Vascular Wall Cells Contribute to Tumourigenesis in Cutaneous Neurofibromas of Patients with Neurofibromatosis Type 1. A Comparative Histological, Ultrastructural and Immunohistochemical Study

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Abstract. *Neurofibromas are benign nerve sheath tumours. They occur sporadically, singly or few in number, and in neurofibromatosis type 1 (NF1), an autosomal inherited disease. These tumours are composed of different cell types, e.g. nerve cells (axons and axon sheaths), Schwann cells, mast cells, and fibroblasts. The local control of tumour growth in NF1 is poorly understood. Identification of cell markers could provide new information on the processes that are involved in tumour growth. Materials and Methods: NF1 patients were diagnosed according to the revised NF1 diagnostic criteria proposed by the US National Institute of Health. Fifteen cutaneous neurofibromas from eight patients (origin: trunk and face) were excised, immediately immersion-fixed in Bouin's fixative and embedded in paraffin. Six micrometre thin sections were incubated with a variety of neuronal markers, connective tissue and glial cell markers, neurotrophic factors and their receptors. In addition, material was fixed, embedded and further processed for light and electron microscopic studies. Results: The tumours were composed of different cell types, e.g. nerve cells (axons and axon sheaths), Schwann cells, mast cells, compartmentalising cells and fibroblasts. Neuronal markers were identified in axons (neuron-specific protein gene product 9.5, PGP9.5), in several cell types (neurofilament protein-200 kDa, NF-200) and glial cells (protein S-100, S-100). In glial cells the immunoreactivity for fibroblast surface protein (FSP) was scanty, low for cyclic 2,3-nucleotide 3'-phosphodiesterase (CNPase), strong for glucose transporter 1 (Glut-1) but lacking for glial fibrillary acidic protein (GFAP). Schwann cells and so-called compartmentalising cells exhibited immunoreactivity for neurotrophin receptor protein TrkA (TrkA) and glial cell-derived neurotrophic factor (GDNF). GDNF receptor α-1 (GFR-α1) exhibited distinct immunoreactivity in single axons, in Schwann cells, and with lower intensity in some perineurial sheet cells. No immunoreactivity was observed for the low-affinity neurotrophin receptor protein $p75^{NTR}$, high-affinity receptor protein TrkB (TrkB), high-affinity receptor protein TrkC (TrkC), the neurotrophin 3 (NT-3), and the brain-derived neurotrophic factor (BDNF). Conclusion: Human cutaneous neurofibromas displayed a pattern of neurotrophic factors and their receptor immunoreactivity, which is characteristic of differentiated non-malignant tumours, and exhibited some differences from that established in developing and differentiated control Schwann cells (probably involved in the pathogenesis of the neurofibromas), as well as tumour cells in the process of differentiation. Neurofibromas are highly vascularized tumours and possess activated endothelial cells and pericytes. We presume that most of the hyperplastic structural components of a neurofibroma are generated from activated pericytes and smooth muscle cells of the small tumour vessels which possess qualities of adult stem cells.*

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Neurofibromatosis type 1 (NF1) is a tumour predisposition syndrome. NF1 patients are prone to developing a variety of tumours during their life, which is responsible for the reduced life expectancy of these patients (1). Soft tumours of the skin, arising from dermal nerve sheath and variable in size and number, are termed 'neurofibromas' (2). These tumours are usually small, soft lesions that protrude from the skin and do

not exceed a growth of up to a few centimetres in diameter. They occur usually in large numbers on the entire integument and are the hallmark of the disease. These cutaneous neurofibromas are thought to persist as benign entities during the entire lifespan and are the cause of severe disfigurement as a consequence of their widespread growth. Schwann cells are thought of as being the tumour cells in these mixed tumours (3, 4). However, there are obviously different precursor cell lines that develop to tumorous Schwann cell-like cells inside the nerve sheath in NF1 (5).

During recent years, a body of evidence has accumulated showing that Schwann cells express numerous biochemical substances which differentially characterize the prenatal and adult Schwann cells. For example, during the early development of the peripheral nervous system, stem and progenitor Schwann cells arise from migrating neural crest stem cells (NCSCs). The progenitor Schwann cells (SCP) produce myelinating and non-myelinating adult Schwann cells. It is believed that non-myelinating Schwann cells are the main cell type within neurofibromas (3, 4) but other cell types are also discussed as the origin of tumour cells in NF1 (5, 6). According to Jessen and Mirsky (7), markers for the Schwann cell lineage progression are: sex determining region Y–box (SOX) 10 (SOX10), established in all developmental stages; activator protein 2α (AP2α), expressed by NCSCs and SCPs and in nerve sheath tumours (8); cadherin 19 (Cad19), expressed only in SCPs; brain fatty acid-binding protein (BFABP), found in SCPs and immature Schwann cells; and glial fibrillary acidic protein (GFAP) and "S100", established in immature Schwann cells and absent from or found at only in very low levels in SCPs. The development of the Schwann cell lineage is influenced by a number of individual factors or factor combinations, such as neuregulin 1 (NRG1), the extracellular matrix (ECM), SOX10, notch, bone morphogenic proteins (BMP) 2 and 4, platelet-derived growth factor (PDGF) plus neurotrophin (NT) 3 and insulin-like growth factor (IGF), endothelin (ET) plus IGF, and fibroblast growth factor (FGF) plus IGF, important for promoting the SCP and immature Schwann cell survival (9-11). In contrast to SCPs, Schwann cells can support their own survival by secreting different factors such as IGF2, NT-3, platelet-derived growth factor-BB (PDGF-BB), leukaemia inhibitory factor (LIF), and lysophosphatidic acid (9), and also nerve growth factor (NGF) and neurotrophin receptor p75 ($p75^{NTR}$) (12, 13) and some angiogenic factors such as vascular endothelial growth factor (VEGF) and mevalonate 5-diphosphatase decarboxylase (MVD) (14).

The origin and true nature of the NF1 tumour cells is still a matter of debate (5). Concerning the origin and development of the tumour cells, different ancestors were taken into consideration, *e.g.* NCSCs, SCPs, immature Schwann cells, boundary cap cells, dedifferentiated mature (adult) Schwann cells, a tumourigenic microenvironment, as well as more primitive cells and non-myelinating Schwann cells (5, 6, 9, 15). In addition, a small number of more primitive cells which are persistently present in *Nf1* knockout mice are discussed. These cells are involved in the recruitment of mast cells in the peripheral nerves, resulting in a secondary disruption of Remak bundles and dysfunction of nonmyelinating Schwann cells (5, 6, 16). In this respect Yang *et al.* (17) established that neurofibromin-deficient Schwann cells secrete a potent migratory stimulus for $Nf1^{+/-}$ mast cells.

The aim of this study was to obtain comparative information about the histology, electron microscopy and the localisation of a number of neuroactive and other substances within the cell components of the neurofibroma in comparison with healthy skin structures with the aim of performing a better characterization of the tumour cells and to get some new ideas on the cells of origin of NF1 tumours.

Materials and Methods

Fifteen cutaneous neurofibromas from different sites (face and trunk) of eight adult patients with NF1 diagnosed according to the NIH criteria for NF1 (18) were excised, immediately fixed in Bouin's solution and embedded in paraffin. Six micrometre thin sections were processed for the immunohistochemical visualisation of the following different antigens:

Neuronal and neuroendocrine markers. Neuron-specific enolase (anti-NSE, 1:200, Sigma, Deisenhofen, Germany), neuron-specific protein gene product 9.5 (anti-PGP9.5, 1:600, Biotrend, Cologne, Germany), neurofilament protein 200 kDa (anti-NF 200, antibody N52, 1:200, NF-H, Sigma), protein S-100 (anti-S-100, Z311, 1:100, Dako, Hamburg, Germany), nestin [anti-nestin (10.2): sc-23927, 1:1100, Santa Cruz Biotechnology, Santa Cruz, CA, USA], and purified mouse anti-nestin (1:100, BD Transduction Laboratories, Franklin Lake, NJ, USA).

Neurotrophins. Brain-derived neurotrophic factor (anti-BDNF, 1:400, Chemicon, Temecula, CA, USA), neurotrophin 3 (anti-NT-3, 1 μg/ml, Chemicon), glial cell-line derived neurotrophic factor (anti-GDNF, 2 μg/ml, Sigma), GDNF receptor-α-1 (anti-GFRα-1, 1:2000, Transduction-Dianova, Hamburg, Germany), low-affinity neurotrophin receptor protein p75 (anti-$p75^{NTR}$, 1:200, Chemicon), high-affinity neurotrophin receptor protein, tropomyosin-receptor kinase (TrkA) (anti-TrkA, 1:100, Santa Cruz), high-affinity neurotrophin receptor protein TrkB (anti-TrkB, 1:100, Santa Cruz), high-affinity neurotrophin receptor protein TrkC (anti-TrkC, 1:100, Santa Cruz).

