

Progesterone Receptor Expression in Neurofibromas¹

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Abstract

Neurofibromas are benign tumors of the peripheral nerve sheath, which occur sporadically and in association with the common familial cancer syndrome, neurofibromatosis type 1. There are intriguing links between the growth of neurofibromas and levels of circulating hormones: neurofibromas often first appear around the time of puberty, increase in number and size during pregnancy, and shrink after giving birth. We examined 59 human neurofibromas for the expression of estrogen and progesterone receptors (PRs), because their ligands, estrogen and progesterone, were attractive candidate hormones. The majority (75%) of neurofibromas expressed PR, whereas only a minority (5%) of neurofibromas expressed estrogen receptor. Within neurofibromas, PR was expressed by non-neoplastic tumor-associated cells and not by neoplastic Schwann cells. We hypothesize that progesterone may play an important role in neurofibroma growth and suggest that anti-progestins may be useful in the treatment of this tumor.

Introduction

Neurofibromas are benign tumors of the peripheral nerve sheath, which have several interesting properties. First, they occur both sporadically and in association with NF1,³ a complex neurocutaneous disorder in which patients suffer from multiple lesions of diverse types including: hyperplasias, hypoplasias, hamartomas, and neoplasms. The gene responsible for NF1 maps to the pericentric region of chromosome 17q and encodes the protein neurofibromin, a member of the Ras-specific GTPase activating protein (RasGAP) family (1). It remains unclear whether or not the *NF1* gene is inactivated in sporadic neurofibromas. Second, neurofibromas invariably contain a variety of cell types found in normal peripheral nerves (axonal processes of neurons, Schwann cells, perineurial cells, fibroblasts, and mast cells). Their cellular heterogeneity is so striking that some in the past argued that neurofibromas represented hyperplasias, not tumors. Recent studies using cultured Schwann cells from human neurofibromas and conditional *Nf1* mouse models have clearly demonstrated that in NF1 patients and in mice with genetically engineered *Nf1* mutations complete loss of the *NF1* gene occurs within Schwann cells (2, 3), confirming that the Schwann cell is the cell of origin of this tumor. Third, there are intriguing links between the growth of neurofibromas and levels of circulating hormones: neurofibromas often first appear around the time of puberty, increase in number and size during pregnancy, and shrink after giving birth, indicating that blood-borne signals (4), such as hormones, may promote tumor growth. Here, we report that the majority of

neurofibromas express the PR, suggesting that progesterone may be the critical hormone that regulates neurofibroma growth.

Materials and Methods

Human Tissue Sample Acquisition. Formalin-fixed, paraffin-embedded tissue sections were obtained from 59 neurofibromas (20 localized cutaneous neurofibromas, 9 diffuse cutaneous neurofibromas, 2 localized intraneural neurofibromas, 12 plexiform neurofibromas, 12 mixed neurofibromas with features of both diffuse and plexiform neurofibromas, and 4 deep-seated neurofibromas), 7 schwannomas, 10 malignant peripheral nerve sheath tumors, and 1 normal peripheral nerve. Thirty six of the patients whose tumor samples were included in the study carried the diagnosis of NF1, based on the clinical criteria outlined by Gutmann *et al.* (5). The study was done with the approval of the Committee for Clinical Investigation of Boston Children's Hospital and the Human Research Committee of Brigham and Women's Hospital.

Immunohistochemistry of Human Tissue Samples. We stained formalin-fixed, paraffin-embedded tissue sections with: (a) a 1:200 dilution of mouse monoclonal antihuman PR antibody (Clone PgR 636; DAKO, Carpinteria, CA); (b) a 1:200 dilution of mouse monoclonal antihuman ER antibody (Clone 1D5; DAKO); (c) a 1:100 dilution of mouse monoclonal antihuman PR antibody (NCL-L-PGR-312; Vector, Burlingame, CA); and (d) a 1:100 dilution of mouse monoclonal antihuman PR antibody (Clone hPRa 2; Lab Vision, Fremont, CA). We blocked endogenous peroxidases using 1% hydrogen peroxide in ethanol for (a) and (b), or 3% hydrogen peroxide in dH₂O for (c) and (d). To unmask antigens we pretreated samples in 10 mM citrate buffer (pH 6.0) for 30 min in a microwave for (a) and (b), or in Trilogy buffer with EDTA (Cell Marque, Hot Springs, AR) for 15 min in an electric pressure cooker for (c) and (d). We detected antibodies (a) and (b) with a secondary antibody conjugated to a peroxidase labeled polymer (EnVision+ System; DAKO), using 3,3'-diaminobenzidine (DAKO) as a substrate for peroxidase. We detected antibodies (c) and (d) with a biotin-conjugated secondary antibody and ABC (Vector), using 3,3'-diaminobenzidine (Vector) as a substrate for peroxidase. Sections were counterstained with hematoxylin.

