenesis of malignant melanoma.

Cell surface localization of Slit2 and its upregulation

Although Slit2 is predicted to be a soluble protein, it has been shown to bind to Glypican-1 and to be localized on the cell surface in the brain, in a heparan sulfate proteoglycan (HSPG)-dependent manner (Liang et al., 1999; Hu, 2001; Ronca et al., 2001). Similarly, we found that the majority of Slit2 was associated with A375 cells in cell culture conditions, even though a small amount of Slit2 was also detected in the supernatants (Figure 1I). Pretreatment of A375 cells with heparin increased the amount of Slit2 in the supernatants and, at the same time, decreased the amount of Slit2 in the whole cell lysates, supporting the HSPG dependence for cell surface localization of Slit2 on A375 cells.

To explore the molecular mechanism governing the formation of the Slit2 gradient, we examined whether cytokines could regulate Slit2 expression in A375 cells. TNF-α (tumor necrosis factor-α) increased the expression of Slit2 (Figure 1I). Similar
findings were also observed using IL-1β (interleukine-1β; data not shown), indicating that the upregulation of Slit2 in response to the secretion of proinflammatory mediators (and perhaps due to hypoxia or expression of oncogenes) could be involved in increasing Slit2 in the center of A375 cell-derived solid tumors, thus resulting in the generation of a gradient.

Attraction of endothelial cells by Slit2

We used several approaches to investigate whether and how Slit could regulate the migration of endothelial cells. We first tested whether Slit2 had any chemotactic effects on the migration of HUVECs using Boyden chamber assay. Similar to bFGF (basic fibroblast growth factor), purified recombinant human Slit2 protein (Figure 1J) induced the migration of HUVECs in a dose-dependent manner (Figure 2A). Preincubation of Slit2 with RoboN (an extracellular fragment of Robo1 that is a known inhibitor for the Slit2/Robo1 interaction; Wu et al., 1999, 2001) or preincubation of HUVECs with R5 (an IgG2a monoclonal antibody to the first immunoglobulin domain of Robo1), but not with an IgG2b control, significantly neutralized the Slit2-induced migration (Figures 2A and 2B). In contrast, RoboN or R5 did not affect the bFGF-induced migration of HUVECs. These results indicate that Slit2 can promote the migration of HUVECs through Robo1.

To determine the functional significance of Robo1, we performed reconstitution experiments using Robo1/293 cells. Slit2 and bFGF both promoted the migration of Robo1/293 cells, but RoboN only neutralized Slit2-induced, but not bFGF-induced, migration of Robo1/293 cells (Figure 2C). Furthermore, bFGF, but not Slit2, triggered the migration of V/293 cells, indicating that HEK293 cells express endogenous receptors for bFGF.

Evidently, Robo1 is essential for the cell migration mediated by Slit2, but not mediated by bFGF, suggesting a specific role for Robo1 in mediating cellular responses to Slit2.

To determine whether Slit2 guided the direction of endothelial cell migration, rather than simply increasing the motility (non-directional or random migration) of endothelial cells, we adopted a technique that had been successfully used for direct observation of axon projection (Song et al., 1997; Ming et al., 2002). This device could generate a microscopic gradient of a specific protein delivered in a picoliter volume through a micropipette by repetitive pressure (Hopper et al., 1999). We loaded a micropipette with Slit2 or bFGF protein and placed its tip at a distance of 100 μm away from the center of individual HUVECs. The entire trajectory of the migrating HUVECs was recorded in a timelapse microscopy. As shown in the examples (Figure 2D), the endothelial cell did not migrate when PBS (phosphate-buffered saline, pH 7.4) was applied. However, it migrated toward the micropipette loaded with Slit2 in a time-dependent manner, indicating that Slit2 was a chemotactic agent for HUVECs. In contrast, HUVECs could migrate toward or away from the micropipette loaded with bFGF in a time-dependent manner (an example of a cell migrating away from bFGF was shown in Figure 2D). The entire sets of experimental data could be presented as the cumulative distribution (%) versus migrated distance (μm; Figure 2E) and, alternatively, as the migrated distance (μm) versus the various treatment (Figure 2F). Cell migration toward a Slit2 gradient established an attractive role for the Slit2-induced endothelial cell migration (Figures 2D–2F). In contrast, bFGF increased the motility of HUVECs by inducing the migration of HUVECs; however, this migration was not directional (Figures 2D–2F).