Glial markers. Galactocerebroside (anti-GalC, Boehringer, Ingelheim, Germany, 5 μg/ml), anti-GFAP (Sigma, 1:100), cyclic 2,3-nucleotide 3'-phosphodiesterase (anti-CNPase, 10 μg/ml, Boehringer), O4 sulfatide (anti-O4, 10 μg/ml, Boehringer), myelin basic protein (anti-MBP, 1:100, Dako, Hamburg, lot no. 108).

Components of the NO/cGMP system. Nitric oxide synthase neuronal/brain type (anti-NOS-I, nNOS, bNOS, 1:1000, Biomol, Hamburg, Germany), nitric oxide synthase inducible/macrophage type

(anti-NOS-II, i-NOS, 1:500, Biomol), nitric oxide synthase endothelial type (anti-NOS-III, eNOS, 1:500, Biomol).

Connective tissue cell markers. Collagen IV (anti-collagen IV, M785, 1:100, Dako), vimentin (anti-vimentin, 13.2, 1:500, Sigma), fibroblast surface protein (anti-FSP, 1B10, 1:500, Sigma-Aldrich).
Carbohydrate transmembrane transporters: glucose transporter 1 (anti-Glut-1, 1:1000, Chemicon).

The aforementioned antigens were visualised with a combination of the peroxidase-antiperoxidase (PAP) and the avidin-biotin-peroxidase complex (ABC) procedure (19), including nickel-glucose oxidase amplification (20).

As controls, sections were used in which: i. the primary, secondary and tertiary antibodies were replaced by phosphate buffered saline (PBS); ii. preabsorption was performed for 24 h of the primary antibodies with their corresponding synthetic peptides at a final concentration of between 1 and 100 µg/ml. We used no counterstaining following the immunohistochemical procedures, as described elsewhere in detail (21).

For histological evaluation, part of the paraffin sections was stained with haematoxylin and eosin. Part of the material was also fixed in 5.5% glutaraldehyde for 2 h and postfixed in phosphate-buffered OsO_4 for 2 h, followed by dehydration and embedding in Epon 812. Semithin sections were stained with toluidine blue-pyronin G. Ultrathin sections, contrasted with uranyl acetate and lead citrate, were observed in a Philips EM 300 electron microscope.

Results

Histology and electron microscopy. The normal structures of peripheral myelinated or non-myelinated nerves are already well described in the literature (5, 22-25). In cutaneous, subcutaneous neurofibromas and intraneural perineurinoma some characteristic features have been established (5, 26-30). Neurofibromas consisted of a mixture of single or bundles of peripheral nerve axons and different cell types situated between them, all embedded in a large amount of collagenous ECM. Two types of axons, myelinated and non-myelinated, were recognised. Correspondingly, bipolar Schwann cells or Schwann cell outgrowths, which in contrast to nerves of healthy individuals did not possess a continuous basal lamina, were established. The number of Schwann cells was generally increased and most of their processes lacked contact with the axons (dissociated Schwann cells). The tumour matrix, in addition, contained hyperplastic connective tissue cells (fibroblasts) and compartmentalising (synonymous: covering, enveloping cells, see below) cells, as well as densely packed collagen and less numerous elastic or reticular fibres or bundles. The collagen bundles ran in different directions. The tumour mass was highly vascularised. A high number of blood vessel profiles (arterioles as well as arterial and venous capillaries) were disseminated within the tumour mass, consisting mainly of one endothelial cell layer and subendothelial smooth muscle cells or pericytes (surrounded by the vessel basal lamina) and an enlarged adventitia. In some tumour areas, different numbers of tactile corpuscle-like bodies (termed 'pseudo-onion bulbs', 'onion bulb-like'-, 'onion-like'- structures, 'Pacinian-like bodies') were seen [Figures 1A and 1B, (31-34)]. They consisted of a centrally located small vessel which was enclosed by a relatively lightly stained concentric fibrous zone, followed towards the periphery by a thicker densely stained lamellar ring containing Schwann and perineurial cells with long processes. Occasionally, similar bodies consisting of one centrally situated myelinated axon with a corresponding covering of Schwann cells, surrounded by a concentric zone of fine collagen fibres (endoneurial type), as well as by a multilayered concentric zone of perineurial cells with long processes with pinocytotic vesicles and subplasmalemmal densities in their cytoplasm covered with basal lamina were observed by electron microscopy [Figure 1C and Figure 2A (35)]. In addition, areas of the tumour matrix were surrounded by densely appearing perineurial cells, consisting of one or several layers of flat elongated cells with different long branching processes running between the collagen bundles. Around some peripheral nerve fibres, well-defined perineurium was established. Similar structures with looser appearance were seen to surround different large areas within the tumour. Small bundles and single cell rows originating from the peripherally situated perineurial coverings invaded deeply into the tumour mass and lay between collagen fibre bundles, single nerve fibre profiles and at the periphery of blood vessels and tactile corpuscle-like structures. Within the nerve fibres, a larger number of Schwann cell processes covered by a basal lamina were recognised. Occasionally, single dispersed mast cells were distributed within the matrix components of the tumour (Figure 2B). Within the matrix of the tumour, hyperplastic connective tissue cells with fusiform appearances and many collagen fibres were seen (22). Some of these cells, which we designated as being compartmentalising (synonyms: covering cells, enveloping cells or co-cells) possessed a triangular form and delivered very long, particularly branching processes (Figures 2C and D). Between the collagen bundles, cells of variable shapes and of different thickness (partially hyperplastic) were seen (Figure 2E). Numerous vascular profiles of different size were also observed. Most of the vessels showed an increased thickness of their *intima* and *media/adventitia* (Figure 3A).

In some sections, segments of the surface of the skin with all known cellular (basal cells, keratinocytes, melanocytes) and fibrous structural components as well as cutaneous glands (sebaceous, sweat), were seen.

Immunohistochemistry.
Neuronal and neuroendocrine markers. Immunoreactivity for PGP9.5 was observed in axons of the tumours only. A stronger staining intensity was exhibited by the axons that were located at the periphery of the tumour in comparison to those situated

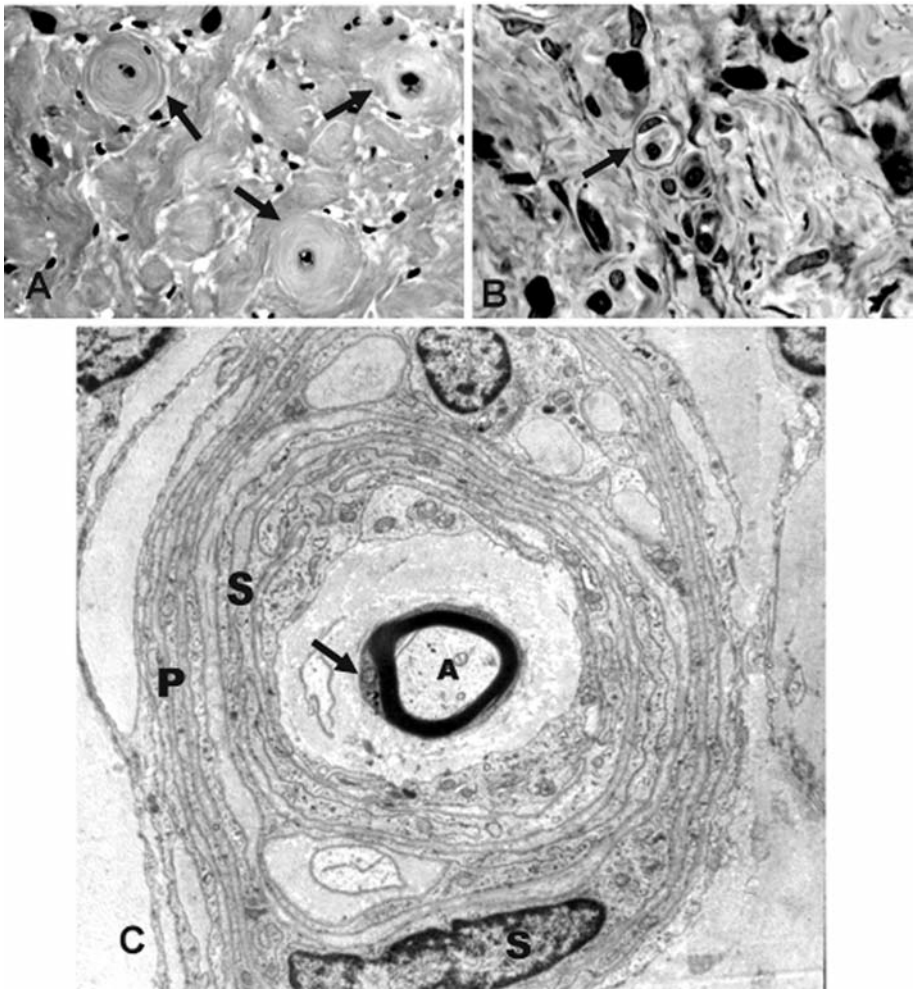

Figure 1. *A: Cutaneous neurofibroma with onion-like (Pacini-like) bodies containing centrally located small blood vessel (arrows), different cell profiles and numerous collagen fibre bundles within the extracellular matrix. Haematoxylin-eosin stain (×630). B: Semithin section of a neurofibroma fragment. The arrow points to an onion-like body with centrally located myelinated nerve fibre. Note the great polymorphism of cell profiles within the tumour (×400). C: Electron micrograph of a lamellar body similar to that seen in B. A, axon with myelin sheath and a myelinating Schwann cell (arrow); A broad zone with collagen of endoneurial type; more peripherally there is a concentric layer of hyperplastic Schwann cell body and numerous processes (S), as well as a layer of concentrically arranged perineurial cell processes (P) (×8000).*

deeply within the tumour. There were also a larger number of immunoreactive axons at the periphery of the blood vessels.

