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Function of endogenous inhibitors of angiogenesis as endothelium-specific tumor suppressors

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Disruption of the systemic angiogenesis balance to favor enhanced angiogenesis is speculated to represent a key step in the growth of tumors. Although a major emphasis has been placed on the increase of angiogenesis stimulators, such as VEGF, on the disruption of the angiogenic balance, the potential role of the physiological levels of endogenous inhibitors of angiogenesis on tumor growth is poorly understood. Here, we use three independent lines of mice deficient in tumstatin, endostatin, or thrombospondin-1 (TSP-1), to address the role that these endogenous angiogenesis inhibitors play in tumor growth. Our experiments demonstrate that normal physiological levels of these inhibitors serve to retard the growth of tumors, and that their absence leads to enhanced angiogenesis and a 2- to 3-fold increase in tumor growth. The tumor-suppressive action of TSP-1, endostatin, and tumstatin correlates with expression of CD36 receptor, $\alpha 5\beta 1$ integrin, and $\alpha v\beta 3$ integrin on proliferating endothelial cells, respectively. Moreover, tumors grow 2-fold faster in the tumstatin/TSP-1 double-knockout mice, compared with either the tumstatin- or the TSP-1-deficient mice, strongly suggesting that ceiling rate of cancer growth is not completely dependent on the genetic defects of cancer cells but also depends on the host-derived tumor microenvironment. Additionally, tumor growth in transgenic mice overproducing endostatin specifically in the endothelial cells (a 1.6-fold increase in the circulating levels; mimicking Down's syndrome condition) is 3-fold slower than the tumor growth in wild-type mice. Collectively, our data suggest that physiological levels of endogenous inhibitors of angiogenesis can serve as endothelium-specific tumor suppressors.

cancer | endostatin | tumstatin | thrombospondin-1

Angiogenesis, the process by which new blood vessels are derived from preexisting capillaries, is considered essential for tumor growth (1, 2). The tumor microenvironment influences the induction of tumor angiogenesis (2–4). The angiogenic switch is turned “on” when levels of angiogenesis stimulators, such as VEGF and bFGF, exceed those of angiogenesis inhibitors (1, 5). Endogenous angiogenesis inhibitors are molecules that are naturally present in the body fluids or tissue and possess antiangiogenic activity, potentially offering a counterbalance for the angiogenesis stimulators, thus maintaining a physiological angiogenesis balance (3, 6).

Thrombospondin-1 (TSP-1) is a secreted glycoprotein found in the extra- and pericellular matrix with an antiangiogenic activity (7). TSP-1 regulates the extracellular milieu by a direct interaction with extracellular matrix proteins, influencing levels of extracellular proteases and activating latent TGF- β . It also functions as a mediator between the extracellular space and the cells through receptor-mediated events (7). TSP-1 inhibits endothelial cell proliferation and migration, thus suppressing angiogenesis (7).

Endostatin is the C-terminal noncollagenous domain of type XVIII collagen, a basement membrane protein found in most vascular basement membranes (3, 8–10). Endostatin inhibits endothelial cell migration and induces apoptosis, thus leading to reduced vascularization of tumors (8). Tumstatin is the noncol-

lagenous domain of type $\alpha 3$ (IV) collagen, a basement membrane collagen found in kidney, lung, testis, and other vascular basement membranes (11–14). Tumstatin inhibits angiogenesis by inducing apoptosis and inhibits endothelial cell proliferation through its binding to $\alpha v\beta 3$ integrin, leading to suppression of cap-dependent protein translation (11, 15, 16).

Endostatin, tumstatin, and TSP-1 exert their effect on the sprouting/proliferating endothelial cells by interacting with their specific receptors on the endothelial cell, thus resulting in changes in the intracellular signaling and inducing antiangiogenic effects (15, 17–20). Although other receptors can be involved, the well studied receptors for endostatin, tumstatin, and TSP-1 on the endothelial cells are $\alpha 5\beta 1$ integrin, $\alpha v\beta 3$ integrin, and CD36, respectively (15, 17–20). Mice deficient in either TSP-1 or tumstatin have been shown to display increased rates of tumor growth because of increased tumor vascularization (21–23). Surprisingly, however, mice deficient in endostatin did not display increased tumor growth (24).