Angiogenic activity of Slit2 and role of Slit2-Robo1 signaling in tumor-induced angiogenesis

To investigate the potential regulation of angiogenesis by Slit2, we first examined whether Slit2 could induce the differentiation, specifically tube formation of HUVECs in vitro. Slit2 increased the generation of tubular networks in a dose-dependent manner (Figures 2G and 2H). Preincubation of Slit2 with RoboN (Figures 2G and 2H), or preincubation of HUVECs with R5 (Figure 2I), neutralized the effect of Slit2, resulting in fewer and shorter tube structures. These results indicate that Slit2 has an angiogenic activity in vitro. It should be mentioned that Slit2 had no detectable activity on the proliferation of HUVECs (data not shown).

Because A375 cells expressed Slit2 and Slit2 induced the migration and the tube formation of endothelial cells in vitro, we tested the pathological significance of Slit2-Robo1 signaling in tumor angiogenesis using the xenografted animal model. For this purpose, we transfected A375 cells with the RoboN plasmid or the plain vector followed by antibiotic selection and single cell cloning. Using this approach, three stable single cell clones expressing RoboN (designated as RoboN/A375_C1, C2, and C3 cells) and one stable cell clone expressing the vector (designated as V/A375 cells) were generated. These clones were characterized by immunoblotting for RoboN, Slit2, and VEGF (β-tubulin as loading control) expression to ensure that the similar amounts of RoboN were expressed in all three C1, C2, and C3 clones and that no alterations of Slit2 and VEGF expression were detected among RoboN/A375_C1, C2, C3, and V/A375 clones (Figure 3A). In addition, they were tested for in vivo growth rates to ensure that they all grew at similar rates in cell culture conditions (Figure 3B).

These transfectants were then inoculated subcutaneously into athymic nude mice. When compared to those from V/A375 cells, tumors resulting from RoboN/A375_C1, C2, and C3 cells had significantly reduced microvessel densities (Figures 3C and 3D). Furthermore, the tumor volumes and masses from Robo1/A375_C1, C2, and C3 cells were all markedly smaller than those from the control V/A375 (Figures 3E and 3F).

As an alternative approach to the RoboN construct, we tested R5, the function-blocking monoclonal antibody against the first immunoglobulin motif of Robo1. RoboN would block Slit signaling by absorbing Slit proteins, whereas R5 should block Robo specifically, thereby providing a complementary approach to inhibit Slit2-Robo1 signaling. We found that, compared to an IgG2a control, R5 clearly reduced tumor microvessel densities and tumor masses (Figures 3H–3M).

To substantiate the above conclusion, we further examined the effects of Slit2 overexpression in tumor angiogenesis and growth. We proposed that Slit2 overexpression could induce the exaggerated tumor angiogenesis and the accelerated expansion of tumor mass. After transfection of A375 cells with the Slit2 plasmid or the plain vector as described above, we generated two stable single cell clones expressing Slit2 (designated as Slit2/A375_C1 and C2 cells) and one stable cell clone expressing the vector (designated as V/A375 cells). They were characterized by immunoblotting for Slit2 and VEGF (β-tubulin

CANCER CELL : JULY 2003

21
**Figure 2.** Slit2-induced migration and tube formation of endothelial cells

Migrations of HUVECs (A and B), Robo1/293 cells, and V/293 cells (C) were measured using Boyden chamber assay. Results were calculated as mean ± SD values from triplicate measurements of three to six separate experiments.