Strong immunoreactivity for NF-200 was found in some slices in a few thick nerve fibres and nerve bundles, as well as in glial-like cells (Figures 3B and C). Within the tumour mass, a lower intensity of immunoreactivity was seen in endothelial cells and smooth muscle cells of the vessel walls, as well as in some compartmentalising cells. NF-200 expression was also shown within the cytoplasm of keratinocytes and in small round cells in the *lamina propria* and epidermis.

Distinct NSE staining intensity was seen in the Schwann cells surrounding myelinated nerve fibre bundles. Endothelial cells and smooth muscle cells of blood vessels were also immunoreactive for NSE. In addition, numerous compartmentalising cells that surrounded differing areas of the tumour mass possessed strong immunoreactivity, most of all in their processes. Basal cells of the epidermis and atypically situated cells of the excretory ducts of sebaceous glands also exhibited moderate immunostaining. Single fusiform cells in the perineurial sheets were also positive. Between the collagen fibres surrounding the nerve fibre bundles, single small cells also displayed immunostaining.

Neurotrophins. We were not able to establish any $p75^{NTR}$, TrkB, TrkC, NT-3, or BNDF immunoreactivity in the material under study.

Immunostaining for TrkA was exhibited by Schwann cells, most of all by their processes surrounding the nerve fibres.

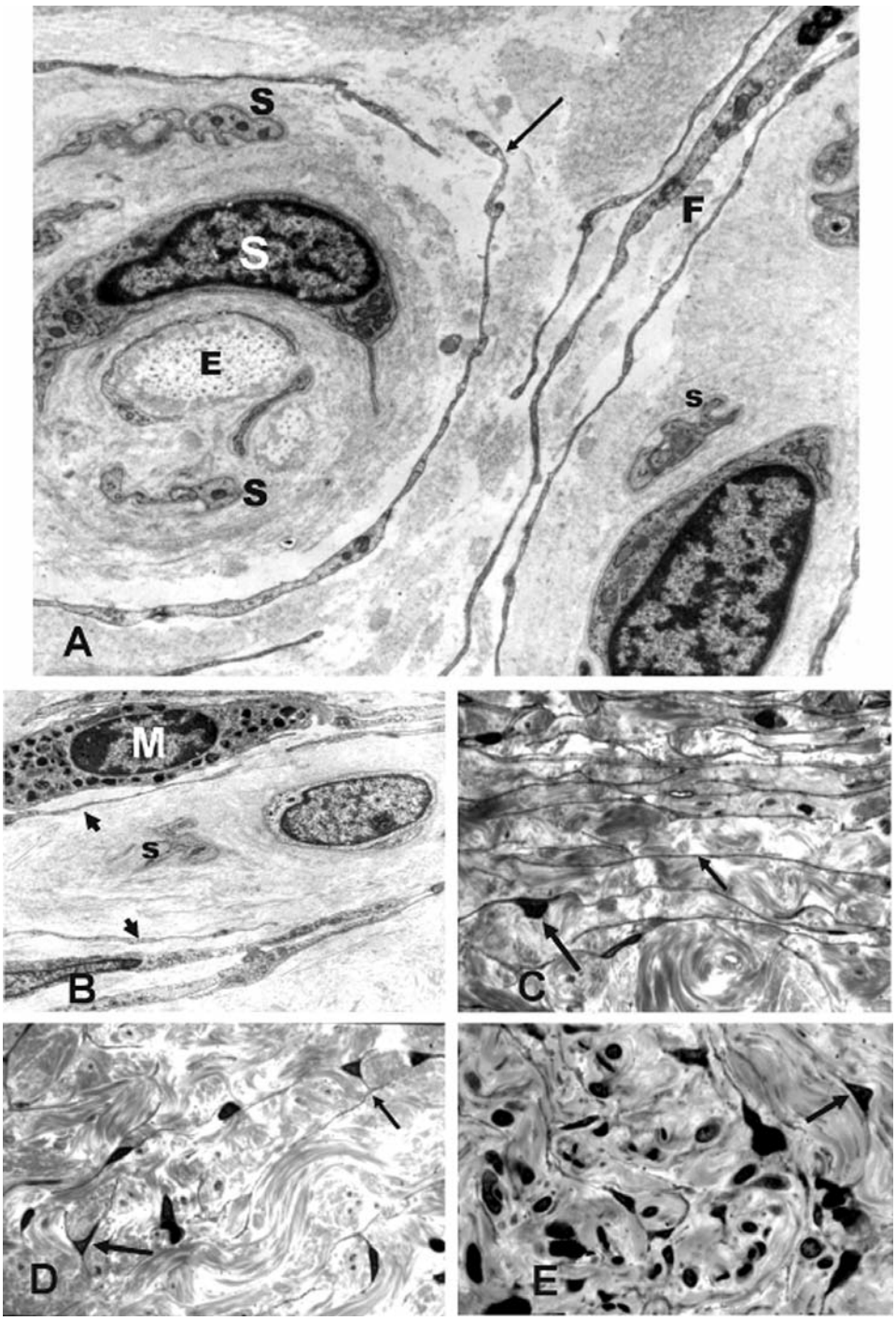

Figure 2. *A: Electron micrograph of a tumour fragment. An elastic bundle (E) is surrounded by a Schwann cell process and within some distance a Schwann cell body (S) can be seen. Dissociated Schwann cell processes (S) and long fibroblastic processes (F) are also situated beneath. The round elastic bundle and Schwann cell processes complex with intermingled collagen fibres surrounded by thin compartmentalising cell processes (arrow) (×10000). B: Electron micrograph of a tumour fragment containing a mast cell with characteristic dense bodies (M) and interstitial matrix with profiles of Schwann cells (S). The large structures are segregated by thin processes of fibroblasts and compartmentalising cells (arrows) (×8000). C-E: Semithin section stained with toluidine blue-pyronin G. C and D: These images show parts of the interstitial matrix containing numerous collagen fibre bundles running in different directions. The bundles are surrounded by parallel running compartmentalising cells (arrows) which posses multiple long, branching processes that communicate with neighbouring compartmentalising cells, thus segregating the matrix in circumscribed areas (×500); E: This image demonstrates the distinct polymorphism of the hypertrophic cellular constituents of the neurofibroma. Triangular cell body of a compartmentalising cell can be seen (arrow) (×500).*

Endothelial cells and smooth muscle cells of small blood vessels exhibited immunoreactivity for TrkA. Moderate staining intensity was also displayed by Schwann cells and compartmentalising cells within the nodules. Immunoreactivity was also seen in the basal epidermal cells, as well as in epithelial cells, of the secretory ducts of the sebaceous glands and of the epithelium of the sweat glands. Numerous cells in the dermis also exhibited strong immunoreactivity.

Immunoreactivity for GDNF was seen within the compartmentalising cells and the Schwann cells as well as the endothelial cells of blood vessels, the smooth muscle cells of the *musculi arrectores pilorum* and the epithelial cells of the excretory ducts of the sebaceous glands.

Immunostaining within the neurofibroma nodules for one of the corresponding GDNF receptors, namely GFRα-1, was distinctly positive in Schwann cells and in single axons. Lower staining intensity was observed in the cells of some perineurial sheets. Distinct immunoreactivity was seen in basal cells of the epidermis and myoepithelial cells of the sebaceous glands.

Glial markers. We were not able to find any immunoreactivity for GFAP in cutaneous neurofibroma by the method used.

S-100 protein was found in a large number of nerve fibres and Schwann cells [Figures 3D (i) and (ii)]. In the nodule itself, the immunoreactivity was of lower intensity and quantity. Numerous small round or oval structures, which were partially in contact with the nerve fibres (endothelial cells and pericytes of small blood vessels and capillaries), exhibited moderate staining intensity. Generally, the S-100 staining was more strongly expressed within the skin dermis and at the periphery of the tumour nodules (Figure 3E).

The strongest immunoreactivity for CNPase within the nodules was displayed by the perineurial cells, Schwann cells, endothelial cells and the innermost sheet of smooth muscle cells of arterioles and small arteries (Figure 3F). Distinct staining intensity was also exhibited by some axons and axon bundles of the peripheral nerves within the tumour mass (Figure 3G). The Schwann cells, some fibroblasts and compartmentalising cells had a lower immunoreactivity. CNPase in the form of a diffuse cytoplasmic staining was observed in endothelial cells, smooth muscle cells and pericytes of blood vessels, as well as in keratinocytes of the epidermis, in dermal connective tissue and in dendritic-like connective tissue cells. Numerous round-like cells also exhibited a positive reaction.

