Promotion of Seminomatous Tumors by Targeted Overexpression of Glial Cell Line-derived Neurotrophic Factor in Mouse Testis

Xiaojuan Meng, Dirk G. de Rooij, Kennet Westerdahl, Mart Saarma, and Hannu Sariola

Developmental Biology [X. M. K. W., H. S.] and Molecular Neurobiology Research Programs [M. S.], Institute of Biotechnology, Väikä Biocenter, FIN-00014 University of Helsinki, Finland; HUCS Diagnostics and Department of Biomedicine, FIN-00014 University of Helsinki, Finland [H. S.]; and Department of Cell Biology, University Medical Center Utrecht, 3584 CX Utrecht, the Netherlands [D. G. d. R.]

Abstract

We show with transgenic mice that targeted overexpression of glial cell line-derived neurotrophic factor (GDNF) in undifferentiated spermatogonia promotes malignant testicular tumors, which express germ-cell markers. The tumors are invasive and contain aneuploid cells, but no distant metastases have been found. By several histological, molecular, and histochemical characteristics, the GDNF-induced tumors mimic classic seminomas in men, representing a useful experimental model for testicular germ-cell tumors. The data also show that a deregulated stimulation of a normal proto-oncogene by its ligand can be an initiating event in carcinogenesis.

Introduction

Mutations causing the constitutive activation of Ret receptor tyrosine kinase, the signaling receptor for GDNF, have been found in thyroid cancers, pheochromocytomas, and MEN2 (MEN2A and MEN2B; Ref. 2). Transgenic mice with the MEN2B mutation of Ret display thyroid C-cell and adrenal chromaffin cell hyperplasia, which progresses into pheochromocytomas (3). Melanomas develop in mice in which melanocytes are targeted to express a Ret mutation that was originally found in papillary thyroid carcinomas (4).

GDNF is a distant member of the transforming growth factor-β superfamily (5). It maintains several sets of neuronal cells, is required for enteric innervation, regulates urogenital branching in the embryo, and controls cell fate decision of undifferentiated spermatogonia in the testis (1, 6). The signal transduction receptor complex for GDNF is composed of Ret receptor tyrosine kinase and a glycosylphosphatidylinositol-linked co-receptor, GFRα1 (1).

GDNF is expressed in the testis by Sertoli cells, the paracrine regulators of spermatogenesis, whereas the GDNF receptors are being expressed by the undifferentiated spermatogonia (6). To test the role of GDNF signaling in spermatogenesis, we have recently targeted full-length human GDNF to the testis by the translation elongation factor 1α promoter, which directs the transgene expression specifically to spermatogonia (6). The GDNF-overexpressing mice do not produce sperm, and all male mice are infertile (6). In short, spermatogenesis is disturbed, and undifferentiated spermatogonia accumulate within the seminiferous tubules at 2–4 weeks of age. The spermatogonial clusters occlude the seminiferous tubules and are dissolved subsequently by apoptosis. The transgenic mice develop testicular atrophy around 8–10 weeks of age. Despite the advanced atrophy, some spermatogonia remain in the periphery of seminiferous tubules. The old transgenic mice develop testicular tumors. We now show that these tumors mimic classic seminomas in men and serve as the first animal model for this tumor type.

Materials and Methods

DNA and Karyotype Analysis. Flow cytometric analysis of the DNA contents of testicular tumor cells was performed by FACScan flow cytometry (Becton Dickinson) after ethidium bromide labeling of the nuclei (CellFIT Cell-Cycle Analysis version 2.01.2). Mouse spleen cells served as diploid cell controls. Karyotyping was done either from frozen tissues or 60-μm sections of paraffin-embedded material.

Histology and Immunohistochemistry. For histology and immunohistochemistry, testes and other tissues were freshly dissected and frozen in liquid nitrogen or fixed in either Bouin or 4% paraformaldehyde for 2–24 h, depending on the size of the sample. The paraffin-embedded samples were sectioned at 5 μm and stained by H&E. For immunohistochemistry with rat monoclonal EE2, TRA98, and CD44 antibodies, paraffin-embedded sections were used. The EE2 and TRA98 antibodies labeling spermatogonia were kind gifts of Dr. Y. Nishimune (Osaka University, Osaka, Japan). The CD44 antibodies labeling lymphocytes were a gift of Dr. Sirpa Jalkanen (University of Turku, Turku, Finland). After deparaffinization, sections were incubated with the EE2, TRA98, or CD44 antibodies at 1:500, 1:1000, and 1:50, respectively. After washes in PBS, the section were incubated with biotinylated anti-rat IgG secondary antibody at 1:500, followed by color detection according to the manufacturer’s instructions (Vector Laboratories, Inc., Burlingame, CA). The enzymatic method for alkaline phosphatase activity on frozen sections was done as described (7).