D: For measurement of directional migration of HUVECs, a protein gradient was applied from a micropipette by the pulsatile application of 0.15 μM Slit2 or 1 μM bFGF. Phase-contrast micrographs of endothelial cells were recorded in a timelapse mode after exposure to the Slit2 or bFGF gradients (min). The arrowhead indicated the direction in which the protein was loaded through the micropipette. Scale bar, 8 μm.

E: The migratory directions induced by Slit2 and bFGF (E) and migrated distances (F) were determined (each dot representing the migratory direction and distance of a single endothelial cell). *, p < 0.05 and **, p < 0.01, Kolmogorov-Smirnov test.

G: Tube formation of HUVECs on Matrigel was visualized by phase-contrast microscopy. Scale bar, 60 μm.

H and I: The effects of Slit2 and bFGF on the tube formation of HUVECs without or with RoboN or R5. Results were calculated as mean ± SD values from triplicate measurements of three separate experiments.

as loading control) expression to ensure that the similar amounts of Slit2 were overexpressed in both C1 and C2 clones and that no alterations of VEGF expression were detected among C1, C2, and V/A375 clones (Supplemental Figure S1A at http://www.cancercell.org/cgi/content/full/4/1/19/DC1). In addition, they were tested to ensure that they all grew at similar rates in cell culture conditions (Supplemental Figure S1B).

After inoculation of these transfectants, tumors resulting from Slit2/A375_C1 and C2 cells had significantly increased microvessel densities if compared to those from V/A375 cells (Supplemental Figures S1C and S1D on Cancer Cell website).

Furthermore, the tumor volumes and masses from Slit2/A375_C1 and C2 cells were all significantly bigger than those from the control V/A375 (Supplemental Figures S1E and S1F). These in vivo results demonstrate the biological significance of Slit2-Robo1 signaling in tumor angiogenesis and growth.

As endothelial cells expressed both Slit2 and Robo1 and Slit2 reportedly inhibited leukocyte chemotaxis (Wu et al., 2001), we measured the leukocyte counts in the blood and in the solid tumors of these xenografted mice. We found no clear differences among these groups (Figures 3G and 3N and Supplemental Figure S1G). The equivalent numbers of leukocytes...
within the tumors were further confirmed by hematoxylin and
eosin (H&E) staining of the tissue sections (data not shown).
Slit2 therefore does not appear to be involved in leukocyte
trafficking into malignant melanoma in vivo.

Expression of Slit2 in human cancers
To explore the general significance of our findings, we examined
the expression of Slit2 in multiple human cancer cell lines origi-
nating from various tissues and organs. Slit2 was expressed
in A375 cells (malignant melanoma), ScaBer cells (bladder
squamous carcinoma), SK-N-SH cells (neuroblastoma), NCI-
H446 cells (small cell lung cancer), T24 cells (transitional cell
carcinoma of urinary bladder), LoVo cells (colon adenocarci-
noma), ZR-75-30 cells (breast cancer), CNE cells (nasopharyn-
geal carcinoma), SMMC-7721 cells (hepatocellular carcinoma),
Acc-2 and Acc-M cells (both were adenoid cystic carcinoma of