A single 5- μ m section of each tumor sample was examined for the presence of ER and PR. Tumors were classified as having "no staining" if <5 positive cells were found per 10 high-power fields, "rare positive cells" if there were between 5 and 20 positive cells/10 high-power fields, "focal positivity" if >20 positive cells were easily identifiable in a single area or multiple discrete areas, or "diffuse positivity" if positive cells were easily identifiable throughout the tumor sample. Cell counting was done in the region with the highest number of positive cells.

Immunofluorescence of Human Tissue Samples. We stained formalin-fixed, paraffin-embedded tissue sections with: (a) a 1:25 dilution of mouse monoclonal antihuman PR antibody (Clone PgR 636; DAKO); (b) a 1:100 dilution of rabbit polyclonal antihuman neurofibromin antibody (sc-67; Santa Cruz Biotechnology, Santa Cruz, CA); and (c) a 1:500 dilution of rabbit polyclonal anticow S-100 antibody (Z0311; DAKO). To unmask antigens we pretreated samples in Trilogy buffer with EDTA for 15 min in an electric pressure cooker. We detected antibody (a) with a rhodamine-conjugated anti-mouse secondary antibody at 1:200 dilution (Jackson ImmunoResearch, West Grove, PA), antibody (b) with a biotin-conjugated anti-rabbit secondary antibody at 1:1000 dilution (DAKO) and streptavidin-conjugated Oregon green at 1:300 dilution (Molecular Probes, Eugene, OR), and antibody (c) with a FITC-conjugated anti-rabbit secondary antibody at 1:1000 dilution (Jackson ImmunoResearch). Sections were then counterstained for 5 min with 4',6-diamidino-2-phenylindole.

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³ The abbreviations used are: NF1, neurofibromatosis type 1; PR, progesterone receptor; MPNST, malignant progression to malignant peripheral nerve sheath tumor; ER, estrogen receptor.

Table 1 PR expression in peripheral nerve sheath tumors

A. Tumors	
Tumor type	No. PR-positive tumors/ total no. of tumors
Neurofibromas	44/59
Localized cutaneous neurofibromas	17/20
Diffuse cutaneous neurofibromas	8/9
Localized intraneural neurofibromas	1/2
Plexiform neurofibromas	6/12
Neurofibromas with extraneural and plexiform features	10/12
Deep-seated neurofibromas	2/4
Schwannomas	1/7
Malignant peripheral nerve sheath tumors	1/10

B. Neurofibromas	
Neurofibroma subset	No. PR-positive neurofibromas/ total no. of neurofibromas in subset
PR-positive localized cutaneous and diffuse cutaneous neurofibromas	25/29
PR-positive plexiform neurofibromas	6/12
PR-positive neurofibromas in males	19/24
PR-positive neurofibromas in females	25/35
PR-positive neurofibromas in patients less than or equal to 20 years of age ^a	14/17
PR-positive neurofibromas in patients greater than 20 years of age ^a	24/30
PR-positive neurofibromas in NF1 patients ^a	9/29
Sporadic PR-positive neurofibromas ^a	14/18

^a Plexiform neurofibromas excluded from the analysis.

