Human Semaphorin 3B (SEMA3B) Located at Chromosome 3p21.3 Suppresses Tumor Formation in an Adenocarcinoma Cell Line

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ABSTRACT

The short arm of chromosome 3 has been shown to exhibit high loss of heterozygosity in several types of cancer including ovarian, kidney, lung, and testicular cancers. In particular, overlapping homozygous deletions in lung cancers have been identified in region 3p21.3. Semaphorin 3B, a gene that resides within this region, has been proposed to be involved in tumorigenesis. To address this hypothesis, we have examined the effects of semaphorin 3B on HEY cells, an ovarian cancer cell line. HEY cells expressing semaphorin 3B exhibited a diminished tumorigenicity in BALB/c nu/nu mice. Semaphorin 3B also severely reduced the anchorage independence of HEY cells. These results demonstrate a role for semaphorin 3B in tumor suppression.

INTRODUCTION

Tumorigenesis results from the abnormal regulation of genes involved in cellular homeostasis. One mechanism involves the alteration in gene expression or mutation of tumor suppressor genes, which are recessive genes where mutations result in a loss of function, e.g., unrestricted cell cycle progression (1). There are several regions within the human genome that when altered, lead to uncontrolled proliferation. One such “hot spot” lies within the short arm of chromosome 3 (3p). Three tumor suppressors localized to this region include the von Hippel-Lindau gene, the MLH1 repair gene, and the FHIT gene (2–4). LOH studies of 3p have identified deletions associated with various cancers (5–7). In particular, the 3p21.3 region exhibits a high LOH in both lung and ovarian carcinomas, suggesting the existence of a putative tumor suppressor gene within this region. In small cell lung cancer, >90% of the tumors exhibit LOH at this site (7, 8). Furthermore, LOH rates >60% are observed in other cancers, e.g., breast, gastric, ovarian, and testicular cancers and renal cell carcinoma (9–14).

Ovarian cancer is one of the leading causes of death in women. Given the high rate of LOH in 3p, several studies have been conducted to identify any ovarian cancer tumor suppressor genes within this region. Monochromosomal transfer studies of 3p by Rimessi et al. (15) have identified three ovarian cancer suppression regions. One region, OCSR-C (8), overlaps with 3p21.3, a region implicated in lung cancer (6, 16–18). Interestingly, two class III semaphorin genes (SEMA3B and SEMA3F) reside in this region and have been proposed to play a role in tumorigenesis (6, 18–20). In normal ovarian tissue, SEMA3B is the predominant semaphorin expressed (20). Semaphorins are a family of signaling molecules initially identified to play a role in axonal guidance and can be classified as either membrane bound (classes 1, 4, 5, and 6) or secreted (classes 2 and 3) (Refs. 21–23). All semaphorins contain ~500 amino acid NH2-terminal sema domains, an immunoglobulin-like domain (with the exception of class 1 semaphorins), and a basic COOH-terminal domain (24). The role of the secreted class 3 semaphorins in axonal guidance has been clearly demonstrated; however, their role(s) in nonneuronal tissue remains to be elucidated (25). The receptors for the class 3 semaphorins are the neuropilin receptors (21, 26, 27). In neuronal tissue, signaling through the neuropilin receptor has been shown to involve a heteromeric complex with the plexins (25, 28). The intracellular signal transduction pathway is less clear but has been shown to involve the Rho family GTPases (29).

Both semaphorin 3B and 3F reside in 3p21.3 (Fig. 1) and exhibit overlapping yet distinct tissue expression patterns (20). Recently, immunohistochemical studies found SEMA3F localization and expression to be altered in lung tumors compared with normal lung tissue (19). These results suggest a role of SEMA3F in tumorigenesis. However, the potential role of SEMA3B in tumorigenesis is less clear. SEMA3B has been found at reduced levels or not expressed in both small cell and non-small cell lung carcinomas, suggesting a role in tumorigenesis (6). Furthermore, mutations resulting in amino acid changes were found; however, the ramifications of these mutations remain to be elucidated (6, 20). On the basis of these observations, we propose that SEMA3B plays a suppressive role in tumorigenesis. To address this hypothesis, HEY cells, a tumorigenic adenocarcinoma cell line, were stably transfected with SEMA3B and examined for tumor suppression abilities. SEMA3B transfectants exhibited decreased tumor formation. Thus, in addition to its role in axonal development, we have identified a tumor suppressive role of SEMA3B.

MATERIALS AND METHODS

Material. The human ovarian cell line, HEY, was used as the tumor cell model and was a gift of Dr. Ron Buick (University of Toronto, Toronto, Ontario, Canada). Briefly, HEY cells were derived from a human ovarian cancer xenograft (HX-62; Ref. 30). α-MEM and the Thermoscript RT-PCR kit were obtained from Life Technologies, Inc. Fetal bovine serum was obtained from HyClone. The mammalian expression vector, pTracer-SV40, and the selection agent, zeocin, were obtained from Invitrogen. Total ovarian RNA was obtained from Clontech. All other chemicals were of reagent grade.

