Activin A Suppresses Neuroblastoma Xenograft Tumor Growth via Antimitotic and Antiangiogenic Mechanisms

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Abstract

The tumor suppressor function of activin A, together with our findings that activin A is an inhibitor of angiogenesis, is down-regulated by the N-MYC oncogene, prompted us to investigate in more detail its role in the malignant transformation process of neuroblastomas. Indeed, neuroblastoma cells with restored activin A expression exhibited a diminished proliferation rate and formed smaller xenograft tumors with reduced vascularity, whereas lung metastasis rate remained unchanged. In agreement with the decreased vascularity of the xenograft tumors, activin A inhibited several crucial angiogenic responses of cultured endothelial cells, such as proteolytic activity, migration, and proliferation. Endothelial cell proliferation, activin A, or its constitutively active activin receptor-like kinase 4 receptor (ALK4T206D), increased the expression of CDKN1A (p21), CDKN2B (p15), and CDKN1B (p27) CDK inhibitors and down-regulated the expression of vascular endothelial growth factor receptor-2, the receptor of a key angiogenic factor in cancer. The constitutively active forms of SMAD2 and SMAD3 were both capable of inhibiting endothelial cell proliferation, whereas the dominant-negative forms of SMAD3 and SMAD4 released the inhibitory effect of activin A on endothelial cell proliferation by only 20%. Thus, the effects of activin A on endothelial cell proliferation seem to be conveyed via the ALK4/SMAD2-SMAD3 pathways, however, non-SMAD cascades may also contribute. These results provide novel information regarding the role of activin A in the malignant transformation process of neuroblastomas and the molecular mechanisms involved in regulating angiogenesis thereof. (Cancer Res 2005; 65(5): 1877-86)

Introduction

Several lines of evidence indicate that tumorigenesis in humans is a multistep process in which genetic alterations drive the progressive transformation of normal human cells into highly malignant derivatives. During this process, tumor cells acquire several capabilities that collectively dictate malignant growth (1). Sustained angiogenesis is one of the acquired capabilities of tumor cells supporting tumor mass growth and providing a gate for metastasis. Indeed, activation of oncogenes and inactivation of tumor suppressors alter the angiogenic switch by up-regulating angiogenic stimulators, like vascular endothelial growth factors (VEGF), and/or down-regulating angiogenic inhibitors such as thrombospondin-1 (2).

Amplification of N-MYC oncogene is a frequent event in advanced stages (III and IV) of human neuroblastomas (3). N-MYC amplification correlates with poor prognosis (4) and enhanced vascularization (5) of human neuroblastomas, suggesting that N-MYC oncogene could activate tumor angiogenesis thereby enhancing neuroblastoma progression. Indeed, overexpression of N-MYC in a neuroblastoma cell line (SH-EP) resulted in an enhanced malignant phenotype of the transfectants (WAC2) allowing them to form well-vascularized tumors in nude mice (6). We have previously screened conditioned media from SH-EP007 and WAC2 cells and found that N-MYC overexpression in WAC2 cells down-regulated the expression of activin A, a protein that inhibited proliferation of cultured endothelial cell and angiogenesis in the chorioallantoic membrane assay (7). In this respect, activin A seemed to be an inhibitor of angiogenesis, a property that has not been previously ascribed to this molecule.

Activin A, a homodimer of two inhibin βA subunits, is a member of the activin/inhibin family, which in turn belongs to the large transforming growth factor-β (TGF-β) superfamily of proteins (8). Activin A transduces its signals via binding to activin type II receptors, ActR-II, and ActR-IIB. The ligand/type II receptor complex then recruits, binds, and transphosphorylates the type I receptor, ActR-IB, also known as activin receptor-like kinase 4 (ALK4). Following activation of its kinase domain, ALK4 phosphorylates and activates selected members of a family of intracellular transducers known as SMADs, a process promoted by SARA-like proteins. On their way to the nucleus, the receptor activated SMADs (referred to as R-SMAD) associate with the related protein SMAD4. SMAD4 is referred to as the Co-SMAD, and is not a transcriptional regulator, controlling the expression of many genes (9).

Activin and inhibin were originally isolated based on their activity to regulate follicle-stimulating hormone release from the anterior pituitary (10). However, activins have been found to exert a large spectrum of biological activities ranging from embryonic mesoderm induction to differentiation of hematopoietic cell lineages and repair processes in skin and brain (11–13). In cancer, activin A inhibits the proliferation of a variety of tumor (and normal) human cell types by blocking cell cycle progression from G1 to S phase (14). Indeed, overexpression of activin A in human prostate cancer LNCaP cells inhibited proliferation, induced apoptosis, and decreased the tumorigenicity of these cells (15). Also, loss-of-function mutations of ALK4 have been identified in human pituitary tumors (16). As a consequence, activin A seems to function as a tumor suppressor, a property shared by the prototype molecule TGF-β, at least in early stages of tumorigenesis (17).
The tumor suppressor function of activin A, together with our findings that activin A is an inhibitor of angiogenesis, which is down-regulated by the N-MYC oncogene, prompted us to investigate in more detail its role in the malignant transformation process of neuroblastomas. Towards this purpose, we have restored activin A expression in neuroblastoma cells expressing high levels of the N-MYC oncogene using stable transfection and expression constructs driven by a cytoomegalovirus (CMV) promoter, which is not down-regulated by N-MYC. The isolated clones exhibited a diminished proliferation rate in culture and formed smaller xenograft tumors with reduced vascularity, whereas lung metastasis rates remained unchanged. Motivated by the reduced vascularity of the xenograft tumors, we have studied the effect of activin A on several critical angiogenic responses of endothelial cells and investigated the molecular mechanisms involved.