Results and Discussion

Several subtypes of neurofibroma have been described (6). While morphologically similar, their clinicopathologic features differ considerably. Localized and diffuse cutaneous neurofibromas affect the dermis and subcutis. Because these proliferations are extraneural, the nerve of origin is difficult to identify. Only a minority, ~10%, of cutaneous neurofibromas are associated with NF1. Localized intraneural and plexiform neurofibromas proliferate intraneurally. Whereas localized intraneural neurofibromas affect a segment of nerve, plexiform neurofibromas involve either a plexus of nerves or multiple fascicles within a large nerve. Like the cutaneous subtypes, the majority of localized intraneural neurofibromas are sporadic. By contrast, plexiform neurofibromas are

found almost exclusively in NF1 patients. Most NF1-associated neurofibromas appear around puberty and increase in number later in life; however, plexiform neurofibromas present in early childhood and are thought to be congenital (4). Importantly, ~5% of plexiform neurofibromas undergo malignant progression to MPNSTs. Massive soft tissue neurofibromas represent the rarest subtype and are restricted to NF1 patients. Microscopically, extraneural and plexiform components are usually present. Despite the enormous size of these lesions, malignant progression is rare.

As a first step toward identifying the critical hormone(s) that regulates neurofibroma growth, we examined 59 human neurofibromas, including neurofibromas of each subtype, for the expression of ER and PR. The majority (75%) of neurofibromas expressed PR (Table 1; Fig. 1 B), whereas only a minority (5%) of neurofibromas expressed ER. Of the neurofibromas that expressed PR, 70% had PR-positive cells distributed diffusely throughout the tumor, 18% had focal areas containing PR-positive cells, and 11% had rare PR-positive cells (see “Materials and Methods”). PR expression was rarely seen in other peripheral nerve sheath tumors (schwannomas and MPNSTs) and was not detected in normal peripheral nerve (Fig. 1, A, C, and D). Previous studies aimed at detecting PR in neurofibromas by measuring progestin binding gave conflicting results (7–10), possibly because very small numbers of neurofibromas were analyzed, and the progestin-binding assays were designed to detect PR at the level of the whole tumor, not the single cell.

The PR ligand progesterone is a steroid hormone known primarily for its role in development and maintenance of the reproductive system. In females plasma progesterone levels are low until the first ovulation occurs, approximately 6–9 months after menarche. Thereafter, plasma progesterone levels fluctuate from 0.3 to 3.0 nmol/liter during the follicular phase of the menstrual cycle to 19.0–45.0 nmol/liter during the luteal phase. The corpus luteum within the ovary is the major source of progesterone during the menstrual cycle and early in pregnancy. After 6–8 weeks of gestation, the placenta takes over as the major source of progesterone, and plasma progesterone levels climb to six times the levels during the luteal phase (11, 12). These changes in plasma progesterone levels correlate well with the periods of rapid growth observed for neurofibromas. Males have low but detectable plasma progesterone levels. The major source of progesterone in males is the testis, where