Here, we demonstrate that genetic loss of individual endogenous inhibitors of angiogenesis leads to a change in the balance between angiogenesis stimulators and their inhibitors (disruption of angiogenesis balance), thus favoring enhanced angiogenesis and increased tumor growth rates. This balance can further be altered by a loss of two inhibitors together, as observed in mice deficient in both tumstatin and TSP-1.

Individuals with Down's syndrome exhibit a significant protection from developing many forms of solid tumors (25, 26). Interestingly, it was noted that due to trisomy-21 and an extra copy of type XVIII collagen on chromosome 21, these individuals circulate ≈ 1.7 -fold more endostatin than do normal individuals (normal; 20.3 ± 11.5 ng/ml vs. Down's syndrome; 38.6 ± 20.1 ng/ml) (26). To test the hypothesis that such mild increase in the circulating levels of endostatin can confer tumor-suppressive effects, we generated conditional transgenic mice to produce endostatin specifically in the endothelial cells using a VE-cadherin promoter. The endostatin transgenic mice producing 1.6-fold higher circulating endostatin, compared with normal mice, were used in tumor growth studies. A 1.6-fold endostatin increase shifts the systemic angiogenesis balance to favor angiogenesis suppression and reduces the rate of tumor growth by 3-fold. Collectively, our studies demonstrate that physiological concentrations of endogenous inhibitors of angiogenesis can function as tumor suppressors.

Materials and Methods

Cell Lines and Gene-Targeted Mice. Lewis lung carcinoma (LLC) and B16F10 melanoma cells were grown at 37°C in 5% CO₂/95%

Abbreviations: TSP, thrombospondin; LLC, Lewis lung carcinoma.

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[‡]Beth Israel Deaconess Medical Center has licensed certain intellectual property surrounding tumstatin to Ilex Oncology, Inc. (San Antonio), and is also an equity owner in Ilex Oncology, Inc.

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transgenic construct to make it possible to induce or suppress the amounts of endostatin produced by cross-breeding to mice expressing the Tet promoter transactivator under the VE-cadherin promoter (28) (Fig. 3A). Endostatin production is regulated by turning the transgene “on” or “off” by providing the double-transgenic mice with tetracycline or doxycycline. When doxycycline is not provided, endostatin transgene expression is at the highest.

In Vivo Tumor Studies. Age- and sex-matched wild-type mice and mice deficient in tumstatin, endostatin, TSP-1, and tumstatin/TSP-1 as well as mice overexpressing endostatin were used for these studies. All mice used were on a C57BL/6 genetic background. More than five mice in each group were used in an experiment, and each experiment was performed at least two or three times. LLC or B16F10 melanoma cells were injected s.c. on the backs of the mice (each 1 or 0.5×10^6 cells per mouse). The tumors were measured as described in ref. 11. The mice were euthanized, and the tumors and plasma were collected at the end of each *in vivo* experiment. All mouse studies were reviewed and approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Immunohistochemistry and Western Blotting. Immunohistochemical staining was performed as described in ref. 21. Four-micrometer frozen sections of tumors or other tissues were incubated with various primary antibodies, i.e., rat anti-CD31 (Pharmingen), rabbit anti-von Willebrand factor (Dako), rat anti- $\alpha 5$ integrin, hamster anti- $\beta 3$ integrin, rat anti-CD36 (Pharmingen), goat anti-endostatin (R & D Systems), rabbit anti-endostatin (15), rabbit anti-tumstatin (21), mouse anti-FLAG (Sigma), and goat anti-TSP-1 (Santa Cruz Biotechnology). In each group, the numbers of CD31-positive endothelial cells of blood vessels were counted at $\times 200$ magnification in a blinded fashion for 10 separate fields and averaged. For double-staining of receptor molecules on tumor vessels, the counting was performed at $\times 200$ magnification in a blinded fashion for five separate fields and averaged. Western blotting of cell lysates was performed as described in ref. 21.

ELISA of Circulating Endostatin. Circulating endostatin levels were measured from plasma obtained from wild-type and endostatin-deficient mice and mice overexpressing endostatin implanted with either LLC or B16F10 tumors. Mice without tumors served as controls. ELISA was performed by using the ChemiKine mouse endostatin sandwich enzyme immunoassay kit (Chemi-con) according to the manufacturer's protocol.