Figure 3. Inhibition of angiogenesis and growth of malignant melanoma
A: Detection of RoboN by the anti-Robo1 Ab in three single cell stable clones of RoboN/A375_C1, C2, and C3, but not in one single cell stable clone of V/A375. Slit2, VEGF, and β-tubulin were also detected by the respective Abs to Slit2, VEGF, and β-tubulin in all these cell clones. No signal was evident with preimmune rabbit IgG (data not shown).
H: R5, but not an IgG 2b control, recognized Robo1. The anti-HA (hemagglutinin) Ab (Clontech), which recognized the HA tag of Robo1 fusion protein in Robo1/293 cells, was used as the positive control.
B and I: Measurement of growth rates for V/A375 cells and RoboN/A375_C1, C2, and C3 cells (B) and for A375 cells in the presence of mouse IgG2b or R5 (I). Results were expressed as the mean ± SD values from triplicate measurements of three separate experiments.
C and J: Immunohistochemical staining of blood vessels within tumors of V/A375 cells and RoboN/A375_C2 cells (C) and of A375 cells treated with mouse IgG 2b and R5 (J) using an anti-CD31 Ab. No positive staining was detected when preimmune IgG was used (data not shown). Scale bar, 20 μm.
D and K: The mean ± SD values of microvessel densities (CD31 staining) were statistically analyzed using the ImageTool software for tumors of V/A375 cells, RoboN/A375_C1, C2, and C3 cells (D) and of A375 cells treated with an IgG 2b control or R5 (K; n = 14 for each group).
E and L: The mean ± SD values of tumor volumes for tumors from V/A375 cells, RoboN/A375_C1, C2, and C3 cells (E) and from A375 cells treated with mouse IgG2b or R5 (L; n = 14 for each group).
F and M: The mean ± SD values of tumor weights for tumors from V/A375 cells, RoboN/A375_C1, C2, and C3 cells (F) and from A375 cells treated with mouse IgG2b or R5 (M; n = 14 for each group).
G and N: Leukocyte counts in blood and in tumors derived from V/A375 cells, RoboN/A375_C1, C2, and C3 cells (G) and from A375 cells treated with mouse IgG2b or R5 (N; n = 14 for each group). *, p < 0.05 and **, p < 0.01, Student’s t test.
Figure 4. Expression of Slit2 in cancers
A: Immunoblotting of cell lysates from various cancer cell lines using the anti-Slit2 Ab and the anti-β-tubulin Ab. The Slit2/293 cells were used as the positive control.
B: Northern blotting of SLIT2 and G3PDH mRNAs using 32P-labeled SLIT2 and G3PDH cDNA fragments. C: Immunohistochemical staining of various human cancers with the anti-Slit2 Ab. Arrows indicated the expression of Slit2 on tumor cells. Scale bar, 10 μm.

salivary gland), and A673 cells (rhabdomyosarcoma; Figure 4A). However, Slit2 was not detected in A549 cells (lung cancer), HeLa cells (cervical epithelial adenocarcinoma), MCF-7 cells (breast adenocarcinoma), and 786-O cells (primary renal cell adenocarcinoma). The expression of Slit2 in these cancer cell lines was confirmed by Northern blotting with 32P-labeled SLIT2 cDNA fragment (Figure 4B). Again, the β-tubulin immunoblotting and the G3PDH Northern blotting were used as the loading controls of input proteins and RNAs (Figures 4A and 4B).

The finding of Slit2 expression in a variety of cancer cell lines is consistent with the recent report of Slit2 expression in prostate cancers using a RT-PCR-based approach (Latil et al., 2003). Along this line of investigation, we examined human samples of malignant melanoma, rectal mucinous adenocarcinoma, invasive breast carcinoma, gastric squamous carcinoma, and hepatocellular carcinoma for Slit2 expression (Figure 4C). We found that in all these cancer samples, Slit2 was expressed in the cancerous tissues, but not in the nearby regions of apparently normal tissues. The positive staining of Slit2 was found in 3 malignant melanomas (n = 7; 70.8% positive), 42 colorectal carcinomas (n = 65; 64.6% positive), and 38 gastric carcinomas (n = 74; 51.4% positive). Notably, Slit2 staining appeared to be more intense in the areas of the tumor, where more cancer cells and blood vessels (visualized by an antibody to vWF) existed within human colon carcinoma (Figure 5A). In contrast, less Slit2 staining was detected where there were fewer cancer cells and vasculatures. A similar correlation was also found in human breast carcinoma (data not shown). Furthermore, Slit2 was absent in normal and hyperplastic colon tissues, began to appear in colon adenomas (3/14; 21.4% positive), and upregulated in colon carcinoma (42/65; 64.6% positive; Figure 5B). These data suggest a correlation among cancerous alterations, Slit2 expression, and the microvessel density within tumors.