Materials and Methods

Generation of WAC2 Cell Transfectants Stably Expressing Activin A. For generation of stable transfectants, a 100 mm dish of 70% confluent neuroblastoma WAC2 cells was transfected with 10 ng of plasmid DNA (CMV-inhibin-βA plus 1 μg pRSV puromycin using the N-[1-(2,3-dioleoyloxy)-propyl]-NNN-trimethylammoniummethyl sulfate transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany). Mock transfectants were generated using the CMV empty vector and the plasmid carrying the puromycin gene. Twenty-four hours later, cells were subcultured into medium containing 0.75 μg/mL puromycin for selection of stable clones. A total of 10 clones (Wact1-10) were expanded and supernatants were analyzed for expression of activin A expression by Western blot analysis. Three highly expressing clones (Wact1, Wact2, and Wact5) were selected and subcloned to ensure homogeneous expression of activin A. The resulting subclones were checked for homogeneous expression of activin A by immunofluorescence.

Cell Proliferation Assays. Cell proliferation assays using bovine brain-derived capillary endothelial (BBCE) cells were previously described (18, 19). For growth curves of the SH-EP007, WAC2, and the WAC2 clones expressing activin A, cells were plated in 12-well plates at 1 × 104 cells per well. Cells were counted every day over a period of 6 days. Fresh medium was added to the cells every second day. In experiments where adenoviruses were used, WAC2 cells were infected for 2 hours (medium replacement), and the next day, the cells were split into 12-well plates at 1 × 105 cells per well. Cells were then counted for 6 consecutive days. The number of cells was determined using a Coulter particle counter (Beckman Coulter, Inc.).

For [3H]methylthymidine incorporation assays, BBCE were seeded in 24-well plates at 8 × 104 cells per well and after an overnight incubation they were treated with 2.5 ng/mL fibroblast growth factor-2 (FGF-2) and 60 ng/mL activin A for 24 hours; for the final 4 hours, cells were pulsed with 1 μCi/well [3H]methylthymidine (ICN Biomedical Inc., Costa Mesa, CA). Culture medium was measured and cells were fixed with ice-cold 10% trichloroacetic acid for 20 minutes at 4°C. Fixed cells were washed thrice with water and solubilized in 0.1 mol/L NaOH overnight at 4°C. In the case of adenoviral infection, BBCE or human umbilical vein endothelial cells were plated in 24-well plates and 24 hours later, cells were infected with adenoviruses as indicated and medium was changed 2 hours after infection. After a further overnight incubation, cells were treated with 2.5 ng/mL FGF-2, 40 ng/mL VEGF, or 60 ng/mL activin A for 24 hours. For the final 4 hours (6 hours for human umbilical vein endothelial cells), cells were pulsed with 1 μCi/well [3H]methylthymidine and processed as above. The radioactivity was counted in a β liquid scintillation counter. Infections with adenoviruses were controlled by observing the green fluorescent protein (GFP) using a Zeiss fluorescence microscope in cases where the GFP was coexpressed, infection was 100%. In the case of adenoviruses which did not coexpress GFP, infection was controlled by indirect immunofluorescence. Western blot analysis was carried out in all experiments.

The effect of SMAD2 on proliferation was evaluated by microinjecting BBCE cells, seeded on 11 mm glass coverslips, with 100 ng DNA of either the constitutively active form of SMAD2 (MYC-SMAD2ca) or the transmembrane region of CD39 fused to GFP (CD39-GFP). The next day, cells were treated with 100 μmol/L bromodeoxyuridine (BrdUrd; Sigma, St. Louis, MO) for 3 hours. Cells were then fixed with 3.7% paraformaldehyde, quenched with 50 mmol ammonium chloride for 15 minutes, permeabli-

zed with 0.1% Triton X-100 for 4 minutes, and nonspecific sites were blocked with 10% donkey serum (Dianova GmbH, Hamburg, Germany). MYC-SMAD2ca and CD39-GFP were detected with 9E10 and GFP antibodies, respectively. Antibodies were postfixed with 3.7% paraformaldehyde and quenched with 50 mmol ammonium chloride for 15 minutes, before treatment with 1.5 mol/L hydrochloric acid for 10 minutes. The proliferating cells were detected with anti-BrdUrd antibody. Primary and secondary antibodies were diluted in 5% donkey serum. Fluorescein- and tetramethylrhodamine isothiocyanate–conjugated donkey anti-mouse, anti-rabbit, or anti-rat IgG secondary antibodies were purchased from Dianova. Coverslips were mounted in Moviol containing 100 mg/mL diazabicyclo(2.2.2)octane (Sigma), and viewed using a Leica TCS-SP scanning confocal microscope, equipped with an argon/krypton laser and Leica TCS software.