Statistical Analysis. All results are shown as mean $\pm$ SEM. Statistical differences between two groups were calculated by using Student's *t* test or Welch's *t* test. ANOVA was used to determine statistical differences between three or more groups. As needed, further analysis was carried out by using *t* test with Bonferroni correction to identify significant differences. $P < 0.05$ was considered statistically significant. *, $P \leq 0.05$ and > 0.01 ; **, $P \leq 0.01$ and > 0.001 ; ***, $P \leq 0.001$.

Results and Discussion

Analysis of LLC and B16F10 Cells for the Production of Type XVIII and Type IV Collagen and TSP-1. Tumor cells have the tendency to produce many different matrix proteins (3). Therefore, when planning to perform experiments to evaluate the role of matrix and matrix-derived endogenous inhibitors of angiogenesis on the growth of tumors, an assessment must be made to determine whether the tumor cells make the endogenous inhibitors of angiogenesis being tested. Fig. 1A demonstrates that, by Western blotting using antibodies to endostatin, the full-length and degradation fragments of type XVIII collagen can be detected

in the cell lysates of LLC cells and a clonal expansion of B16F10 cells (clone 1). A full-length $\alpha 1$ chain of type XVIII collagen can be detected at ≈ 200 kDa, and the noncollagenous domain also can be detected at ≈ 40 kDa, presumably because of proteolytic activity (Fig. 1A). A different clonal population of B16F10 cells (clone 2) does not produce type XVIII collagen (Fig. 1A). Similarly, LLC cells make significant amounts of TSP-1, but not the precursor of tumstatin, the $\alpha 3$ chain of type IV collagen (data not shown) (29). Both clones of B16F10 cells do not produce TSP-1 (data not shown) (29). Collectively, these results demonstrate that a careful analysis for production of angiogenesis inhibitors by the tumors cells must be performed to avoid compensation by cancer cells in the host-deficient environment.

Systemic Disruption of Angiogenesis Balance Influences Tumor Growth. To study the effect of genetic loss of endogenous inhibitors of angiogenesis in shifting the angiogenic balance favoring increased angiogenesis, we studied tumor growth and vascularization in mice deficient in TSP-1, tumstatin, and endostatin, and both TSP-1 and tumstatin. Mice were injected s.c.

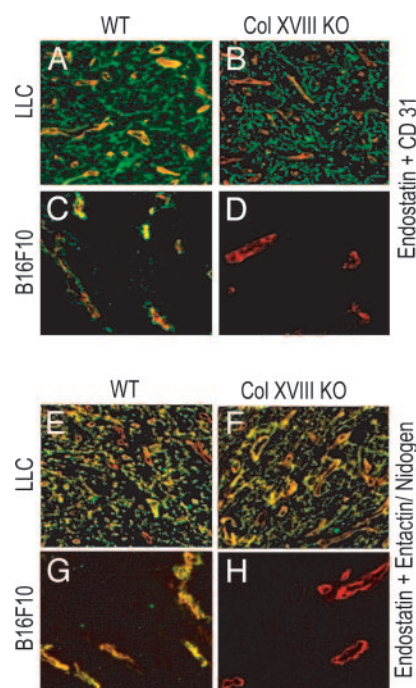


Fig. 2. Expression of endostatin in LLC and B16F10 tumors. (A–D) Double-staining of endostatin (green) and CD31 (red) of LLC (A and B) and B16F10 (clone 2) (C and D) tumors implanted on wild-type and type XVIII collagen-deficient mice. LLC tumors show intense expression of endostatin in the tumor stroma. No expression of endostatin can be observed in the vascular basement membranes (host-derived) in the LLC tumor on the type XVIII collagen-deficient mice (B), whereas on the wild-type mice, endostatin is found in the tumor stroma and the vascular basement membranes (A). (C) B16F10 tumors show endostatin staining only in the vascular basement membranes when implanted on wild-type mice. (D) No endostatin staining can be observed when these cells were implanted on the type XVIII collagen-deficient mice. (E–H) Endostatin (green) and entactin/nidogen (generic basement membrane marker) (red) staining of LLC (E and F) and B16F10 (G and H) tumors implanted on wild-type and type XVIII collagen-deficient mice. (E) In LLC tumors implanted on wild-type mice, colocalization of endostatin and entactin/nidogen can be seen on the tumor stroma and vascular basement membranes. (F) Hardly any colocalization can be seen in the vascular basement membranes on the LLC tumors implanted on the type XVIII collagen-deficient mice. (G) In B16F10 tumors, colocalization can be seen in the vascular basement membrane of tumors implanted on wild-type mice. (H) No endostatin staining can be observed when these cells were implanted on the type XVIII collagen-deficient mice.