In Situ Hybridization. cRNA in situ hybridization was performed as described (8). Antisense and sense 35S-uridine-labeled cRNAs to GDNF, Ret, and GFRα1 were as described (6). WTI probe was from Dr. Jordan A. Kreidberg (Department of Pediatrics, Harvard School of Medicine, Boston, MA). The L-Fng probe and β2-HSD probe were as described (9, 10). In short, the hybridization temperature was 52°C, and slides were exposed at 4°C for 2 weeks. The slides were photographed with an Olympus Provis microscope equipped with a CCD camera (Photormetrics Ltd.). In the Photoshop 4.0 program, the dark-field images were inverted, artificially stained red, and combined with the bright-field image. Sense controls did not show labeling above background (data not shown).

Immunoprecipitation and Western Blotting of Phosphorylated Ret. Homogenization of samples (either freshly dissected or frozen tissues) was performed in lysis buffer (50 mM HEPES, 1% Triton X-100, 50 mM sodium chloride, 50 mM sodium fluoride, 10 mM sodium PPi, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM sodium vanadate). After 30 min incubation on ice, the homogenate was centrifuged at 13,000 χ g for 2 min, and the supernatant was analyzed by either immunoprecipitation-Western blot or Western blotting. The lysates were immunoprecipitated with rabbit polyclonal antibody to human Ret cross-reacting with mouse Ret (Ref. 11; the Ret antibody was a gift from Dr. M. Santoro, University of Naples, Naples, Italy), run on a 7.5% SDS-PAGE gel, and blotted onto Hybrid ECL membrane (Amersham). Ret protein was blotted by the Ret antibody, the phosphorylation of Ret was detected by monoclonal phosphotyrosine antibody (Transduction Laboratory).
Mitogen-activated Protein Kinase and AKT Phosphorylation Assays.

Total protein lysates mentioned above were separated on a 12% SDS-PAGE gel, blotted, and probed with polyclonal antibodies to phosphorylated forms of either ERK1/2 (Promega) or AKT (protein kinase B; New England Biolabs). The blots were then reprobed with the antibodies, which recognize both the phosphorylated and unphosphorylated forms of ERK1/2 (Promega) or AKT (New England Biolabs). The absorbance of the bands was determined using a phosphorimager and a TINA program.

Results and Discussion

The long-term consequence of the continuous overexpression of GDNF by spermatogonia was followed until 15 months of age (n = 37 mice >5 months in FVB and NMRI strains). We first analyzed the atrophic testes of 19 transgenic mice between 6 and 11 months of age. One 7-month-old mouse testis showed small groups of spermatogonia-type cells invading the interstitium (data not shown). All other testes showed typical atrophy at these ages. Thereafter, the transgenic mice frequently developed macroscopic testicular tumors starting at 1 year of age (16 of 18 mice; Fig. 1). In contrast, neither testicular tumors nor microinvasive germ-line cells were observed in the same wild-type mouse strains at any age (n > 200). In most transgenic mice, the testicular tumors were bilateral (56%), and all tumors were histologically uniform. They were composed of round cells with only scant cytoplasm, and the cells invaded the interstitial tissue of the testes, leaving the seminiferous tubules mostly untouched. In the largest tumors, tumor cells also colonized seminiferous tubules, and fibrous bands between tumor nodules were commonly present. The lymphocytes in the tumors were detected by CD44 antibody. Only single CD44-positive lymphocytes were seen (data not shown). The mitotic index of the tumors was constantly high (~10 mitotic figures/40× magnification), and the mitotic figures were often atypical. No distant metastases were found by the autopsy or histological analysis, indicating low or nil metastatic potential of the tumor cells or a follow-up time that was too short.