Involvement of phosphatidylinositol-3 kinase (PI-3K) in Slit2-induced migration and tubulogenesis of endothelial cells
In an effort to explore downstream molecular mechanisms of the Slit2-Robo1 signaling in vascular endothelial cells, we screened a variety of chemicals for their effects on the migration and the tube formation of HUVECs induced by Slit2. We found that Wortmannin and LY294002 (both PI-3K inhibitors; Toker and Cantley, 1997; Fruman et al., 1998), but not KT5823 (an inhibitor of protein kinase G), attenuated Slit2-induced migration, but not bFGF-induced migration, of HUVECs in a dose-dependent manner (Figures 6A and 6B). Both inhibitors also attenuated directional migration (Figure 6E) and tubulogenesis induced by Slit2 (Figures 6C and 6D). Biochemically, Slit2, but not bFGF, could activate PI-3K in HUVECs, resulting in the increased radioactivity of 2Pi-ATP-labeled inositol phospholipids (Figures 6F and 6G). These data demonstrate that activation of PI-3K in HUVECs by Slit2 is required for directional migration and tube formation of vascular endothelial cells.

Figure 5. The correlation of Slit2 expression with MVD alterations and Slit2 expression in carcinoma stage
A: Immunohistochemical staining of human colon carcinoma with the anti-Slit2 and anti-vWF Abs. No Slit2 staining for the normal or hyperplastic colon tissues. Scale bar, 50 μm. B: Immunohistochemical examination of the tissue sections from human colon adenoma (weak staining) and carcinoma (strong staining) using the anti-Slit2 Ab. Arrows indicated the expression of Slit2 on cancer cells and arrowheads indicated the microvessels within the solid tumors. Scale bar, 100 μm for the upper panel and 50 μm for the lower panel.
Discussion

Our results have concluded that in addition to its well-characterized functions in axon guidance and neuronal migration, Slit2 can attract vascular endothelial cells and promote tumor-induced angiogenesis. This strengthens the idea of fundamental conservation of guidance mechanisms for all somatic cells (Wu et al., 2001; Rao et al., 2002). Our results also indicate that Slit2 expressed in solid tumors can communicate with Robo1 expressed in endothelial cells, revealing a pathway mediating tumor-induced angiogenesis. Because we can inhibit tumor growth in vivo by specific neutralization of the Slit2-Robo1 interaction with either the extracellular part of Robo1 or R5, the function-blocking monoclonal antibody for Robo1, it not only indicates the importance of Slit-Robo signaling in tumor-induced angiogenesis and tumor growth, but also provides novel molecular tools for attenuating tumors. Taken together, our results have significant implications in both basic mechanisms and clinical applications.

Migratory behavior is distinct among different cell types,
Experimental procedures are consistent with the notion that other angiogenic factors are to be essential for the angiogenic switch, the expression of VEGF malignant melanoma (Figure 3), it will be interesting to test the after. Although VEGF-mediated signaling is generally believed pletely reduces the microvessel density and the tumor mass of genetically reviewed in Wong et al., 2002). It is thus curious that Slit2 has been found to function as a chemoattractant for vascular endothelial cells. Previous studies by Poo and colleagues have shown that the same guidance cue can either attract or repel the same axons depending on intracellular cAMP and cGMP levels (Song et al., 1997). It will be interesting to test whether cAMP and cGMP play a role in endothelial cell migration and angiogenesis, in addition to their roles in endothelial responses and pathological processes.