nous inhibitors of angiogenesis to the growth of tumors. We used mice deficient in TSP-1, endostatin, tumstatin, and both TSP-1 and tumstatin to assess tumor growth. Tumors in all genetically altered mice grew marginally faster than did tumors in the wild-type mice until day 13 after tumor cell implantation (Fig. 1D). After day 13, tumors on all mice accelerated their growth, but the tumors on the mice deleted for angiogenesis inhibitors grew significantly faster than did tumors in the wild-type mice (Fig. 1E). Interestingly, B16F10 tumors on mice deficient in both TSP-1 and tumstatin grew at a 2-fold faster rate than did tumors on mice deficient in either inhibitor alone (Fig. 1E). The blood vessel density in the tumor tissue correlated with tumor size (Fig. 1F).

Collectively, these results indicate that the alteration within the angiogenic balance due to a reduction in the levels of individual endogenous inhibitors of angiogenesis leads to increased tumor growth, and that this effect is cumulative when two endogenous inhibitors of angiogenesis are deleted together in mice. Additionally, these results also indicate that the TSP-1, endostatin, and tumstatin serve as tumor suppressors. Such host-derived tumor-suppressive actions of angiogenesis inhibitors are potentially critical in controlling tumor growth, and when deleted, they raise the ceiling rate of cancer growth. This finding suggests that the ceiling rate of tumor growth and cancer progression is determined not just by the genetic defects accumulating in the cancer cell but also by host-derived factors.

The LLC tumor cells in the type XVIII collagen-deficient mice show robust expression of endostatin, whereas the B16F10 tumor cells do not (Figs. 1C and 2A–H). These results indicate that the endostatin produced by the LLC cells themselves was sufficient to counteract the lack of this inhibitor in the host animal. It was described in ref. 24 that tumors derived from B16F10 melanoma cells and T241 fibrosarcoma cells did not grow faster on mice deficient in endostatin, compared with wild-type mice. Here, we demonstrate that production of endostatin varies between cancer cell lines and also subclones of the same cancer cell line, including the T241 fibrosarcoma (data not shown). Hence, it is possible that the cell lines used in the earlier studies might produce endostatin, masking the effect on tumor growth. In this regard, recently B. R. Olsen's group convincingly demonstrated that mice deficient in endostatin exhibit increased angiogenesis (24, 30), further supporting the data that, in the complete absence of type XVIII collagen (host and cancer cell), increased angiogenesis and tumor growth are feasible.

A 1.6-Fold Overexpression of Endostatin in the Circulation, Mimicking the Elevated Levels in Down's Syndrome Individuals, Leads to Significant Reduction in Tumor Growth. To further verify the hypothesis that circulating endogenous angiogenesis inhibitors influence the angiogenic balance, we generated transgenic mice that overexpress endostatin under a tetracycline-inducible Tet CMV promoter (Fig. 3A). Mice overexpressing endostatin appear normal and breed. By breeding the transgenic mice to mice that express the Tet promoter transactivator under the VE-cadherin promoter (Fig. 3A and C), we obtained double-transgenic mice with moderately increased levels of circulating endostatin (Fig. 3D). *In vitro* transfection experiments using 293 human embryonic kidney cells demonstrate that endostatin production can be regulated by tetracycline or doxycycline (Fig. 3B). The circulating levels were 1.6-fold higher in the transgenic mice than in the wild-type mice ($P = 0.03$) (Fig. 3D). When B16F10 cells (clone 2) were implanted on the endostatin transgenic mice, significant suppression of tumor growth was observed, compared with the wild-type mice (Fig. 3E). The suppression of tumor growth was associated with significant reduction in the number of blood vessels in the tumor (Fig. 3F). Increased expression of endostatin (with a BM-40 signal peptide for the export of protein) in the endothelial cells of the transgenic mice did not reveal an increase