Zymographic and Cell Migration Assays. Zymographic assays were carried out essentially as described earlier (20, 21), whereas for endothelial cell migration evaluation, the wounding method was used (22). BBCE cells were used in both assays.

Reporter Assays for Activation of Transcription. For SMAD3-dependent transcription, multiple copies of the SMAD-binding element fused to luciferase (23) was used. The promoters of p21 and p27 fused to luciferase (p21-luc and p27-luc) were used for assessing the effect on cyclin-dependent kinase (CDK) inhibitors. BBCE cells were used in all assays, transfection was carried out using LipofectAMINE-Plus (Life Technologies GmbH, Karlsruhe, Germany) and luciferase measurements were carried out according to the Promega E4030 kit.

Xenograft Experiments in Mice. Adult (8–10 weeks old) female BALB/c nu/nu mice were used as host animals. For tumor growth rate evaluation, ~ 1.0 × 106 of WAC2 transfectants in 10 μL of Ca2+- and Mg2+-free HBSS were inoculated intradermally in the flanks of mice (24). Tumor volume (V) was calculated as V = (π/6)ab2, where a is the longer and b is the shorter of two perpendicular diameters, measured with calipers. The volume at 4 weeks after the cell inoculation was used as the parameter for tumor growth rate. Tumor-induced angiogenesis was assessed by using an intradermal angiogenesis assay (25). Inoculation of WAC2 transfectants was carried out as for the tumor growth rate evaluation, and mice were killed on day 7 after inoculation. For the lung colonization assay, aliquots of 3.0 × 105 of WAC2 transfectants suspended in 0.2 mL of HBSS were inoculated into the lateral tail vein of mice and the mice were sacrificed and autopsied 5 weeks after the inoculation. The lungs were removed and the number of colonies were determined by using a stereomicroscope (24).

Statistical Analysis. Statistical evaluation of xenograft assays in mice was done by nonparametric analysis using the Mann-Whitney test followed by t test. Two-sided tests and a significance criterion of P < 0.05 were used. In all other cases, three or more groups were compared using the one-way ANOVA test followed by post hoc analysis and LSD correction and significance criterion P < 0.05. Statistical analysis was done with the Statistical Package for the Social Sciences 10.0 statistical software (SPSS, Inc., Chicago, IL).

Results

Restoration of Activin A Expression in Neuroblastoma Cells Inhibits Their Proliferation. We have previously shown that activin A is (a) down-regulated by the N-MYC oncogene in neuroblastoma cells and (b) is an inhibitor of angiogenesis in the chicken chorioallantoic membrane assay (7). As the TGF-ß/ activin family members are known to inhibit cell proliferation;
together, these observations implied an important role for activin A in the neuroblastoma malignant transformation process. To investigate this issue, we have reintroduced the activin A gene into WAC2 cells by stable transfection using a construct containing the entire coding region of the βA-subunit of human inhibin under the control of a CMV promoter, which is not down-regulated by the N-MYC oncogene. We were able to select three clones expressing activin A at various levels (Wact1, Wact2 and Wact5; Fig. 1A). All three clones exhibited dramatically decreased proliferation rates compared with the parental WAC2 cell line (Fig. 1B), their proliferation rate being even lower compared with SHEP007, which expresses normal levels of the N-MYC oncogene. Moreover, the decrease in proliferation rate correlated well with the expression levels of activin A in each cell line (Fig. 1A and B). Similarly, WAC2 cells infected with activin A-expressing adenoviruses exhibited a considerably decreased proliferation rate (data not shown). Thus, forced overexpression of activin A in WAC2 cells inhibits their proliferation in vitro.

**Xenograft Tumors from Neuroblastoma Cells with Restored Activin A Expression Grow Slower, Exhibit Reduced Angiogenesis, and Have Unchanged Metastatic Potential.** Next, we evaluated the growth of the activin A-expressing neuroblastoma clones as xenograft tumors in mice. The tumor volumes of all activin A-expressing WAC2 clones were significantly reduced in mice sacrificed 4 weeks after intradermal implantation compared with the control Wvect2 and Wvect3 WAC2 clones (Fig. 1C). To assess whether inhibition of angiogenesis contributed to the slower growth of the xenograft tumors, the number of capillaries of xenograft tumors was quantified in another set of mice. Indeed, 7 days following intradermal implantation in the flanks of immune-deficient mice, the number of capillaries was statistically significantly decreased in clones Wact1 and Wact5 compared with that of the control clones (Fig. 2A). At the same time point, the weight and volume of the tumors were decreased in a statistically significant manner in all activin A-expressing WAC2 cells (Wact1, Wact2, and Wact5; Fig. 2B and C). Thus, the activin A-expressing neuroblastoma WAC2 cells form slow-growing xenograft tumors with reduced angiogenesis. However, when the metastatic potential of Wact1, Wact2, and Wact5 cells was evaluated, there was no statistical difference in the number of lung colonies compared with the control neuroblastoma Wvect2 and Wvect3 clones (Fig. 2D).