GalC: In some cases, within the matrix of the tumour nodules, Schwann cells and numerous connective tissue cells displayed moderate immunostaining. Together with the axons, GalC immunoreactivity of strong intensity was exhibited by endothelial cells, smooth muscle cells and pericytes of the blood vessels, as well as compartmentalising cells within the endoneurium of peripheral nerve fibres and the ECM (Figure 3H). Some of the perineurial cells also exhibited lower immunostaining. The keratinocytes of the epidermis, as well as round small-sized cells in the dermal connective tissue and some other (fibroblast-like) connective tissue cells were also strongly stained.

Immunoreactivity for MBP was found within the myelin sheaths of both large and tenuous nerve fibres inside the nodules, as well as in a large number of dissociated Schwann cells. However, not all nerve fibres of a bundle were immunoreactive. In addition, the antibody used displayed a strong staining intensity of the epithelial cells of the sebaceous glands. Cells of the sebaceous gland excretory ducts were also stained positive. In addition, smooth muscle cells of arterioles possessed low immunoreactivity.

Immunoreactivity for the glial marker O4 was observed in the Schwann cells and within the myelin sheaths of the nerve fibres. Epithelial cells of the sebaceous glands also exhibited some positive staining. In general, the distribution pattern strongly resembled the localisation of MBP immunoreactivity.

Components of the NO/cGMP system. Constitutive NOS I was stained positively within the myelinating Schwann cells surrounding axons of the nerve fibre bundles. Low immunostaining was observed within the endothelial cells of some small vessels. In the larger nodules, compartmentalising cells also displayed a positive immunoreactivity. On the other hand, the eNOS (NOS III) was positive in a number of axons, in compartmentalising cells and in endothelial cells of small blood vessels. A strong reaction product deposit was observed in the cells of the *arrector pili* muscles. Epithelial cells of the sebaceous glands and their myoepithelial cells exhibited a strong staining intensity, as did smooth muscles of larger arterioles and single axons of peripheral nerves. Only macrophages in circumscribed areas of single nodules exhibited NOS II immunoreactivity.

Connective tissue cell markers. Strong immunoreactivity for collagen type IV was observed at the periphery of the tumours, where numerous lamellae were positively stained. The staining resembled the pattern observed after Glut-1 staining of the perineurium. Immunoreactivity of lower staining intensity was found within structures of the tumour mass. In this location, most immunostaining was observed in the periphery of blood vessel walls. The basal lamina and the myoepithelial cells of the sweat glands also exhibited some staining.

Vimentin immunoreactivity was found within the nodules in numerous connective tissue cells, the endothelial cells of blood vessels, as well as in compartmentalising cells with bifurcating cell processes (Figure 4 A). Numerous connective tissue cells surrounding the sweat and sebaceous glands, as well as numerous dendritic-like cells within the basal keratinocyte layer and the *lamina propria* of the epidermis were strongly stained.

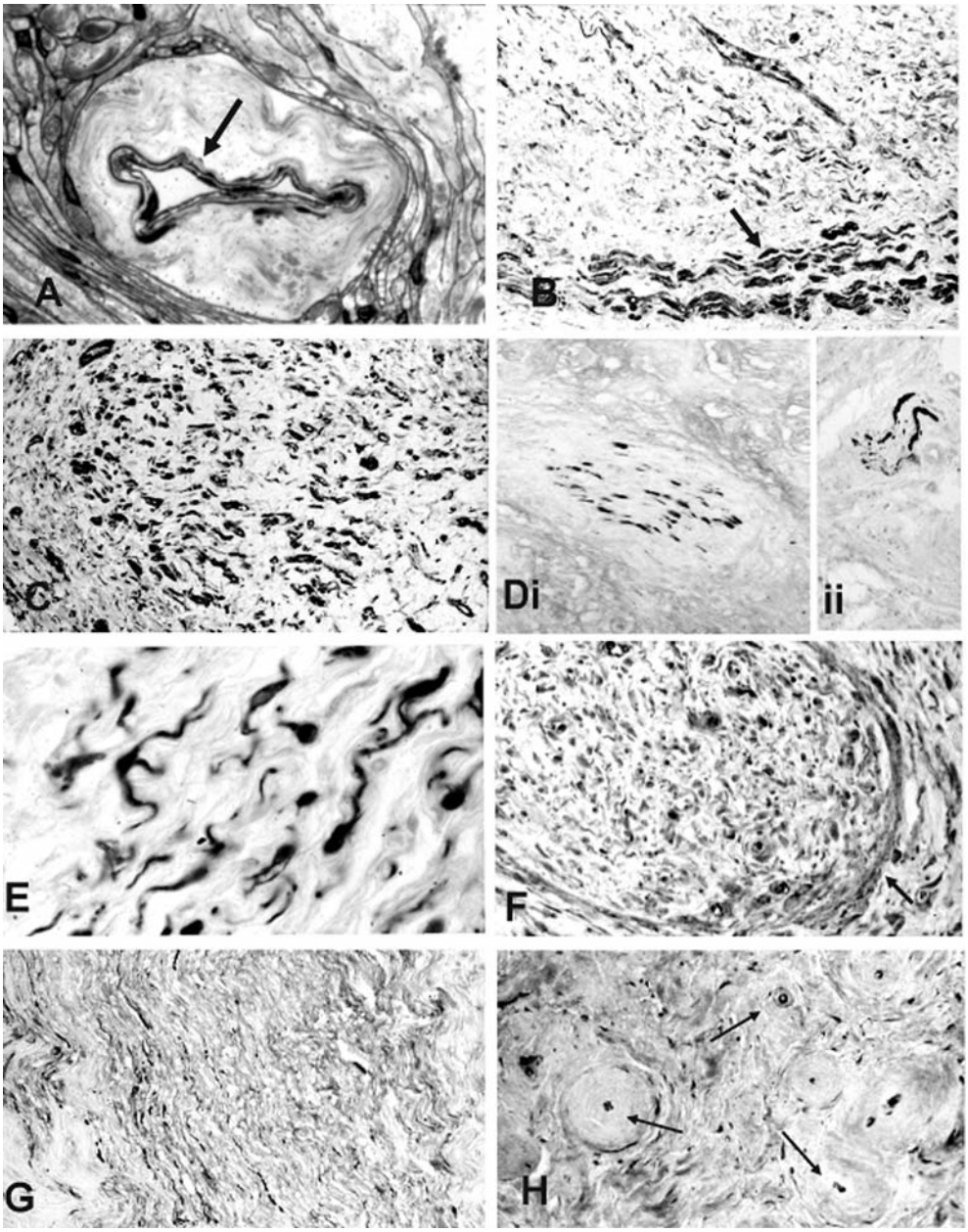

Figure 3. *A: Semithin section of a larger blood vessel with thicker intima (arrow), media and adventitia, surrounded by processes of compartmentalising cells and perineurial cells (×500). B and C: Immunohistochemical visualisation of neurofilament 200 (NF 200). The axons of randomly distributed and bundles of nerve fibres possess strong staining intensity (arrow). Endothelial cells and some Schwann cells show distinct immunoreactions to NF-200 antibody (×630). D and E Immunostaining for S-100 protein in axons of nerves located within the neurofibroma [D (i) and (ii), ×400], as well as in Schwann-like cells and dendritic cells within the skin dermis bordering the tumour (E, ×630). F and G: Immunoreactivity for cyclic 2,3-nucleotide 3'-phosphodiesterase (CNPase) in cross- and longitudinally sectioned peripheral nerves within a cutaneous neurofibroma. Stronger immunoreactivity is seen in some vessel profiles, the axons of the nerve bundle and in some perineurial cells at the periphery of the nerve (arrow) (×500). H: Immunostaining for galactocerebroside (GalC). The strongest immunoreactivity is exhibited by the blood vessel profiles situated in the centre of the onion-like bodies (arrows). Other single positive cells are distributed randomly within the interstitial matrix of the tumour (×400).*

Immunoreactivity for FSP was seen in endothelial cells. Numerous connective tissue cells of the subdermal layer (Figure 4 B) as well as cells of the epidermis were stained. A fine granular reaction product was seen in compartmentalising cells. In some sections mast cells were also stained.