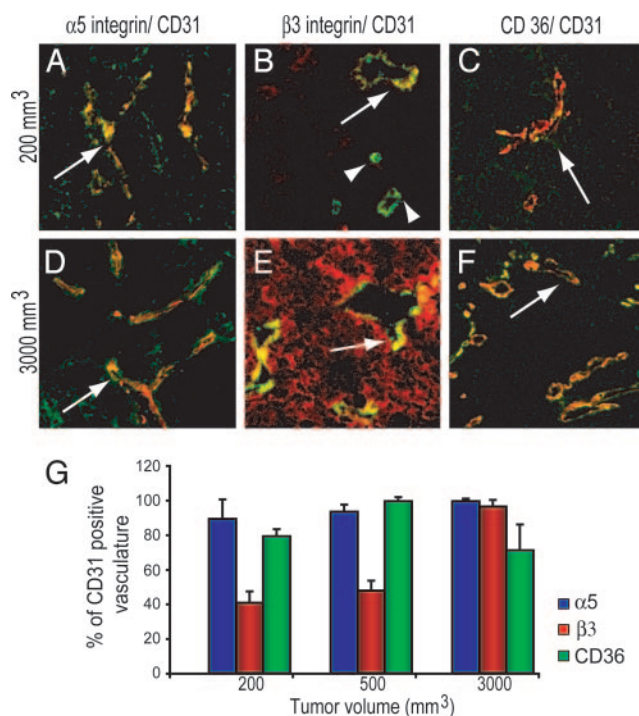


Fig. 4. Double-staining of CD31 and the receptors for endostatin, tumstatin, and TSP-1 on B16F10 tumor blood vessels of different sizes and quantification of the percentage of receptor-positive vessels (percent of all CD31-positive vessels). Tumors were implanted on wild-type mice. Arrows indicate colocalization of the receptor and CD31 staining, and arrowheads indicate vessels negative for the receptor molecule. (A, D, and G) The endostatin receptor $\alpha 5$ integrin is expressed at constant levels regardless of the size of the tumor. (B, E, and G) The tumstatin receptor $\beta 3$ integrin is initially expressed at lower levels, but increased expression on the blood vessels is observed as the tumor size increases. (D) As the tumor grows larger, strong expression of $\beta 3$ integrin also is seen in the tumor. (C, F, and G) The expression TSP-1 receptor CD36 is high at all stages of tumor growth.

in the tissue-associated endostatin, suggesting that the tumor-suppressive effect observed in these mice is associated with a demonstrable increase in the circulating endostatin levels and not tissue-associated levels (Fig. 3 G–N).

These results clearly demonstrate that increasing the circulating endostatin levels by 1.6-fold leads to a shift in the angiogenic balance, subsequently affecting the tumor growth rate. This finding provides an experimental proof for the hypothesis that a 1.7-fold increase in circulating endostatin concentration in the individuals with Down's syndrome might offer a protection against growth of solid tumors (25, 26). Collectively, our results also show that a disruption of systemic angiogenesis balance either by deletion of an endogenous inhibitor of angiogenesis or by an overproduction of an endogenous inhibitor of angiogenesis can lead to altered tumor growth.

The Growth of B16F10 Tumors in Mice Correlates with the Expression of Functional Receptors for TSP-1, Endostatin, and Tumstatin. Recent studies have suggested that the activity of TSP-1, endostatin, and tumstatin depend on the expression CD36, $\alpha 5\beta 1$ integrin, and $\alpha \nu \beta 3$ integrin on proliferating endothelial cells, respectively (15, 21, 29). Therefore, in the present study, we assessed the expression of these molecules on the endothelial cells at different stages of tumor growth. We double-labeled tumors of different sizes (200, 500, and 2,000 mm³) implanted on wild-type mice with the endothelial cell marker CD31 and $\beta 3$ integrin, $\alpha 5$ integrin, or CD36 receptors. Interestingly, both $\alpha 5$ integrin and CD36 were expressed at very high levels on tumors starting at 200 mm³ and