**Activin A Inhibits Key Angiogenic Responses of Endothelial Cells.** The suppressed vascularity of the activin A-expressing neuroblastoma xenograft tumors confirmed our previous observations that activin A is an inhibitor of angiogenesis in vivo (7). Because the process of angiogenesis involves degradation of basement membrane, invasion of endothelial cells to the surrounding matrix, and subsequent proliferation, we have investigated the effect of activin A on these processes. Zymographic analysis of conditioned media and endothelial cell extracts revealed that activin A (60 ng/mL) inhibited both basal and FGF-2-induced u-PA activity (Fig. 3A). Moreover, activin A inhibited basal and FGF-2-induced migration of endothelial cells to denuded areas of wounded BBCE monolayers (Fig. 3B). Likewise, activin A (50 ng/mL) inhibited basal and FGF-2-induced [3H]methylthymidine uptake in BBCE cells (Fig. 3C). Similar effects of activin A have been observed also on VEGF-induced proliferation (data not shown). Previously, it has been shown that activin A inhibits basal growth of different endothelial cell types (26). Collectively, these data indicate that activin A inhibits several important steps of the activation phase of angiogenesis, i.e., degradation, invasion, and proliferation.

**Inhibition of Endothelial Cell Proliferation by Activin A Is Mediated via ALK4 Activation and Underlined by Over-expression of CDK Inhibitors and Down-regulation of VEGF Receptor-2.** Although ActRIIB can bind both ALK4 and ALK2 type I receptors, only ALK4 is activated following activin A recognition by ActRIIB, ALK2 being part of ActRIIB/bone morphogenetic protein complexes (9, 27). Infection with adenoviruses expressing a constitutively active form of ALK4 (ALK4T206D) inhibited VEGF- (Fig. 4A) and FGF-2-induced (Fig. 4B), as well as basal (serum-induced) endothelial cell proliferation (Fig. 4A and B). Identical results were obtained when endothelial cells were infected with adenoviruses expressing activin A (Fig. 4A and B). On the contrary, constitutively active ALK2 (ALK2Q207D) enhanced both basal and FGF-2-induced endothelial cell proliferation (Fig. 4C). The result implies that activation of ALK4 receptor is the most likely mediator of the effects of activin A on endothelial cell proliferation. The
result further suggests that activation of different type I receptors by the numerous members of the TGF-β superfamily may exhibit opposing effects.

Up-regulation of CDK inhibitors, CDKN1A (p21), CDKN2B (p15), and CDKN1B (p27) is the hallmark of the cytostatic effect of TGF-β in various cells leading to inactivation of G1 phase CDKs (28). Activin A induced weakly, but in a statistically significant manner, transcription of the CDKN1A promoter in endothelial cells irrespective of whether activin A was given transiently (exogenous addition) or continuously (infection with adenoviruses expressing activin A; Fig. 5A). Activin A induced transcription of CDKN1A promoter also occurred in the presence of mitogenic factors such as FGF-2 (Fig. 5B). Similarly, activin A weakly induced CDKN2B (data not shown) and CDKN1B promoters (Fig. 5C) in endothelial cells. All

Figure 2. Xenograft tumors derived from activin A-expressing neuroblastoma WAC2 cells exhibit decreased vascularization; unaltered lung metastasis rate of activin A-expressing WAC2 cells. A-C, BALB/c nu/nu mice were injected intradermally with WAC2 cells transfected with activin A-containing vector (Wact1 n = 20, Wact2 n = 19, and Wact5 n = 20) or the empty control vector (Wvect2 n = 19 and Wvect3 n = 16). Mice were sacrificed 1 week after inoculation and the capillary numbers (A), weight (B), or volume (C) of the tumors derived from the activin A-transfected cells were determined and compared with those from the control cells. D, WAC2 cells transfected with activin A containing vector (Wact1 n = 9, Wact2 n = 10, and Wact5 n = 10) or the empty control vector (Wvect2 n = 10 and Wvect3 n = 9) were injected in the tail vein of BALB/c nu/nu mice. Lung colonization was quantified by counting the surface colonies at day 35 after injection. ( ), different mice; inset, statistical significance.