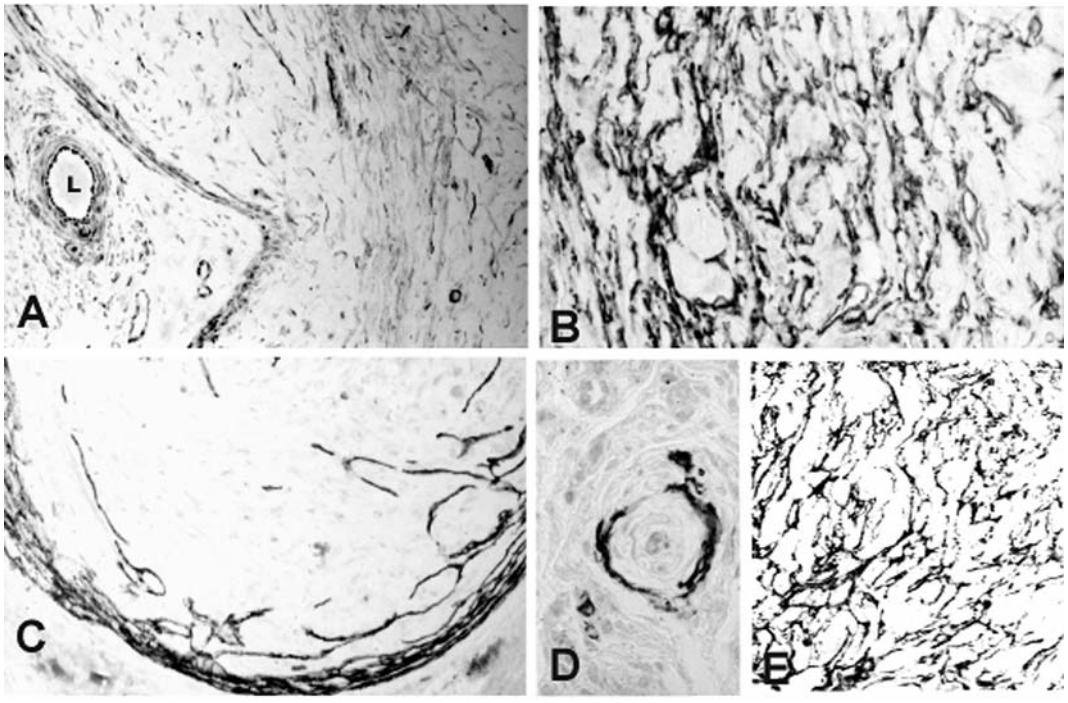

Figure 4. *A: Vimentin immunopositive endothelial cells surrounding a vessel lumen (L), a perineurium profile of a nerve and numerous connective tissue cells (fibroblasts, fibrocytes) of the matrix (×400). B: Visualization of fibroblast surface protein (FSP) immunoreactivity in endothelial cells of the vessels, in connective tissue cells and in compartmentalising cells that surround different large areas of collagen (×500). C-E: Immunoreactivity for glucose transporter 1 (Glut-1) is seen in the perineurium of the nerve bundles (C; ×500) and at the periphery of an onion-like body (D; ×500). Some of the perineurial cells invaginate deeply toward the endoneurium of the nerves (C) and in some areas of the matrix they possess a network-like appearance surrounding the collagen fibre bundles (E; ×400).*

Carbohydrate transmembrane transporters. Immunoreactivity for Glut-1 protein was found mostly in the perineurial sheets of larger nerves (Figure 4 C), some onion-like bodies (Figure 4 D) and, following microwave pre-treatment of the sections, in the perineurial sheets of centrally situated small nerve fibres of the nodules In general, the perineurial sheets that covered the nodule exhibited a strong immunoreactivity. In contrast, the sheets surrounding the nerve fibre bundles had lower staining intensity and were composed of a lower number of lamellae. In single nodules, there was only one lamella at the periphery of a single nerve fibre bundle that exhibited staining intensity comparable with that of the peripheral perineurial sheets. The endoneurium of some axons was separated by deeply invaginated branches of the perineurium. In larger nodules there was distinct staining of processes of the covering cells that, in addition to the perineurial cells, surrounded differing large areas of the tumour matrix (around the collagen bundles) (Figure 4 E). Generally the immunoreactivity of these cells was of lower intensity compared to the cells of the perineurial sheets. Interestingly, the myoepithelial cells of sebaceous glands were also strongly Glut-1-positive.

Nestin. Immunoreactivity for nestin (originally a marker for neural stem cells; now detected within most organs of developing and adult vertebrates in activated and proliferating stem/progenitor cells) was detected exclusively in the microvasculature (small arterioles, arterial and venous capillaries, and venules) of all neurofibromas under study. The reaction product was observed preferentially within the pericytes and smooth muscle cells in a wide capillary network of the tumours (Figure 5 A), as well as in arterioles and venules within the tumour matrix (Figures 5 B-D). In some vessels, positive pericytes probably in the process of detachment and migration from the vascular wall, could be seen (Figure 5 C). The migrating pericyte exhibits a lower staining intensity, probably reflecting initial commitment and further differentiation of this stem/progenitor cell. Occasionally, lamellate structures resembling the Vater-Pacini corpuscles (onion-like bodies) were recognized, which possessed a centrally situated nestin-positive microvessel, as well as concentrically running capillaries at its periphery (Figure 5 E). Within cross- or longitudinally sectioned profiles of small peripheral nerves, a number of nestin-positive microvessels which ran parallel to the nerve fibres were also recognized (Figure 5 F). Sporadically, nestin-positive

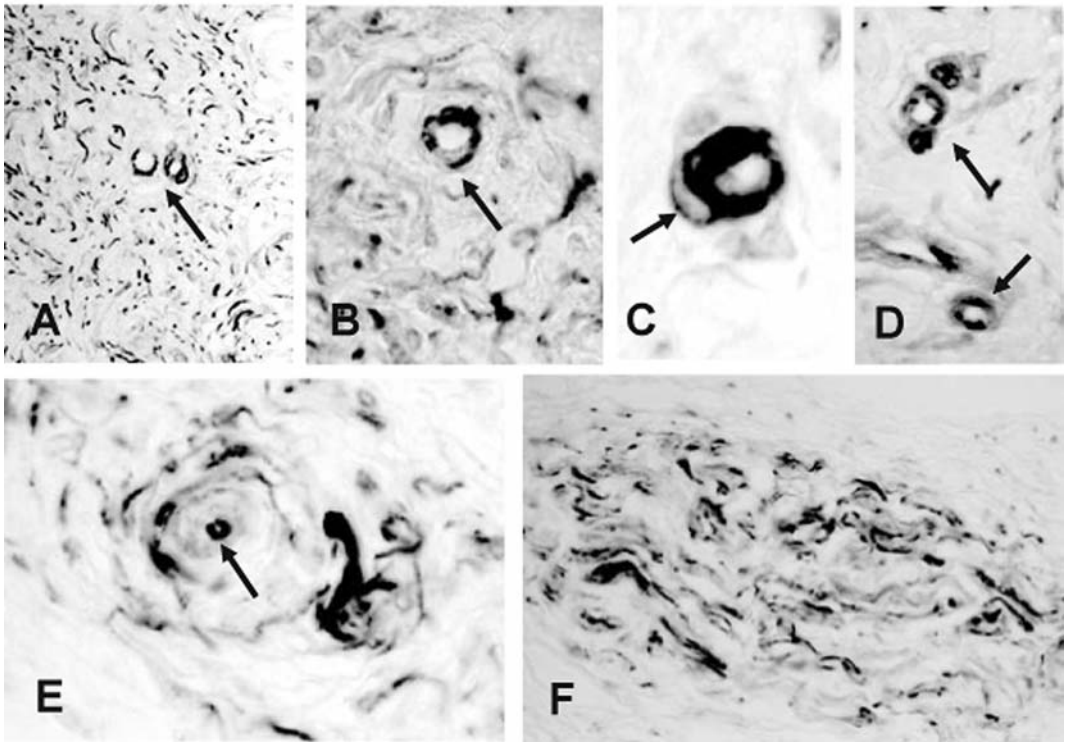

Figure 5. *Immunohistochemical visualisation of nestin in paraffin sections of neurofibromas. A: Low magnification (×250) of a portion of a neurofibroma with a densely developed microvasculature network. The small positively stained structures represent capillary profiles. The arrow points to an arteriole/venule with nestin-positive pericytes. B-D: High magnification view of positively stained pericytes and smooth muscle cells (arrows) in the wall of blood vessels within the tumour mass. In C, the arrow points toward a pericyte that is probably in the process of detachment and migration towards the tumour matrix. Note the lower staining intensity. E: Structure resembling a lamellar (Vater-Pacini, onion-like) body with centrally situated nestin-positive vessel and concentrically running capillaries at its periphery. F: One obliquely-section of a small peripheral nerve, with numerous nestin-positive vessels running parallel to the nerve fibres. B, D and F, ×500; C and E, ×600.*

endothelial cells but with significantly lower staining intensity were visualized.

Discussion

This study shows that the expression profile of neurotrophins and their receptors in cutaneous neurofibromas differs inside the tumours and even between cells of the same type (12, 13, 36). The neurofibroma consists of a mixture of cells, predominantly being composed of hyperplastic Schwann cells, perineurial cells, compartmentalising cells and fibroblasts, the population of each cell type differing according to the individual case, differentiation and functional status and topography within a given tumour. Immunostaining without counterstaining allowed the application of highly diluted primary antibodies and produced a distinctive reaction pattern. The cells of origin of neurofibromas are still a matter of debate. At present, there is some evidence that the primary neoplastic element in neurofibromas is derived from Schwann cells or their precursors (3-5).