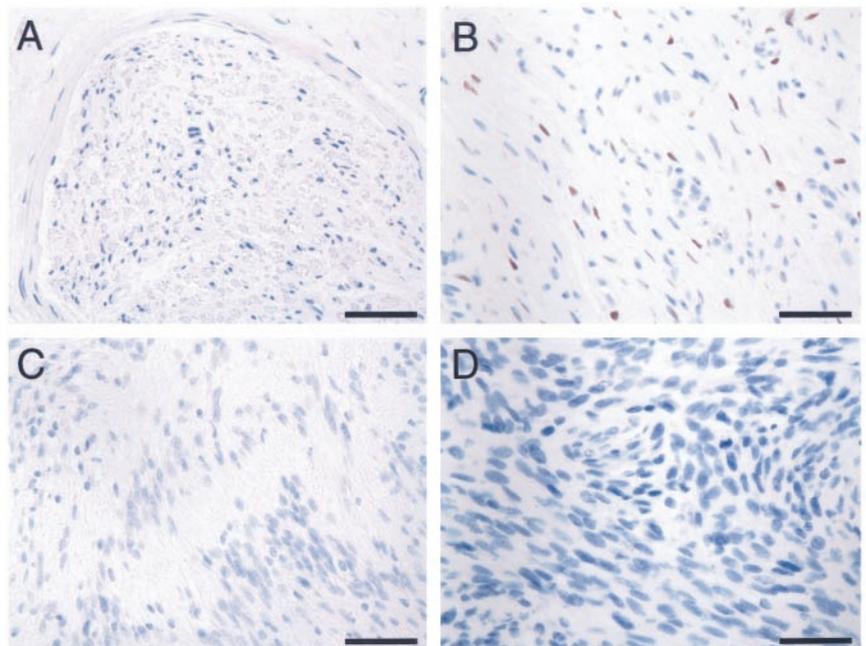


Fig. 1. PR is expressed in human neurofibromas. Immunohistochemistry with an anti-PR antibody demonstrates that scattered cells within neurofibromas have nuclear staining for PR (B). The majority of schwannomas (C) and malignant peripheral nerve sheath tumors (D), as well as normal peripheral nerve (A) are negative for PR. Scale bars = 50 μ m.

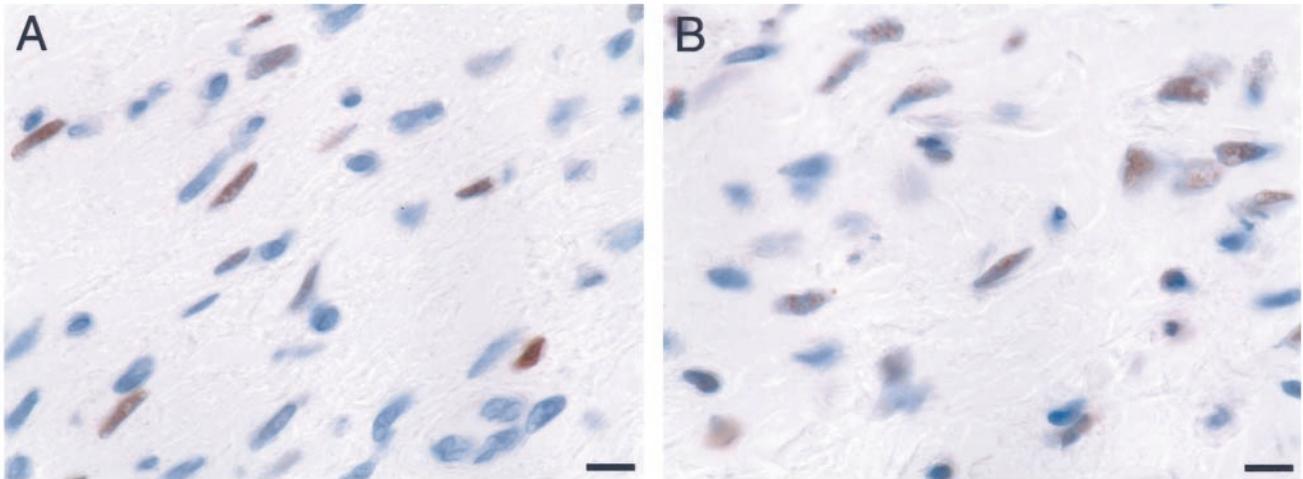


Fig. 2. Isoforms A and B of PR are expressed in human neurofibromas. Immunohistochemistry with antibodies specific for isoform A of PR (A) or isoform B of PR (B) demonstrates that scattered cells within neurofibromas have nuclear staining for both isoforms. Scale bars = 10 μ m.

progesterone serves as an intermediate in the synthesis of testosterone (13). To date, a systematic analysis of the growth rate of neurofibromas under various hormonal conditions has not been performed. If progesterone is the critical hormone regulating neurofibroma growth, we would predict that the increase in tumor growth rate at puberty would be more dramatic in females than males and that an increase in tumor growth rate might also be found in females taking oral contraceptives containing progesterone.

PR expression was found in every subtype of neurofibroma (Table 1). However, the cutaneous subtypes were more frequently positive for PR (86%) than was the plexiform subtype (50%). This difference is statistically significant with a $P < 0.025$ and correlates with the clinical observation that the cutaneous subtypes first appear around the time of

puberty, whereas the plexiform subtype is thought to be congenital. The frequency of PR positivity in neurofibromas was not affected by sex, age, or NF1 disease status. Because plexiform neurofibromas are found almost exclusively in NF1 patients, have a strong tendency to occur early in childhood, and are more likely to be PR negative, the data from the 12 plexiform neurofibromas in our study was not included in the analysis of the effects of age and NF1 disease status on PR positivity.