Figure 3. Activin A inhibits angiogenic responses of endothelial cells. A, BBCE cultures were exposed to medium only, FGF-2 only, activin A only, or FGF-2 plus activin A. Infection experiments, BBCE cells were first infected with adenoviruses expressing either GFP alone or GFP plus activin A, and then incubated in the presence or in the absence of FGF-2. Cell extracts and conditioned medium were subjected to zymographic analysis. Zymograms were photographed under dark ground illumination after overnight incubation. B, BBCE cells were seeded in 24-well plates, and when confluent, the monolayer was wounded and images of the wounded area were taken. Wounded cultures were exposed to medium only, FGF-2 only, activin A only, or FGF-2 plus activin A. After 8 hours, cells were photographed again. Cells crossing the wounded area were counted and expressed as the number of cells per millimeter of wound. Activin A decreased migration of endothelial cells in a statistically significant manner both in the absence (P < 0.013) and presence (P < 0.001) of FGF-2. C, BBCE cells were seeded in 24-well plates and 24 hours later cells were treated with FGF-2 (2.5 ng/mL) in the presence or in the absence of activin A. Cells were pulsed with [3H]methylthymidine, fixed with trichloroacetic acid, and solubilized in NaOH. The radioactivity was counted in a scintillation counter. Activin A decreased thymidine incorporation statistically significantly both in the absence (P < 0.001) and presence (P < 0.001) of FGF-2. Experiments were carried out in triplicate and repeated twice (A) or thrice (B and C).
three promoters were also induced by TGF-β (data not shown). Induction of CDK inhibitors could, in principle, explain the inhibitory effect of activin A on both basal and VEGF-/FGF-2-induced endothelial cell proliferation. However, as activation of G1 phase CDKs is determined by the balance between antimitogenic (induction of CDK inhibitors) and mitogenic (induction of cyclin D) signals (29), we sought evidence regarding a possible direct interception of activin A on the mitogenic pathways of VEGF. Indeed, ALK4T206D inhibited VEGF-induced phosphorylation of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases (Fig. 5D) in endothelial cells by down-regulating VEGF receptor 2 expression (Fig. 5E). Thus, activation of ALK4 down-regulates the expression of VEGF receptor-2 receptors inhibiting the mitogenic pathways of VEGF and, thus, potentiating the cytostatic effects of activin A via induction of CDK inhibitors.

Constitutively Active Forms of Both SMAD2 and SMAD3 Inhibit Endothelial Cell Proliferation. Because the ALK4 receptor phosphorylates both SMAD2 and SMAD3 proteins, we sought evidence regarding the relative contribution of each of these pathways in inhibiting endothelial cell proliferation. Towards this end, we have generated constructs expressing the constitutively active forms of SMAD2 and SMAD3 (designated SMAD2ca and SMAD3ca) by substituting the serine residues of the SSMS phosphorylation site with glutamic or aspartic acids (30–32). These constructs were tested regarding their function and specificity using SMAD-binding element fused to luciferase and FAST/ARE reporter assays (data not shown). As we were unable to generate recombinant adenoviruses expressing SMAD2ca, we have addressed the role of SMAD2ca in endothelial cell proliferation by micro-injecting BBCE cells with the SMAD2ca construct or a control construct expressing the transmembrane domain of CD39 fused to GFP (CD39-GFP) and evaluating the incorporation of BrdUrd in the microinjected cells. Using indirect immunofluorescence, we have observed that a smaller percentage of cells microinjected with SMAD2ca was incorporating BrdUrd compared with the cells that were microinjected with CD39-GFP. Upon quantitation, it was evident that microinjection of SMAD2ca caused a statistically significant decrease in BrdUrd incorporation compared with CD39-GFP (Fig. 6A). Likewise, infection of BBCE cells with a recombinant adenovirus expressing the SMAD3ca caused a statistically significant decrease in thymidine incorporation both in the presence and absence of activin A (Fig. 6B). Thus, both SMAD2 and SMAD3 pathways can contribute to the inhibition of endothelial cell proliferation by activin A.

Activin A-Induced Inhibition of Proliferation Is Only Partly Mediated via the SMAD Pathway. As several non-SMAD pathways have been identified for TGF-β/activin A ligands (17), we needed to determine whether SMAD-dependent pathways are solely responsible for the activin A-induced inhibition of endothelial cell proliferation or whether non-SMAD pathways might contribute to this effect. To this purpose, we have infected BBCE cells with adenoviruses expressing the dominant-negative forms of either SMAD2 (Ad-SMAD2Δc) or SMAD4 (Ad-SMAD4Δc) and investigated the ability of activin A to inhibit proliferation of the infected cells (Fig. 7). Whereas activin A was able to inhibit proliferation of non-infected and control-infected BBCE cells (Ad-control), infection with the adenoviruses expressing the dominant-negative forms of SMAD2 (Ad-SMAD2Δc) and SMAD4 (Ad-SMAD4Δc) released the inhibitory effect of activin A by a modest 20% (Fig. 7A and B). Both Ad-SMAD2Δc and Ad-SMAD4Δc adenoviruses were well expressed (Fig. 7C) and significantly suppressed induction of the SMAD-binding element fused to luciferase construct by activin A (Fig. 7D). Thus, despite the fact that both SMAD2 and SMAD3 pathways are capable of mediating antimitotic signals on endothelial cell proliferation, other non-SMAD pathways seem to contribute to the inhibitory effect of activin A on endothelial cell proliferation.
A (Wact1, Wact2 and Wact5) grew at a much lower rate as xenograft that activin A might act as a tumor suppressor in neuroblastoma. proliferation of several normal and tumor cells (14), and suggests agreement with the reported inhibitory effect of activin A on the N-MYC oncogene. The stable neuroblastoma cell transfectants expressing high levels of activin A (Wact1, Wact2, and Wact5) exhibited a significantly reduced proliferative potential, although the N-MYC oncogene was still overexpressed. This result is in agreement with the reported inhibitory effect of activin A on the proliferation of several normal and tumor cells (14), and suggests that activin A might act as a tumor suppressor in neuroblastoma.