In the discussion on the origin of Schwann cells (or Schwann cell-like tumour cells) the focus lies on derivation from the neural crest vs. the capacity of blood vessel cells (pericytes and smooth muscle cells) to build progenitor cells, and the eventual mast cell involvement. Parrinello and Lloyd presume de-differentiated Schwann cells to be the cells of origin giving rise to neurofibromas (15) because no original Schwann cell progenitors were found in differentiated NF1 tumours (37-39). However, newly published results provide evidence for the existence of neurofibroma-derived precursor cells (40). These cells were revealed in human cutaneous neurofibromas to express multipotency-associated biomarkers. After isolation and maintenance in cell cultures, it was established that these tumour cells express nestin and have the potential to differentiate into Schwann cells, neurons, epithelial cells and adipocytes. Thus, Jouhilahti *et al*. (40) concluded that these cells may be derived from multipotent cells of the hair roots (40). However, this theory does not explain the development of neurofibroma in the hairless skin. In conclusion, multipotent $NF1^{+/-}$ precursor cells can give rise

to some of the different cell types existing in neurofibromas. The first part of the discussion will focus on single findings in the context of the literature followed by a second part presenting a hypothesis concerning the stem cell concept of tumourigenesis of neurofibromas in NF1 based on the above detailed findings.

Neuronal and neuroendocrine markers. Immunostaining for PGP9.5 was confined to the axon in this study. PGP9.5 is an ubiquitin hydrolase abundantly expressed in the nervous system. In normal sciatic nerves of rat myelinated and non-myelinated axons, PGP9.5 was expressed, but virtually none of the Schwann cells were stained. However, after nerve degeneration following transection, the denervated Schwann cells showed intense staining for PGP9.5 (41), indicating the ability of Schwann cells to synthesize this protein following adequate stimuli. Schwann cell immunoreactivity for PGP9.5 is documented in nerve sheath tumours of NF1, in particular in neurofibroma (42) and MPNST (43). However, PGP9.5 is not a specific marker of neural and nerve sheath tumours (44). Recently, SOX10 was established as providing more specific results (45).

The expression pattern of NSE and NF200 concerning Schwann cells is in accordance with results on normal peripheral nerves described in earlier studies (46-48).

Neurotrophins. In this study the expression of neurotrophins and their receptors varied considerably in cutaneous neurofibromas. Neurotrophins and their receptors are expressed in normal skin, as shown by Bronzetti *et al*. (49). These authors found $p75^{NTR}$ and TrkA-like immunoreactivities in the epidermis (basal keratinocytes), sweat glands, Schwann cells and perineurial cells, among others (49). On the other hand, it was reported in another study that normal Schwann cells lack a functional Trk receptor (50) but express $p75^{NTR}$ (low-affinity NGF-receptor) before axonal contact is completed and before myelination commences (51, 52). The expression of $p75^{NTR}$ progressively decreases throughout the maturation of Schwann cells, probably due to a negative feedback mechanism following axonal contact (53).

Bonetti *et al*. (53) revealed $p75^{NTR}$ in sporadic Schwannomas and referred to the report of Pintar *et al*. (54) that neurofibromas of von Recklinghausen's disease also express $p75^{NTR}$. $p75^{NTR}$ expression was as high as 93% in NF1-related neurofibromas (55). We were not able to confirm this finding. However, the expression of NGF and receptors in Schwann cells of neurofibromas in NF1 probably varies in different growth states (54).

The neurotrophin BDNF is an endogenous regulator of the myelinating process during the development of the peripheral nerves. The enhancement of myelin formation by BDNF is a long-term lasting effect mediated by $p75^{NTR}$ (56, 57) and by the main receptor TrkB (58). In this study, neither BDNF and its receptors (TrkB, $p75^{NTR}$) nor NT-3 and its major receptor TrkC were detectable. The lack of factor and receptor expressions in cutaneous neurofibromas fits into this concept of impaired myelination capacity in neurofibroma Schwann cells and the capacity of these nerve sheath cells to thrive in the absence of growth factor receptors relevant for the maintenance of cell metabolism. However, EGFR up-regulation was reported in *Nf1* mutant Schwann cells in transgenic mice (59). TrkA, the receptor for NGF, was identified in Schwann cells of cutaneous neurofibromas. Local release of NGF is relevant for the outgrowth of neurites in normal Schwann cells and Schwann-like cells of neurofibroma in NF1 (12, 36). ^{125}I-NGF binds to subsets of Schwann-like cells of cultured neurofibromas (36). In normal Schwann cells, steady-state mRNA levels were found for NGF, BDNF and LNGFR ($p75^{NTR}$), but not for NT-3, TrkA, TrkB and TrkC (37). NGF supports the maintenance of the differentiated phenotype of cultured Schwann cells but does not stimulate Schwann cell proliferation (38).

NF1 encodes for a ras-GTPase-activating (GAP) protein (neurofibromin) for the NGF-receptor TrkA. Neurofibromin appears to negatively modulate TrkA signalling through p21ras during neuronal development (60). TrkA expression was noted in perineurial cells, Schwann cells, endoneurial fibroblasts and large axons of the nerve trunks supplying the digital skin (61). Thus, TrkA expression in NF1 appears to simulate the Schwann cell phenotype. However, this finding was not uniformly confirmed (50). *NF1* mutations promote survival after NGF withdrawal (62). In this study, we found TrkA expression in Schwann cells and compartmentalising cells cells. TrkA expression is constitutive in peripheral nerves, even under different pathologies, but shows sensitivity to transection (63). The expression of TrkA appears to be variable in experimental settings of neurofibroma-associated tumours (64) and shows dependency on $p75^{NTR}$ recycling in normal Schwann cells (65).

Neurotrophins play a key role in the myelination process (66). $p75^{NTR}$ is a positive modulator of myelination (67). $p75^{NTR}$ was not expressed in cutaneous neurofibroma of NF1 patients in this study. This membrane-bound receptor is capable of binding to all neurotrophins [NGF, BDNF, and NT-3 to NT-5 (68)], but with low-affinity. Binding of neurotrophins to $p75^{NTR}$ induces apoptosis. This pathway is dependent on p53 (69). Obviously, a transcription factor is necessary for the expression of this receptor in Schwann cells following adequate stimuli [*e.g.* nerve injury (70)]. Myelin formation is inhibited in the absence of functional $p75^{NTR}$ and enhanced by blocking TrkC activity (67). Furthermore, BDNF enhances Schwann cell myelination *via* $p75^{NTR}$ (67). Therefore, $p75^{NTR}$ expression is crucial in both survival and death of Schwann cells (71). It is likely that absence of $p75^{NTR}$ in neurofibromas impairs apoptosis of tumorous cells. However, our findings are in contrast to

previous reports on immunohistochemical evidence for $p75^{NTR}$ in human neurofibroma of NF1 patients and the potentiation of growth of neurofibroma *in vitro* by application of exogenous NGF (13). $p75^{NTR}$ expression was also found in other benign nerve sheath tumours [*e.g.* sporadic schwannomas of the colon and rectum (73)]. On the other hand, $p75^{NTR}$ receptor expression might be impaired by further proteins (74). Concerning TrkA, our results are generally in agreement with the findings of Vega *et al*. (61) for the immunoreactivity of the nervous and non nervous tissue structures. Our results concerning the immunoreactivity of neurotrophins and their receptors in the cellular constituents of the skin (epidermal keratinocytes, nerve bundles, sweat glands and sensory corpuscles) are at odds with previous findings by Montaño *et al*. (75).

GDNF and GFR-α1 are involved in neurotrophic activities and the immune system (76). According to Iwase *et al*. (77), GDNF exerts its biological effects by activating its receptor GFR-α1 and the neural cell adhesion molecule (NCAM), which all are expressed by neonatal Schwann cells. GDNF is important for the migration of Schwann cells and enhancement of the early stages of myelin formation. Both GDNF and GFR-α1 were expressed in Schwann cells of neurofibromas [and in control Schwann cells and skeletal muscles (78)]. GDNF was discussed as a candidate modifier in NF1 phenotype (79). GFR-α1 is highly expressed in adrenergic mouse pheochromocytoma cells from heterozygous *Nf1* knockout mice and these cells respond to GDNF (64). Our findings support a role for GDNF and its receptor in the regulation of neurofibroma growth. However, further studies are needed to identify possible roles of these factors in the progression of the disease.

Glial markers. GalC: In this study, only few Schwann cells inside the neurofibroma nodules were immunoreactive for GalC. In an earlier *in vitro* study, Schwann-like cells from subcutaneous and plexiform neurofibroma were co-cultured with foetal rat dorsal root ganglion neurons in order to determine whether these tumorous cells are competent at differentiating in the presence of axons. In this experimental setting, after three weeks of maintaining Schwann-like cells (SLC) in culture with axons, some spindle-shaped cells expressed GalC. These authors concluded that their results demonstrate that axons induce SLC to down-regulate surface NGFR ($p75^{NTR}$) and to express some myelin components in a qualitatively normal fashion (80).