Because NF1 patients often develop multiple neurofibromas, we were able to determine whether the pattern of PR expression was synchronized across tumors from individual patients. In 10 of 11 of patients with multiple neurofibromas, the pattern of PR expression was synchronized. For example, 1 patient had 3 localized cutaneous neurofibromas that grew rapidly during pregnancy and were removed shortly after giving birth (at

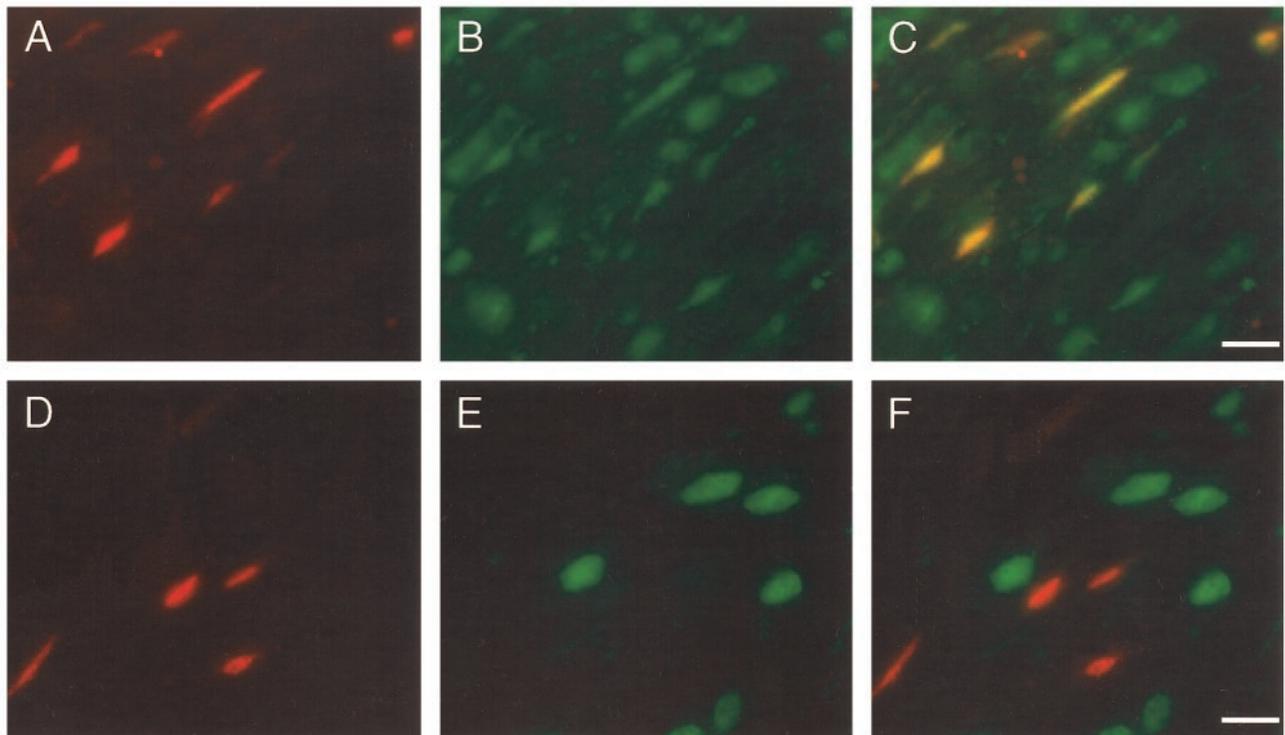


Fig. 3. Neoplastic Schwann cells within neurofibromas do not express PR. PR immunofluorescence alone (A). Neurofibromin immunofluorescence alone (B). Overlay of PR and neurofibromin immunofluorescence showing the presence of cells expressing both PR and neurofibromin (C). PR immunofluorescence alone (D). S-100 immunofluorescence alone (E). Overlay of PR and S-100 immunofluorescence demonstrating that PR and S-100 do not colocalize. Scale bars = 10 μ m.

a time when progesterone levels would have recently decreased). All 3 of the neurofibromas in this patient were negative for PR. The finding of synchronized PR expression in multiple neurofibromas from individual patients suggests that systemic hormone levels may influence local PR expression within neurofibromas. Alternatively, the genetic background of individual patients may determine PR status.