Quantitative Real-Time RT-PCR. SEMA3B gene expression was quantified using Taqman chemistry on an ABI 7700 SDS machine (Perkin-Elmer) as described by manufacturer. The primers and probes used for detection of SEMA3B were: 5′-GCGACACCCCACTTGCATCA-3′/5′-CGTGGAGAA-GACGGCATAAG-3′, and the probe was 5′-6-carboxyfluorescein-CTC-CAGGATGTGTTTCTGTTGTCCTCGC-6-carboxytetramethylrhodamine-3. Briefly, reverse transcription was performed at 48°C for 30 min using the Multiscribe reverse transcriptase (Perkin-Elmer). Samples were then denatured at 95° for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were analyzed on the SDS software version 1.7.

SEMA3B cDNA Isolation and Cloning into Expression Vector. The full-length cDNA of SEMA3B was obtained by RT-PCR using human ovary total RNA. The Thermoscript RT-PCR kit was used for this procedure. Briefly, cDNA corresponding to the total mRNA was reverse transcribed using an...
expression was normalized across samples by dividing the absolute values by the glyceraldehyde-3-phosphate dehydrogenase expression values from each sample. Bars, SD.

RESULTS

The Semaphorin 3B Gene Suppresses Tumor Formation in Athymic Nude Mice. SEMA3B is highly expressed in ovarian tissue; however, its expression level in HEY cells, an ovarian adenocarcinoma, is unknown. To evaluate the expression level, quantitative RT-PCR was performed. SEMA3B expression in total human ovarian RNA (Ambion) was determined to be 0.918 arbitrary units (Fig. 1B). In contrast, the SEMA3B expression in HEY cells was 0.038 arbitrary units, ~25-fold less than that observed in the total human ovarian RNA. The substantial decrease in the expression of SEMA3B in the ovarian adenocarcinoma cell line is analogous to the observations in lung carcinomas and suggests a role for SEMA3B in ovarian tumorigenesis. To examine whether SEMA3B has a role in tumorigenesis, HEY cells were stably transfected with a SEMA3B cDNA clone. The SEMA3B cDNA was isolated by amplification from total human placenta RNA (Clontech) and cloned into the pTracer-SV40 mammalian expression vector (Invitrogen). Stably transfected expressing SEMA3B from the introduced cDNA were identified by RT-PCR using a forward primer specific to the vector backbone and a reverse primer specific for SEMA3B (Fig. 2A). Use of the vector-specific forward primer allowed us to identify only transfectants that were expressing SEMA3B from the pTracer expression vector. To assess the tumor suppression properties of SEMA3B, five stable HEY clones were injected into five male athymic nude mice, and tumor growth was monitored in intervals of 3–5 days for 38 days. Mice receiving injections of two distinct sets of control HEY cells containing the pTracer-SV40 vector alone exhibited exponential tumor growth after 5 days for 38 days. Mice receiving injections of two distinct sets of control HEY cells containing the pTracer-SV40 vector alone exhibited exponential tumor growth after 5 days for 38 days. Mice receiving injections of two distinct sets of control HEY cells containing the pTracer-SV40 vector alone exhibited exponential tumor growth after 5 days for 38 days. Mice receiving injections of two distinct sets of control HEY cells containing the pTracer-SV40 vector alone exhibited exponential tumor growth after 5 days for 38 days.
a RT-PCR product because they did not contain the pTracer-SEMA3B construct (Fig. 2D, Lanes 1 and 2). In comparison, RNA isolated from the tumors formed from the SEMA3B transfectants also did not result in a RT-PCR product, indicating the loss of SEMA3B expression from the pTracer-SEMA3B construct (Fig. 2D, Lanes 3–10).

In vitro, cancer cells lose contact inhibition and acquire anchorage independence. To examine the anchorage independence, both controls and SEMA3B transfectants were grown on soft agar (Fig. 3). Over a 3-week period, control HEY cells formed several hundred foci (Fig. 3A). In contrast, the number of foci formed from the SEMA3B transfectants was reduced up to 10-fold (Fig. 3B).

SEMA3B Alters the Proliferation Rate of HEY Cells. One mechanism by which SEMA3B may reduce tumor formation is by affecting cell proliferation. To examine this possibility, we performed an XTT metabolic assay. This assay involves the reduction of the tetrazolium salt (XTT) by viable cells. The assay principle is based on the fact that proliferating cells are more metabolically active than resting cells. Both control and SEMA3B transfectants were plated at the same density and grown for 24 h in α-MEM containing either 0.5 or 10% FBS (Fig. 4). The metabolic activity of the control HEY cells grown in medium containing 10% FBS is >2-fold higher than those grown in 0.5% FBS. In contrast, the SEMA3B transfectants only exhibited a 1.2–1.6-fold change. Importantly, no cell death was observed in the cells grown in medium containing 0.5% FBS, as determined by trypan blue exclusion (data not shown). Because no cell death was observed, we consider the metabolic activity to reflect proliferative properties of the cell population. Thus, the cell proliferation rate of SEMA3B transfectants is reduced by ∼36% compared with controls (Fig. 4B).