All previous studies of Slits in neurons and leukocytes indicate that Slits are repulsive or inhibitory for migrating cells (recently reviewed in Wong et al., 2002). It is possible that Slit2 has been found to function as a chemoattractant for migrating cells. Previous studies by Poo and colleagues have shown that the same guidance cue can either attract or repel the same axons depending on intracellular cAMP and cGMP levels (Song et al., 1997). It will be interesting to test whether cAMP and cGMP play a role in endothelial cell migration and angiogenesis, in addition to their roles in endothelial responses to Slit2.

We have found that PI-3K is involved in endothelial cell responses to Slit2. It will be interesting to compare the pathways mediating attractive responses in endothelial cells to those in neurons, although the intracellular transduction pathway for Slit-Robo signaling in neuronal repulsion is only beginning to emerge (Bashaw et al., 2000, Fritz and VanBerkum, 2000, Wong et al., 2001). For example, it would be interesting to know whether PI-3K inhibitors, such as Wortmannin and LY294002, could prevent the repellant action of Slit2 on axon guidance and neuronal migration, and the inhibitory action of Slit2 on leukocyte chemotaxis.

Extensive studies of genetically engineered mouse models of spontaneous tumorigenesis and pathological examinations of human cancers have delineated discrete stages of carcinogenesis, including pre-angiogenic hyperplasia/dysplasia/carcinoma in situ (CIS), angiogenic dysplasia/CIS, and small tumor and large tumor/invasive carcinoma (Hanahan and Folkman, 1996). Among these stages, angiogenesis is switched on during the premalignant stage of tumor development, persisting thereafter. Although VEGF-mediated signaling is generally believed to be essential for the angiogenic switch, the expression of VEGF and its cognate receptors, flt-1 (VEGF-R1) and flk-1 (VEGF-R2), appears to be constitutive; no alterations in the expression of these molecules can be clearly detected (Bergers et al., 1999, 2000, Inoue et al., 2002). The disappointing results from the clinical trial using several inhibitors for VEGF and its receptors are consistent with the notion that other angiogenic factors are playing critical and indispensable roles in tumor angiogenesis (Kerbel and Folkman, 2002). Our finding of the Slit2 expression on cancerous tissues (Figure 5B) suggests that Slit2 is a novel player in tumorigenesis.

It should be pointed out that we have screened and found the increased Slit2 expression in a number of tumor cell lines and primary tumors in this study. However, there are two other Slits (Wong et al., 2002), which have not yet been tested in tumors. It is possible that Slit1 and Slit3 could also be expressed in some tumors. Similarly, we have so far focused on Robo1, whereas there are three other Robos. Considering the possibility that different Robos could be involved in angiogenesis, we suspect that RoboN (made from the extracellular part of Robo1) may block all three Slits and that the R5 antibody (made by immunization of and screening against the first immunoglobulin domain of Robo1) may block other Robos as well as Robo1.

The result of immunohistochemical staining suggests that Slit2 protein is expressed in a center-to-periphery gradient in solid tumors. It will be interesting to investigate whether this gradient contributes to the exaggerated angiogenesis in the center of solid tumors and what is the mechanism for forming and maintaining the Slit2 gradient. It has been reported that Slit2 associates with HSPGs on Glypican-1 expressed on the neuronal cells in the central nervous system. Likewise, the gradient formation of Hedgehog requires Tout-velu, an enzyme involved in HSPG biosynthesis (Bellaiche and Perrimon, 1998). Further, overexpression of the glypican HSPG, called Dally-like, leads to higher than normal levels of extracellular Wingless (Wg) accumulation (Tsuda et al., 1999). In contrast, clones defective in HSPG biosynthesis bind to lower than normal levels of extra-cellular Wg and are deficient in Wg signaling (Baeg et al., 2001). Notum, a member of the $\omega/\beta$-hydrolase superfamily, influences the distribution of Wg by modifying HSPG Dally-like and Dally (Giradex et al., 2002). Our finding that pretreatment of A375 cells with heparin increases the amount of Slit2 in the supernatants and, at the same time, decreases the amount of Slit2 in the whole cell lysates also supports the association of Slit2 with HSPGs on cancer cells. It remains to be determined whether Slit2 is associated with Glypican-1 HSPGs expressed on cancer cells, whether regulation of Glypican-1 HSPGs alters the temporal and spatial distribution of Slit2 on cancer solids, and whether Tout-velu and/or Notum could affect the surface localization of Slit2 by modulating Glypican-1 HSPGs.