When tested in mice, all neuroblastoma clones expressing activin A (Wact1, Wact2 and Wact5) grew at a much lower rate as xenograft tumors, compared with the control stable transfectants (Wvest2 and Wvest3). Importantly, the vascularity of the resulting tumor xenografts was significantly reduced in the activin A-expressing neuroblastoma clones. Moreover, the activin A-expressing WAC2 cell clones did not exhibit any statistical increase in the number of metastatic lung colonies, compared with control. These results are distinct from what is known about the role of TGF-β in cancer. Indeed, despite the suppressive effects on cell proliferation, TGF-β exhibits a tumor-promoting role at later stages of neoplasia (17, 33). TGF-β is oversecreted in most human carcinomas (34, 35), where it can cause epithelial-mesenchymal transition of tumor cells or surrounding normal epithelia leading to enriched tumor stroma and more invasive tumor cells with increased ability to metastasize (36, 37). Moreover, TGF-β suppresses immune cell activation against the tumor and induces tumor angiogenesis (17, 38). Our data suggests that activin A might have distinct effects on

Discussion

In a previous study, we have shown that N-MYC oncogene amplification in neuroblastoma cells abolishes expression of activin A (7) suggesting that activin A might contribute to the malignant phenotype of this solid tumor. Towards this end, we have reintroduced activin A cDNA in neuroblastoma cells under the expression of a promoter (CMV), which is not down-regulated by the N-MYC oncogene. The stable neuroblastoma cell transfectants expressing activin A (Wact1, Wact2, and Wact5) grew at a much lower rate as xenograft tumors, compared with the control stable transfectants (Wvest2 and Wvest3). Importantly, the vascularity of the resulting tumor xenografts was significantly reduced in the activin A-expressing neuroblastoma clones. Moreover, the activin A-expressing WAC2 cell clones did not exhibit any statistical increase in the number of metastatic lung colonies, compared with control. These results are distinct from what is known about the role of TGF-β in cancer. Indeed, despite the suppressive effects on cell proliferation, TGF-β exhibits a tumor-promoting role at later stages of neoplasia (17, 33). TGF-β is oversecreted in most human carcinomas (34, 35), where it can cause epithelial-mesenchymal transition of tumor cells or surrounding normal epithelia leading to enriched tumor stroma and more invasive tumor cells with increased ability to metastasize (36, 37). Moreover, TGF-β suppresses immune cell activation against the tumor and induces tumor angiogenesis (17, 38). Our data suggests that activin A might have distinct effects on

Figure 5. Activin A increases the activity of cyclin-dependent kinase inhibitors and inhibits the mitogenic signals from VEGF. A and B, BBCE cells were left uninfected or were infected with recombinant adenovirus expressing GFP alone (Ad-Control) or GFP plus activin A (Ad-Activin A) or GFP plus ALK4T206D (Ad-ALK4T206D), and 2 hours after infection, cells were transfected with p21-luc and CMV-β-gal (A). The next day, cells were placed in 0.2% serum and 8 hours later noninfected BBCE cells (columns 1 and 2) were treated with activin A (50 ng/mL). Another set of BBCE cells were transfected with p21-luc and CMV-β-gal and, 24 hours later, cells were treated with FGF-2 only or with activin A only or with FGF-2 plus activin A (B). Relative light units were counted after an additional 16-hour incubation. Relative light units of p21-luc were statistically significantly increased by activin A (P < 0.001) in nontreated cells. Similarly, relative light units of p21-luc were statistically significantly increased in cells infected with adenoviruses expressing either activin A (P = 0.001 and P = 0.002 with a multiplicity of infection of 15 and 25, respectively) or ALK4T206D (P = 0.001) compared with cells infected with control adenoviruses. Moreover, activin A statistically significantly increased relative light units of p21-luc even in the presence of FGF-2 (P = 0.004). C, BBCE cells were transfected with p27-luc, CMV-β-gal, and either control vector or SMAD3Δc constructs and 24 hours later were placed in 0.2% serum for an additional 8 hours. Cells were left untreated or treated with 50 ng/mL of activin A for 16 hours before measuring relative light units. All relative light units (A-C) were standardized for transfection efficiency using the values of the β-galactosidase activity. Relative light units of p27-luc were statistically significantly increased by activin A (P = 0.009) and this increase was statistically significantly reduced in cells transfected with SMAD3Δc (P = 0.006). D and E, human umbilical vein endothelial cells were infected for 2 hours with recombinant adenoviruses expressing either GFP alone (Ad-Control) or GFP plus ALK4T206D (Ad-ALK4T206D). The next day, cells were stimulated with VEGF (50 ng/mL) and incubated for a further 24 hours before collecting the cell lysates. The phosphorylation of extracellular signal-regulated kinase 1/2 and the expression of extracellular signal-regulated kinase 1/2 (D) and VEGF receptor-2 (E) was assessed by immunoblotting with anti-p-p44/42, anti-p44/42 and anti-VEGF receptor-2 antibodies, respectively. Experiments were carried out in triplicate (A-C) and repeated twice or thrice.
malignant transformation compared with TGF-β; expression of activin A is suppressed at late stages of neuroblastomas and restoration of its expression leads to xenograft tumors that grow slower, exhibiting reduced angiogenesis and unaltered metastatic potential. Alternatively, the involvement of activin A in the malignant transformation process of neuroblastoma is specific for this tumor. Indeed, neuroblastoma is developmentally different from epithelial cancers in which most of the effects of TGF-β have been studied. It is worth noting that when activin A expression was restored in Kelly neuroblastoma cells, these cells exhibited reduced growth and vascularity when grown as xenografts in mice fully supporting our observations.4