S-100: Immunoreactivity for S-100 in peripheral nerves is restricted to neurons (61) and normal or neoplastic Schwann cells (61, 81, 82). S-100 immunoreactivity was confined in this study to nerve sheath cells, both in the tumour and additionally in adjacent structures (*e.g.* glands). S-100β labels Schwann-like cells in neurofibroma, whereas fibroblasts and perineurial-like cells appear to be negative (5).

GFAP: Expression of GFAP appears at a relatively late stage of Schwann cell development. GFAP is detectable when immature Schwann cells are formed (83). GFAP is down-regulated in those Schwann cells that form myelin (84). GFAP was not detected in cutaneous neurofibroma in this study. The GFAP detection rate in neurofibroma appears to vary considerably [0 (85), 11% (86), and 40% (87)]. On the other hand, GFAP expression in NF1 might differ in central and peripheral nerve sheath cells (88). Immunocytochemistry on sections of cortex and cerebellum of unaffected and NF1 individuals showed that GFAP was up-regulated in brains of three NF1 patients. GFAP content/cell and the number of GFAP-immunoreactive astrocytes were increased in brains of NF1 patients as compared to the controls (88). Individuals affected with NF1 harbour increased numbers of GFAP-immunoreactive cerebral astrocytes (89). GFAP binds integrin v8, which initiates mitotic signals soon after damage by interacting with fibrin. Consistently, mitogen-activated protein kinase phosphorylation was reduced in crushed *GFAP*-null nerves (83).

MBP, O4: O4 is a marker of myelinating cells and is used to identify possible precursors of Schwann cells in culture (72). This study demonstrates the expression of proteins expressed in myelinating Schwann cells (O4, Myelin, and MBP) at least in some areas of the tumour. Overexpression of transcripts associated with neuroglial differentiation, including MBP, was found in NF1-associated MPNST (90). Aberrant growth and differentiation of oligodendrocyte progenitors was found in an experimental setting of neurofibromatosis type 1 mutants, where glial cell marker O4 was co-expressed with the progenitor marker nestin in $Nf1^{-/-}$ mice. Furthermore, mutant progenitors also abnormally expressed the glial differentiation markers O4 and GFAP in this study (72). Cutaneous neurofibromas in NF1 express O4.

CNPase: CNPase was predominantly found in epidermal keratinocytes, in the connective tissue, in endothelial and smooth muscle cells of blood vessels. Endothelial cells and the innermost sheet of smooth muscle cells of arterioles and small arteries showed the strongest staining intensity within the tumour. Schwann cells and the connective tissue cells showed a lower immunoreactivity for CNPase. CNPase is an important enzyme that visualises myelinating cells. CNPase activity is far higher in myelin from the central nervous system (CNS) than from the peripheral nervous system (PNS). The function of CNPase in myelinating cells is unclear. It was suggested that CNPase might function to some extent as an adhesion factor to make myelin compact (91). CNPase immunoreactivity in endothelial and smooth muscle cells of vessels is an endogenous control for antibody immunoreactivity (92).

Components of the NO/CGM system. The role of NOS in peripheral nerves is still unclear. NOS I, II and III were investigated in the facial nerves of guinea pigs. Both

constitutive NOS isoforms and soluble guanylyl cyclase (sGC) were detected in bipolar cells of geniculate ganglion of normal animals, but NOS II was not detected in normal peripheral nerves (93). NOS III was detected in blood vessels and predominantly localised in the perineurial sheath and less in the endoneurium (93). Experimental toxic damage of the nerve revealed neo-expression of NOS II in the perikarya of the geniculate ganglion and in the perineurial sheath. It was concluded that NOS II expression might be a contributing factor to facial nerve palsy *via* NO-mediated overstimulation of nerve fibres (viscero-afferent and motor) and dysregulation of facial nerve blood vessels (93).

NOS subtypes exhibited different spatial patterns of immunoreactivity in cutaneous neurofibroma. NOS I expression was detected in myelinated and non-myelinated nerve fibres and in myelinating Schwann cells (94). The expression pattern of NOS I in neurofibroma was similar to that found in normal peripheral nerves. Identification of NOS II in neurofibroma was apparently not investigated up to now. NOS-II was not expressed in untreated, healthy peripheral nerves and resident T-cells in a nerve compression model (median nerve) of dogs. Macrophages, but not Schwann cells, exhibited NOS II (iNOS) expression after nerve compression (95). NOS II was also identified in macrophages of schwannomas (96). In the present study, NOS II expression was restricted to macrophages in the perineurium. Recently, a cellular neurofibromin-associating protein, *N*(G),*N*(G)-dimethylarginine dimethylaminohydrolase (DDAH), a known cellular NO/NOS regulator, was identified. It was speculated that DDAH modulation might interfere with cellular function of neurofibromin implicated in NF1 pathogenesis (97). In addition, there is evidence that the synthesis of NGF and NT-3 by Schwann cells mediated by the NO-sGC-cGMP pathway prevents neuronal apoptosis (98).

Connective tissue cell markers. Schwann cell myelination is induced by contact with components of the basement membrane (99), consisting of collagens, laminins and other molecules. Collagen IV is synthesised by Schwann cells from neurofibromas (100). Immunoreactivity for collagen IV in Schwann cells of neurofibroma declined from the periphery to the centre of the nodule. This difference probably indicates a progressive loss of synthesizing capacity of cells located in the central parts of the tumour, possibly due to reduced oxygenation of cells or impaired perfusion and drainage.

The expression pattern of vimentin and FSP concerning Schwann cells is in accordance with results on normal peripheral nerves described in earlier studies (46-48). Triolo *et al*. (83) established that vimentin binds integrin α5β1, which regulates proliferation and differentiation later in regeneration of peripheral nerves, and may compensate for the absence of GFAP in mutant mice.

Glut-1. Glut-1 has a temporo-spatial expression pattern in the human peripheral nerves (101). Expression of Glut-1 is variable in oral neurofibromas with spatial differentiation (102). Hirose *et al*. (103) found only a small number of Glut-1-positive cells in neurofibromas. These cells were also epithelial-membrane antigen (EMA)-positive and arranged in perineurium-like structures. The alterations of Glut-1 expression may represent up-regulation of the protein within hypoxic zones inside mesenchymal tumours, irrespective of type. Indeed, Glut-1 is obviously not specific for perineurial differentiation (104). Immunoreactivity of integumental glands to Glut-1 in neurofibromas was also noted by others (104), and revealed for sweat glands adjacent to cutaneous neurofibroma in this study.

There are various presumptions on the origin of the tumour cells in neurofibromas.

As seen, a cutaneous neurofibroma contains mainly three cell types, namely Schwann cells, perineurial cells and fibroblasts. In addition, compartmentalising cells and mast cells, as well as endothelial and smooth muscle cells/pericytes of the vasculature, also need to be taken into consideration (see below) (5). The possibility exists that most of these cell types originate from a common adult pluripotent stem cell. As revealed, such cells were found in peripheral nerves and isolated in culture, where they were able to differentiate into cell types of all three embryonic cell layers originating from the epiblast (namely the ectoderm, the intraembryonic mesoderm and the endoderm) and the hypoblast [which forms the extraembryonic mesoderm and the yolk sack, allantois and amnion: (105)]. As Jessen and Mirsky (7) mentioned, early in embryonic development, peripheral nerves consist of compact axons columns and tightly associated Schwann cell precursors arising from the neural crest (9, 106). These early nerves do not possess connective tissue, protective covering, nor their own blood vessels. These components arise later, between the 14th-15th embryonic day and the third postnatal week, when the embryonic nerves reach their targets (24). It also becomes evident that the structures building a peripheral nerve originate from cells of the neural crest or more primitive stem cells that are able to differentiate to both neural crest cell precursors and adult stem cells with multi-lineage progenitor qualities (9, 105, 107-109). Moreover, Morrison *et al*. revealed the existence in peripheral nerves during late gestation of neural crest stem cells (110), which in adult humans may contribute to the origin of tumours in the peripheral nervous system. Another possibility is also discussed, namely that in response to injury, myelinating Schwann cells de-differentiate and can re-enter the cell cycle and build Schwann progenitor cells and immature Schwann cells (7, 11). It seems that in this process, the

microenvironment plays a special role that changes from pro-differentiative to pro-carcinogenic (15). In addition, the loss of axonal contacts of the Schwann cells appears to be an important factor in the process of tumourigenesis (15).