The results presented here have demonstrated the role of Slit2–Robo1 signaling in tumor angiogenesis and have evaluated the significance of this signaling pathway in the pathogenesis of cancers. However, whether this discovery can be translated to the diagnosis, treatment, and prevention of malignant tumors remains to be determined. For example, can the immunohistochemical staining of Slit2 be used as a reliable marker for the diagnosis of certain cancers? Are the measurements of Slit2 in the blood and the urine useful for screening certain carcinomas? Considering that the neutralization of Robo1 activity incompletely reduces the microvessel density and the tumor mass of malignant melanoma (Figure 3), it will be interesting to test the efficacy of a combined therapy using inhibitors for both VEGF and Slit2 for treatment of cancers. It is also interesting to test whether Slit2 has any synergistic effects on the angiogenic activities of VEGF, FGFR, ephrin, and other known angiogenic factors.

Experimental procedures

RT-PCR and Northern blotting

Primary HUVECs were cultured as previously described (Geng et al., 1990). RT-PCR was performed as before (Ma and Geng, 2000). Primers used were human Slit2 sense (5'-GAC GAG GGA TCC CAT ATC GCG GTA GAA CTC-3') and antisense (5'-GGA CAC CTC GAG CTT ACC GCA CTG-3'), human ROBO1 sense (5'-CCT ACA CAG ATG ATC TTC C-3') and antisense (5'-CAG AGC AGC CTG CAG CTC-3').
AGC TTT CAG TTT CCT C-3; human β-ACTIN sense (+) 5'-ATG GAT GAT GAT ATC GCC GC-3' and antisense (-) 1227 5'-CTA GAA GCA TTT TGG GTG G-3'. Total RNAs from A375 cells and the primary culture of HUVECs were also probed with the 32P-labeled human Slit2 (1 kb), rat ROBO1 (2.1 kb), or human G3PDH (1.3 kb) cDNA fragments.

**Cell lines**

Stable human embryonic kidney 293 cell lines expressing full-length human Slit2 with a c-myc tag at its carboxyl terminus (Slit2/293 cells), the extracellular portion of rat Robo1 (RoboN/293 cells) and full-length of rat Robo1 (Robo1/293 cells) with a HA tag at their carboxyl termini, and the plasmid vector (V/293 cells) were established as previously described (Li et al., 1999; Wu et al., 1999, 2001).

**Antibody generation, immunoblotting, and immunostaining**

GST and His-tag fusion proteins of Slit2 (encoding 57–207 or 1272–1593 amino acids of human Slit2) and Robo1 (encoding 1–168 or 961–1217 amino acids of rat Robo1) were constructed into pGEX-4T-1 (Amersham Pharmacia Biotech) and pET-30a (+) (Novagen) vectors and expressed according to the manufacturers’ protocols. The purified GST fusion proteins were used as the antigens to immunize rabbits and mice for generation of anti-Slit2 and anti-Robo1 polyclonal and monoclonal antibodies. The purified His-tag fusion proteins were used for affinity isolation of the polyclonal antibodies and for ELISA assay during the screening of monoclonal antibodies. Equal amounts of cell lysates (routinely verified by β-tubulin immunoblotting; Sigma) were used for immunoblotting. For experiments of TNF-α or heparin treatment, A375 cells were cultured in the presence of either 300 units/ml TNF-α, 50 μg/ml heparin, or both for 12 hr. Antibodies against CD31 (PharMingen; a 1:50 dilution for cryostat sections), vWF (Antibody Diagnos...