The reduced angiogenesis observed in the activin A-expressing neuroblastoma xenografts confirms our previous observations that activin A inhibits angiogenesis in the chick chorioallantoic membrane assay (7). In clear distinction, TGF-β is known to be angiogenic in both the rabbit corneal and the chick chorioallantoic membrane assays (39, 40). Indeed, increased expression of TGF-β1 in transfected prostate carcinoma or Chinese hamster ovary cells enhanced tumor angiogenesis in immunodeficient mice (41, 42) and local administration of neutralizing antibodies to TGF-β1 strongly reduced tumor angiogenesis (42). The inducing role of TGF-β on angiogenesis is further supported by targeted inactivation of the TGF-β1 (43) or TβRII genes (44), which results in embryonic lethality owing to defective vasculogenesis and angiogenesis. Angiogenesis-defective phenotypes are also seen in mice with null mutations of the genes encoding TβRI ALK-5 (45), ALK-1 (46), Smad5 (47, 48), and endoglin (49, 50). On the contrary, inactivation of activin A gene has not been reported to cause any defects in angiogenesis (51).

It is difficult to reconcile the differential in vivo activity of activin A on angiogenesis compared with that of TGF-β. In our experience, activin A inhibited production of proteolytic enzymes, migration, and proliferation of cultured endothelial cells, properties that are also shared by TGF-β (20, 39). Activin A transduces its signals via activation of the ALK4 receptor (9, 27), and, indeed, infection of endothelial cells with adenoviruses expressing the constitutively active form of ALK4 (ALK4T206D) inhibited basal and growth factor-induced endothelial cell proliferation. Towards this end, activin A and/or its constitutively active ALK4 receptor (ALK4T206D) induced the transcriptional activation of cell cycle–inhibiting genes, such as CDKN1A (p21), CDKN2B (p15) and CDKN1B (p27), and down-regulated VEGF receptor-2 expression in endothelial cells, properties also exhibited by TGF-β (28, 52). On the other hand, a constitutively active form of ALK2 (ALK2Q207D), a type I receptor activated by ActRIIB/bone morphogenetic protein-7 complexes (27), exerted a mitotic effect on endothelial cells. In this respect, our results confirm recent indications suggesting that certain bone morphogenetic proteins are inducers of angiogenesis (53, 54). The differential activity of ALK4 and ALK2 receptors on endothelial cell proliferation is reminiscent of the opposing actions of the two TGF-β type I receptors ALK1 and ALK5 (TβRI). ALK1 signaling via SMAD1 increases proliferation and migration of endothelial cells, whereas ALK5 signaling via SMAD2/3 suppresses these activities (55, 56). Similarly, ALK2 activates SMAD1/5/8, whereas ALK4 signals via SMAD2/3. The opposing activities of the various type I receptors of the TGF-β superfamily could potentially explain the differential activity exerted by TGF-β and activin A on endothelial cells in in vivo conditions. Perhaps the ALK1 pathway predominates in vivo, conveying the proangiogenic effects of TGF-β, whereas ALK5 activation is dominant in vivo inhibiting angiogenic endothelial cell responses. Likewise, the balance between ALK1 and ALK5 activation might provide a possible basis for understanding the dose dependency of several of TGF-β responses (40, 46, 55). Interestingly, ALK5 is required for ALK1 activation by TGF-β (56) providing an explanation regarding the fact that both ALK1 and ALK5 cause an angiogenesis-defective phenotype in knockout mice. Activin A activates only the ALK4 receptor and thus it is not surprising that activin A has consistent in vitro and in vivo effects on endothelial cell responses and angiogenesis.