There are two isoforms of PR, PR-A and PR-B (14), which are transcribed from distinct estrogen-inducible promoters. In most contexts PR-B acts as a transcriptional activator, whereas PR-A acts as a transcriptional repressor. Both PR-A and PR-B contain an NH₂-terminal inhibitory domain; however, PR-B contains an extra 164 amino acid domain at the extreme NH₂ terminus that is thought to mask the inhibitory domain. PR-A is a transcriptional repressor of PR-B as well as of estrogen, glucocorticoid, androgen, and mineralocorticoid receptors. PR-A can heterodimerize with PR-B and, therefore, may inhibit PR-B directly. However, PR-A cannot heterodimerize with ER, suggesting that the mechanism of transcriptional interference is indirect and may involve binding to corepressors. In most PR-expressing cells, PR-A and PR-B are present in equimolar amounts. There are exceptions, such as uterus, and breast and endometrial tumors, which have been shown to have low PR-B levels. Because the pharmacological response to antiprogestins is likely determined by the relative expression of the two isoforms, we performed immunohistochemistry on the human neurofibromas with antibodies specific for each isoform, and detected strong expression of both PR-A and PR-B (Fig. 2).

Within the PR-positive neurofibromas not every cell expresses PR (Fig. 1). This is to be expected given the cellular heterogeneity of neurofibromas. To address the question of which cell type within the tumor expresses PR, we performed double immunofluorescence on five neurofibromas from NF1 patients (Fig. 3). Neoplastic Schwann cells from these tumors should express the Schwann cell marker S-100 (15) but lack the *NF1* gene product neurofibromin. We found that in contrast to neoplastic Schwann cells, the PR-expressing cells contained neurofibromin and lacked S-100, indicating that they represent non-neoplastic tumor-associated cells.

The precise identity of the non-neoplastic PR-expressing cells within neurofibromas remains unclear. One possibility is that the PR-expressing cells are fibroblasts or perineurial cells. However, to our knowledge neither of these cell types has been shown to express PR. A second possibility is that the PR-expressing cells are neighboring wild-type or *NF1*^{+/-} Schwann cells. PR has been detected in primary Schwann cell cultures from rats (16), and Schwann cells synthesize their own progesterone (17), suggesting the presence of an autocrine loop. One of the functions of progesterone in the peripheral nervous system is to promote remyelination of regenerating nerves by binding to PR on Schwann cells and stimulating transcription of the transcription factor Krox-20 (18), which, in turn, stimulates transcription of several myelin protein genes. Given their origin from peripheral nerves, neurofibromas result in nerve injury, which may lead to the recruitment of neighboring Schwann cells to help in repair. These neighboring Schwann cells may up-regulate PR in an effort to promote remyelination. It has been proposed that Schwann cells involved in nerve repair dedifferentiate and lose mature Schwann cell markers (19, 20), which could explain the absence of S-100 in the PR-expressing cells. To affect tumor growth, the PR-expressing cells may produce paracrine factors that increase the proliferation and/or survival of the neoplastic Schwann cells; characterizing such paracrine interactions may reveal new cellular targets for drug development.

Currently, surgical resection is the only means of treating neurofibromas (6). However, complete surgical resection is not always possible for large lesions or for NF1 patients who may have hundreds to thousands of lesions. Furthermore, surgical resection of intraneural neurofibromas

(localized intraneural, plexiform, and massive soft tissue subtypes) requires sacrifice of the parent nerve leading to significant neurological deficits. Malignant transformation to MPNSTs occurs in a significant fraction of plexiform neurofibromas. Treatment for MPNSTs consists of surgical resection followed by radiation therapy. Even with aggressive treatment the prognosis is poor with 10-year survival rates ranging from 23 to 34% (6). Antiprogestins, such as Mifepristone (RU486), are being used currently for the treatment of other hormonally responsive tumors that express PR, including breast carcinoma and meningiomas (21). We propose that antiprogestins may be useful for the treatment of neurofibromas as an alternative to surgery, to reduce the size of lesions so that smaller surgical procedures could be performed, and/or to slow malignant progression of plexiform neurofibromas.

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