The germ-cell origin of tumor cells was confirmed by two germ-cell-specific antibodies, EE2 and TRA 98. The EE2 antibody recognizes a cell membrane protein on spermatogonia (12), and it labeled all tumor cells (Fig. 1, d–f). Another germ line-specific antibody, TRA98 preferentially labeling gonocytes and spermatogonial nuclei (13), showed the same pattern as EE2 (data not shown). A possible Leydig or Sertoli cell contribution to the tumors was excluded by a Leydig cell marker, 3β-HSD (10), and Sertoli cell marker, WT1 (14). 

Like the intratubular spermatogonial clusters in young transgenic mice (6), the tumor cells expressed the GDNF transgene, Ret and GFRα1 (Fig. 1, g–i), as well as elevated levels of hyperphosphorylated Ret protein as detected by immunoprecipitation-Western blotting (Fig. 2). AKT (protein kinase B) and mitogen-activated protein kinases ERK1/2, among others, are phosphorylated upon Ret activation (15). We found increased phosphorylation of both ERK1/2 and AKT in the tumors as compared with the wild-type testis (Fig. 2). Because the Ret-mediated transforming effect in both MEN2A and MEN2B cancer syndromes is critically dependent on the activation of the AKT pathway (16–18), its high activity in the GDNF-induced seminomas indicates that AKT is involved in the oncogenesis of these experimental tumors as well.

Three tumors were analyzed by flow cytometry, and they exhibited an aneuploid karyotype. A distinct triploid peak was always observed, whereas a considerable number of tumor cells appeared to be hypodiploid. No peak at the tetraploid or higher level was found. When the

Fig. 1. Development of seminomatous tumors in old transgenic mice targeted to overexpress GDNF in testes. a, seminiferous tubules in a normal adult testis. The spermatogonia are situated at the peripheral rim of the tubules. b, a testis from a transgenic mouse at 4 weeks of age. Note the clusters of undifferentiated spermatogonia within seminiferous tubules. c, solid sheets of invasive spermatogonia-type cells in a transgenic mouse developing the testicular tumor at 1 year of age. The tumor cells are uniform; they have clear cytoplasm, well-defined cell margins, and a central nucleus with clumped chromatin. Inset, a tripolar mitotic figure. d, fibrous band between tumor nodules. d–f, the EE2 antibody labeling shows the spermatogonial identity of the tumor cells. g, wild-type testis. e, a 4-week-old transgenic testis with clusters of spermatogonia within seminiferous tubules. f, the germ-line markers are also expressed by the testicular tumors. In situ hybridization for the GDNF transgene (g), Ret (h), and GFRα1 (i) in the testicular tumors are shown. g–i, the red color depicts the signals of in situ hybridization and marks the shape of a seminiferous tubule. Left panel, wild-type testis; middle and right panels, testes from GDNF-overexpressing mice. Scale bar: a and b and d–i, 100 μm; c, 33 μm.
DNA ploidy was analyzed in young transgenic testes without tumors, no aneuploidy was found (n = 10 mice between 3 and 10 months of age; Fig. 3). However, mitoses with a tripolar organization indicating a triploid DNA content were occasionally observed in the spermatogonia of atrophic testes. Such cells might represent CIS cells, but they were apparently too few to be detected by flow cytometry.

Further characterization of the tumor phenotype was done with alkaline phosphatase reaction and with molecules involved in Notch signaling. Placental alkaline phosphatase is expressed by the embryonic germ-line cells, the gonocytes (7, 19–23), and it is normally down-regulated postnatally (Fig. 4a). The tumor cells showed a strong alkaline phosphatase reactivity, and in addition, it was also seen in a few cells in the spermatogonial clusters of young transgenic mice (Fig. 4, b and c). Notch has been implicated in the pathogenesis of leukemia, cervical and colon carcinoma, and Alagille syndrome (24). During embryogenesis, the Notch signals regulate the patterning and cell fate decisions of different tissues (25, 26). Notch1 and Notch2 were expressed by wild-type and transgenic spermatogonia in young and old mice and by the tumor cells (data not shown). L-Fng is a modulator of Notch signaling. It inhibits Notch activation via the ligand Serrate but enhances Notch activation via Delta (25). L-Fng was expressed in wild-type testes only within a narrow time window during prepuberty, and it became undetectable at the onset of spermatogenesis (Fig. 4d). In contrast, the intratubular spermatogonial clusters in young transgenic mice and the tumors invariably expressed L-Fng (Fig. 4, e and f). The continuous expression of L-Fng that is normally down-regulated at puberty and the reappearance of alkaline phosphatase by the transgenic spermatogonia indicate that they initially maintained prepubertal features that gradually regressed to or were replaced by an embryonic gonocytic phenotype.