The closest *in vivo* equivalent of an embryonic stem cell is an early germ cell (105, 112, 113). These stem/progenitor cells are disseminated within the whole organism and are recognised as adult mesenchymal stem cells (114-117), multipotent adult progenitor cells (118-122), unrestricted somatic stem cells (123), marrow-derived adult multi-lineage inducible cells (124), monocyte-derived multipotential cells (125, 126), as well as peripheral blood multipotential mesenchymal progenitors capable of regenerating epidermis (127) and non-haematopoietic tissue committed stem cells or very small embryonic/epiblast-like cells (5, 113, 128-131). It is important to note that in most cases, it is difficult to determine the exact structural features of the cell type surrounding the nerve because of the existence of transitional cell forms between Schwann cells, endoneurial fibroblasts and perineurial cells and the detachment of the Schwann cells from the nerve (35, 132). Parrinello and Lloyd (15) suggest that different Schwann cells are the cells of origin of neurofibromas. In this respect, a typical Schwann cell phenotype with extremely long and multiple cytoplasmic processes were described by Rosenbaum *et al*. (133). Previously, similar phenotypes were encountered for neurofibroma-derived fibroblasts (134) and for Schwann cells derived from neurofibromin-deficient mice (135). Moreover, Joseph *et al*. (108) emphasised that neural crest stem cells and Schwann cells are able to differentiate towards fibroblasts and *vice versa*, and mesenchymal stem cells are able to transdifferentiate toward a Schwann cell lineage under the induction of dorsal root ganglia (136). It is well known that the growth behaviour of neurofibroma-derived Schwann cells by itself is insufficient to explain tumourigenesis in NF1. One reason for that may be the loss of GFAP which causes defective proliferation of Schwann cells and delayed nerve regeneration after damage (83). Additional genetic and environmental events seem to be necessary to induce Schwann cells in neurofibromas to hyperproliferate. In this respect, Kim *et al*. showed that an elevation of intracellular cAMP leads to enhanced proliferation of neurofibromin-deficient Schwann cells (135). The surrounding somatic tissue, and most of all the mast cells, seems to be important for the initiation and progression of neurofibromas in NF1 (5, 137).

Neurofibromas are highly vascularised. The structural components of the neurofibromas are probably produced by proliferating vascular stem/progenitor cells, such as smooth muscle cells and pericytes.

As mentioned above, there is a clear discrepancy between the large number of Schwann cells and their decreased proliferation rates in NF1. This discrepancy might be explained by the possibility that it is not the Schwann cells themselves, but rather their cells of origin, namely the vascular wall stem cells, which proliferate and differentiate very fast towards initially committed SCP or Schwann cells in varying stages of differentiation. This may be also true for the fibroblasts and the perineurial cells as well as for some other cell types. There is also evidence that angiogenesis plays a major role in the transformation of plexiform neurofibroma into MPNST in children with NF1 (138).

Neurofibroma-derived, but not normal, Schwann cells are invasive and promote angiogenesis (133, 139). As our findings show, neurofibromas represent massively vascularized tumours (140, 142). The induction of the tumour vasculature, the 'angiogenic switch', is needed for the tumour propagation and progression (144, 145). Mesenchymal stem cells frequently correlate with blood vessel density (115). In analogy with the results for the regeneration of the Leydig cells in the testis (21, 145), the presumption arises that structural components of the tumour microvasculature can play an essential role in the origin and functional properties of the cells within neurofibroma formations. In this respect, the pericytes and the smooth muscle cells of the tumour microvasculature may be the leading stem cell types which are responsible for the proliferation and differentiation of the Schwann cells, perineurial cells, fibroblasts and cells of the vasculature participating in the processes of vasculogenesis and angiogenesis in neurofibromas [morphofunctions and stem cell characteristics of pericytes are reviewed in detail elsewhere (145-147)]. This idea is supported by results in the literature which show that smooth muscle cells of the vasculature, rather than Schwann cells, are the proliferating cells in NF1 vasculopathy (141). Ozerdem found that angiogenesis is accelerated in NF1 due to hyperproliferation of pericytes and endothelial cells (148; see also (149) for developing ovine *corpus luteum*). Moreover, as Xu *et al*. (150) revealed, NF1 regulation of RAS plays a critical role in vascular smooth muscle proliferation after injury. VEGF also seems to mediate the vasculogenesis. This view is supported by the results that VEGF and VEGFR-immunoreactivity exists within the pericytes and endothelial cells of vessels in the early *corpus luteum* (149). At the same time NO may increase VEGF expression by vascular smooth muscle cells and pericytes, thus contributing to enhanced angiogenesis (145). Recent findings reveal that for the proliferation activity of vascular cells in $Nf1^{+/-}$ mice, in addition to the MAPK activation, an imatinib (Cleevec™)-sensitive molecular pathway is involved (152). It is interesting to note that neurogenesis and angiogenesis are closely associated processes that appear in the same spatiotemporal frame (152-154). New findings by Jouhilahti *et al*. describe the presence in neurofibromas of multipotent neurofibroma-derived precursor cells (NFPs) and discuss the possibility that these cells, which express nestin, can differentiate into Schwann cells, neurons, epithelial cells, and

adipocytes (155, 156). Furthermore, these authors presume that these stem cells may be derived from the multipotent cells of the hair roots, which are closely associated with microscopic neurofibromas. However, as seen in the images of the work of Jouhilahti *et al*. (155, 156), the hair roots are also closely associated with small blood vessels which may be the real source of the multipotent precursor cells.

In our opinion, the pericytes in all organs represent a population of cells that resemble primitive stem cells (21, 145, 146, 157-159). Pericytes are capable of giving rise to cell types of the three main embryonic layers, namely the ectoderm, mesoderm and endoderm, that are produced by the epiblast of the blastocyst (115, 160, 161). Recent results reveal that pericytes (bone marrow stromal stem cells, rat adipose tissue-derived stem cells etc.) may differentiate towards Schwann cells (162), neural stem cells and neurons (163-168), different mesenchymal stem and daughter committed cells (169). It is evident that some of the mesenchymal stem cells [descendants from the pericytes (170)] are apparently multidifferentiated in addition to being multipotent (167) and represent neural crest stem cells, their descendant boundary cap cells and progenitor cell derivatives (171-176). As we, and others, presumed, the pericytes represent a population of dormant stem cells situated within a vascular stem cell niche (145). Under different physiological and pathological situations, the pericytes can be activated to proliferate and migrate out from the niche and differentiate toward different cell types depending on the environmental (epigenetic) influences. Thus, pericytes as stem cells can give rise to a great number of variable progenitor cells and cells in variable stages of differentiation, providing the entire variability of cells within the perivascular space that migrate toward the interstitial space and the parenchyma of different organs (6, 116, 177-179). Pericytes of the CNS also demonstrate macrophage morphology and functional properties (180, 181). Bone-marrow mesenchymal stromal cells grafted in solid cancer express pericyte markers and show perivascular location that indicates these cells act as pericytes within tumours (170, 182). Adipose tissue derived stem cells may differentiate to Schwann cell-like cells (183, 184) and, interesting enough, adipocytes arise from progenitor cells (mural cells, pericytes) of the adipose vasculature (183, 185). The pericytes have the most potent adipogenic potential (186). Moreover, the combination of endothelial progenitor cells with adipose stem cells in ischemic tissues shows a distinct higher neovascularization capacity in comparison to a single stem cell application (187).

Concerning neurofibroma, a typical structure that we found was the numerous Pacinian corpuscle-like or onion bulb-like lamellate structures (see Results). Most of these structures contain centrally situated capillaries and arterioles from which pericytes and smooth muscle cells and a large variety of other cell types may arise. Both the pericytes and the smooth muscle cells of the microvasculature may be descendants of the neural crest. Moreover, it became evident that neural crest stem cells can generate endoneurial fibroblasts in addition to Schwann cells (108). As mentioned above, Jouhilahti *et al*. were able to isolate precursor cells from dissociated human cutaneous neurofibromas which expressed nestin and were able to differentiate to Schwann cells, neurons, epithelial cells, and adipocytes (155, 156).

In addition, it was recently established that normal and cancer stem-like cells can arise *de novo* from more differentiated cell types (188). The transient induction of the four reprogramming factors octamer binding transcription factor 4, SOX2, Kruppel-like factor 4, and c-MYC) can efficiently transdifferentiate fibroblasts into functional neural stem/progenitor cells (189). New findings reveal that in adult species, a small population of tissue-resident vascular precursor cells that are able to undergo vasculogenesis exists (*de novo* vessel formation), although in adults, most vessels are formed by angiogenesis. It is interesting to note that these cells express the Schwann cell protein zero (190).

Conclusion

This combined study on the cutaneous neurofibroma in humans confirms the great variability of the structural components of the tumours and emphasizes the variable immunohistochemical images which reflect the differentiation stage of the cells, as well as the narrow relationship between the origin of the tumour cells and the tumour vasculature. In accordance with the established stem/progenitor properties of the pericytes and the vascular smooth muscle cells of the microvasculature, we propose a vascular origin of the different structural components of the neurofibromas. The multipotent qualities of the pericytes explain their capacity to develop a broad progeny towards structures arising from the three embryonic layers, namely ectoderm, mesoderm and endoderm. The reason for the difficulties in the recognition of the origin of the variable cell phenotypes of the tumours provide the very fast process of transdifferentiation of the stem cells (pericytes and smooth muscle cells) which leads to the arrest of most genes characteristic for the stem cells and the sequential activation of genes regulating the commitment and differentiation of the daughter cells through intermediate forms toward the final phenotypes of the neurofibroma. To our opinion, this fact explains the recognition and definition of large numbers of perivascular mesenchymal stem/progenitor cells, which in fact represent the progeny of the vascular wall stem cells differentiating under variable epigenetically/environmental conditions.

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