SEMA3B Increases the Susceptibility of HEY Cells to Cytotoxic Environments. To further characterize the effects of SEMA3B, we performed the XTT assay under various cytotoxic conditions. The degree of cytotoxicity can be determined using this assay because cytotoxic factors reduce the rate of tetrazolium salt cleavage.
that corroborate this result: (a) 3p21.3 exhibits >60% LOH in ovarian carcinomas and >90% in small cell lung carcinomas, suggesting that a tumor suppressor(s) resides within this region; (b) SEMA3B expression appears to be absent or altered in both small cell and non-small cell lung carcinomas (5, 6, 20); and (c) we found that SEMA3B expression in an ovarian carcinoma cell line is ~25-fold less than the expression in total RNA from human ovarian tissue. Moreover, mutations in SEMA3B have been identified in tumor cell lines (6, 20). In this study, we demonstrate that expression of SEMA3B decreases both tumorigenicity and cell proliferation rates. Furthermore, the characteristic anchorage independence of cancer cells is severely diminished by SEMA3B expression.

Suppression of Tumorigenesis by SEMA3B May Occur through Multiple Mechanisms. Our data indicate that there is an intrinsic change in the cells that have been transfected with semaphorin 3B. Perhaps the most significant difference is that transfected cells no longer exhibit anchorage-independent growth. This coupled with the result of decreasing the metabolic activity or viability of the cells (increased cell death; Refs. 31, 32). It is important to note that this measure of cytotoxicity or cell death is inclusive of cells that undergo both apoptosis and necrosis. Control and SEMA3B transfectants were grown for 24–48 h in the absence or presence of Taxol, a genotoxic agent that interferes with microtubule remodeling, leading to programmed cell death. As shown in Fig. 5A, control HEY cells containing only the pTracer-vector exhibit a ~22% decrease in metabolic activity after a 24-h exposure to Taxol. The metabolic activity decreases by ~40% after 48 h. In contrast, a ~35–59% decrease in metabolic activity is observed in the SEMA3B transfectants after 24–48 h, respectively. In the presence of Adriamycin, another genotoxic agent that inhibits topoisomerase activity, both controls and SEMA3B transfectants exhibited a similar behavior (Fig. 5B). This is analogous to previous observations that HEY cells exhibit resistance to cisplatin, another DNA interacting agent (30).

Serum starvation also induces cell death but in a more obscure pathway than Taxol and Adriamycin. Under serum starvation, control HEY cells did not display a significant decrease in metabolic activity until 48 h (40%; Fig. 5C). In contrast, the metabolic activity of the SEMA3B transfectants decreased by 40–80% over 24–48 h, respectively. In sum, the presence of SEMA3B increased the cytotoxic effects of Taxol and serum starvation but not Adriamycin on HEY cells.

DISCUSSION

Cancer progression is a multistep process that requires the mutation and/or alteration of expression of multiple genes. Here we demonstrate that SEMA3B, a gene that resides in 3p21.3, suppresses tumor formation in an ovarian carcinoma. There are several lines of evidence...
decrease in metabolic rate would suggest that semaphorin 3B expression brings the cells more in line with normal cell activity. Class 3 semaphorins are secreted proteins initially identified to play a role in axonal migration (33). However, the widespread expression profile in adult tissues suggests that other functions exist. The most extensively studied semaphorin is SEMA3A. SEMA3A has been shown to be a repellent, causing the collapse of axons. This repellent nature appears to be through both alterations in cell migration and therefore the induction of apoptosis in progenitor cells migrating toward the SEMA3A gradient (33–36). The effects of the class 3 semaphorins are mediated through the Np receptors, Np-1 and Np-2. For SEMA3A, Np-1 seems to be a major component of its signaling pathway. Interestingly, the Np receptors also serve as coreceptors for several isoforms of VEGFs (37–40). VEGF is known to bind two receptor tyrosine kinases, the kinase domain region (KDR) and fms-like tyrosine kinase (Flk-1). When the Np receptors are coexpressed with KDR, VEGF affinity and mitogenic activity are enhanced (37, 40). VEGF is known to be a potent angiogenic factor as well as a mitogenic factor and has been found to be an essential initiator of tumor angiogenesis. Thus, SEMA3A functions, in part, to competitively inhibit VEGF.

This has been well demonstrated by several laboratories (37, 38, 40). Similar to SEMA3A, SEMA3B also binds to Np-1 and Np-2 with high affinity (41). HEY cells express Np-2. Thus, SEMA3B action in tumorigenesis may also involve inhibition of tumor angiogenesis. On one level, SEMA3B works in an autocrine fashion to decrease cell proliferation and promote cell anchorage dependence. Concomitantly, VEGF action may be down-regulated through direct sequestration of both Np-1 and Np-2 receptors, preventing vascularization of the tumor tissue. In sum, our demonstration of the tumor suppressive role of SEMA3B combined with its wide range of expression suggests that SEMA3B is involved in homeostasis in nonneuronal tissue, and regulation may lead to uncontrolled proliferation and tumorigenesis.

REFERENCES

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