TGCTs are the most common solid tumors in young men, and their frequency is increasing (21). The tumors are grouped in two entities, seminomas and nonseminomatous TGCTs. A few testicular malignancies are comprised of mixed seminomatous and nonseminomatous areas (22). Seminomas, accounting for approximately half of all TGCTs, are further subdivided in two distinct subtypes. Classic seminoma is composed of fairly uniform medium-sized cells with clear cytoplasm and well-defined cell borders (22). Human classic seminoma is supposed to originate from CIS cells. They arise early in development, resemble gonocytes, the immature germ cells, and proliferate but are unable to differentiate. Both CIS cells and classic seminomas express placental alkaline phosphatase (7, 19–23). The other variant, spermatocytic seminoma, does not only show homogeneous fields of gonocyte-type cells but also large, often gigantic, cells that are supposed to represent spermatocytes or type B spermatogonia, and this rare tumor variant usually does not show alkaline phosphatase reactivity (7, 19–23). All classic and most spermatocytic seminomas...
show an aneuploid DNA content, except tetraploidy, or higher DNA ploidy, which is a characteristic of spermatocytic seminomas, whereas the triploid karyotype is characteristic of classic seminomas (7, 19, 20). Several important characteristics of the GDNF-induced tumors resemble those of classic human seminomas rather than spermatocytic seminomas: (a) the tumors consisted of a gonocytic cell type without the thread-like chromatin and giant cells that are typical for spermatocytic seminomas; (b) no peaks at the tetraploid or higher level were found in the flow cytometric analysis. Instead, a triploid peak was present, which is a characteristic karyotype in classic seminomas; (c) all GDNF-induced tumors were alkaline phosphatase positive similar to the classic seminomas. Such cells are only sporadically observed in spermatocytic seminomas; (d) the development of the GDNF-induced tumors resembled that of classic seminomas. In the transgenic mice, immature gonocyte-like germ cells, which seem to be comparable with CIS cells in humans, were present before tumors appeared. Human CIS cells are also alkaline phosphatase positive and are unable to undergo differentiation, slowly replacing the seminiferous epithelium (7, 19–23). We found clusters of undifferentiated spermatogonia at 2 weeks of age and the first microinvasive spermatogonia at 7 months of age, further indicating that the pathogenesis of these tumors can be traced back to a young age. Although these experimental murine TGCTs and human seminomas are similar in several respects, there are also differences, such as the high frequency of bilateral tumors in mouse (unilateral in man), the appearance of tumors at old age in mouse versus at young age in man, the tumor development in the context of severely distorted spermatogenesis in mouse versus normal spermatogenesis in man, and the absence of large lymphocyte infiltrates in mouse, whereas these are present in most but not all classic seminomas (7, 22, 23). Although no distant metastases were found, several features suggest that the tumors in the GDNF-overexpressing mice were malignant. The tumors were invasive, aneuploid, and atypical mitotic figures were frequent. In fact, human seminomas are infrequently metastatic. Twenty % of patients present with retroperitoneal lymph node involvement and <5% with distant metastases (27). However, the lack of metastases suggests that the mutations leading to distant colonization of the tumor cells are not necessarily the same as those that cause the local invasive behavior. Spontaneous seminomas are extremely rare in animals (28). Although seminomatous tumors have been reported in mice, dogs, rams, bucks, rabbits, and possibly bulls, almost invariably these tumors have been characterized as spermatocytic seminomas (29). In contrast, the morphology, histochemical and molecular characteristics, and aneuploidy of the GDNF-induced tumors are similar to human classic seminomas. Thus, these mice represent the first transgenic animal model for this tumor type that is the most common TGCT in men. It was shown recently that GDNF and its receptors are expressed by human seminomas (11), but their role in the pathogenesis of the human testicular tumors remains to be established. The high frequency of tumors in this mouse model provides further evidence that a deregulated stimulation of a normal receptor tyrosine kinase by its normal ligand has carcinogenic potential. This risk should also be taken in consideration when GDNF-related therapies are designed for amyotrophic lateral sclerosis, Alzheimer’s or Parkinson’s disease (1).

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References


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