Assuming that ALK4 and ALK5 convey the antiangiogenic effects of activin A and TGF-β, respectively, then SMAD2/3-regulated
genes must account for most of these effects. However, it is not clear whether both SMAD2 and SMAD3 proteins participate in the antiangiogenic effects. We have generated constitutively active forms of either SMAD2 or SMAD3 proteins, and indeed, both were capable of inhibiting endothelial cell proliferation. To this end, physical and functional interactions between SMAD2, SMAD3, and SMAD4 proteins with Sp1 transcription factor have been reported (57, 58). Moreover, although mainly SMAD2 and SMAD4 genes are mutated in cancer (59, 60), CDKN2A/p16, CDKN2B/p15, CDKN1A/p21, SMAD3, p107, E2F4/5, and DR1 form cytosolic complexes which, following ligand activation, translocate to the nucleus down-regulating transcription of C-MYC gene (62). C-MYC is not only a transcriptional activator of proliferation (63, 64), but also inhibits transcription of the C-MYC gene (62).

The SMAD pathway is not solely responsible for inhibition of endothelial cell proliferation. A. BBCE cells were infected with adenoviruses expressing GFP alone (Ad-Control), or GFP plus SMAD3A (Ad-SMAD3A) for 2 hours. The next day, cells were stimulated with activin A (50 ng/mL) for 24 hours. Cells were pulsed with [3H]methylthymidine, fixed with trichloroacetic acid, and solubilized in NaOH. The radioactivity was counted in a scintillation counter. B. BBCE cells were infected with adenoviruses expressing LacZ alone (Ad-Control), or LacZ plus SMAD4A (Ad-SMAD4A) for 2 hours. The rest of the treatment was similar to (A). C. lysates of BBCE cells, infected as indicated above, were analyzed by immunoblotting. The expressions of SMAD3A and SMAD4A were detected with an anti-Flag antibody. D. BBCE cells were infected with adenoviruses expressing GFP alone (Ad-control), or GFP plus SMAD3A (Ad-SMAD3A), LacZ plus SMAD4A (Ad-SMAD4A), and 2 hours after infection, cells were transfected with SMAD-binding element fused to luciferase and CMV-β-gal. The next day, cells were placed in 0.2% serum and 8 hours later were treated with activin A (50 ng/mL) and relative light units were counted after an additional 16 hours of incubation. Transfection efficiencies were standardized using β-gal values. The inhibition by Ad-SMAD3A and Ad-SMAD4A compared with Ad-Control is statistically significant (P < 0.001). Experiments were carried out in triplicate (A-C) and repeated thrice.

Figure 7. The SMAD pathway is not solely responsible for inhibition of endothelial cell proliferation. A. BBCE cells were infected with adenoviruses expressing GFP alone (Ad-Control), or GFP plus SMAD3A (Ad-SMAD3A) for 2 hours. The next day, cells were stimulated with activin A (50 ng/mL) for 24 hours. Cells were pulsed with [3H]methylthymidine, fixed with trichloroacetic acid, and solubilized in NaOH. The radioactivity was counted in a scintillation counter. B. BBCE cells were infected with adenoviruses expressing LacZ alone (Ad-Control), or LacZ plus SMAD4A (Ad-SMAD4A) for 2 hours. The rest of the treatment was similar to (A). C. lysates of BBCE cells, infected as indicated above, were analyzed by immunoblotting. The expressions of SMAD3A and SMAD4A were detected with an anti-Flag antibody. D. BBCE cells were infected with adenoviruses expressing GFP alone (Ad-control), or GFP plus SMAD3A (Ad-SMAD3A), LacZ plus SMAD4A (Ad-SMAD4A), and 2 hours after infection, cells were transfected with SMAD-binding element fused to luciferase and CMV-β-gal. The next day, cells were placed in 0.2% serum and 8 hours later were treated with activin A (50 ng/mL) and relative light units were counted after an additional 16 hours of incubation. Transfection efficiencies were standardized using β-gal values. The inhibition by Ad-SMAD3A and Ad-SMAD4A compared with Ad-Control is statistically significant (P < 0.001). Experiments were carried out in triplicate (A-C) and repeated thrice.

In conclusion, restoration of activin A expression in neuroblastoma cells exhibiting high expression levels of N-MYC oncogene results in a reduced growth rate of these cells in culture. Moreover, xenograft tumors, derived from the high activin A-expressing neuroblastoma cells, exhibit a strong decrease in tumor size and vascularity, whereas lung metastasis remained unaffected. In this respect, activin A differs substantially from TGF-β, up-regulation of which in late stages of cancer leads to increased angiogenesis and metastasis. Indeed, the latter properties of TGF-β pose impediments regarding potential therapeutic applications of TGF-β in cancer. Activin A might be devoid of the adverse effects of TGF-β and, therefore, might be a more attractive agent for therapeutic interventions. Evidently, restoration of activin A expression might be beneficial for late neuroblastomas. However, its role in other more common cancers merits further investigation.
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The Antitumor Activity of Activin A

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