Enhanced Expression of NGF Receptors in Multiple Sclerosis Lesions

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Abstract. The receptor for nerve growth factor (NGF) comprises a 75-kDa (NGFR\textsuperscript{p75}) and a tyrosine kinase A (TrkA) subunit. In view of conflicting opinions on the identity of glial targets of NGF in human central nervous system (CNS), we examined the cellular distribution of both NGF receptor subunits in normal CNS and in chronic multiple sclerosis (MS) lesions. For this, we compared the pattern of recognition of 2 monoclonal antibodies (mAbs) and a polyclonal antiserum to NGFR\textsuperscript{p75}. Only the 2 mAbs specifically recognized NGFR\textsuperscript{p75}, while the polyclonal antiserum showed widespread reactivity. In normal CNS and silent MS lesions, immunohistochemistry with anti-NGFR\textsuperscript{p75} mAbs and for TrkA revealed perivascular cell reactivity. At the edge of chronic active MS lesions, selective NGFR\textsuperscript{p75} staining was prominent on reactive astrocytes, while throughout the lesion, NGFR\textsuperscript{p75} was expressed on microglia/macrophages. The vast majority of mature or precursor oligodendrocytes did not express NGFR\textsuperscript{p75}. Both NGF receptors were co-expressed on a subset of inflammatory cells. Immunoreactivity for NGFR\textsuperscript{p75} on glial and immune cells did not correlate with the distribution of apoptotic figures, as detected by TUNEL. Thus, expression of NGF receptors in active MS lesions suggests a role for NGF in regulating the autoimmune response at both immune and glial cell levels.

Key Words: Apoptosis; Astrocytes; Microglia; Multiple sclerosis; NGF receptors; Oligodendrocytes.

INTRODUCTION

Nerve growth factor (NGF) is a survival/differentiation factor for neuronal and glial cells as well as for a subset of immune system elements. The receptor for this neurotrophin is composed of a low-affinity subunit of 75 kDa (NGFR\textsuperscript{p75}) and a high-affinity tyrosine kinase type A (TrkA) (1–3). The trophic influence of NGF depends on the presence of both receptors; TrkA was initially considered to be the biologically active molecule, the effect of which was potentiated by the low-affinity subunit (3). However, subsequent studies demonstrated that NGFR\textsuperscript{p75} is also able to deliver Trk-independent signals (1, 2). In this regard, in vitro investigations have demonstrated that the selective recruitment of NGFR\textsuperscript{p75} may induce either cell death (4–7), or anti-apoptotic effects through the activation of the transcription factor, NFkB (8, 9).

In recent years, compelling evidence from experimental and clinical studies has underscored an important role for NGF in central nervous system (CNS) autoimmune diseases (10). Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are diseases associated with inflammatory infiltration of CNS white matter and consequent autoimmune destruction of myelin and of the myelin-forming cell, the oligodendrocyte (11). Up-regulation of NGFR\textsuperscript{p75} and/or NGF has been found in situ both in MS and in EAE (12–15). In addition, NGF treatment has been shown to exert protective effects in primate EAE (15).

In order to understand better the molecular mechanisms mediating the NGF response in CNS autoimmune conditions, a fundamental issue is the identification of glial cell targets of NGF and their differential pattern of NGFR\textsuperscript{p75} and TrkA expression. In fact, receptor profile is known to influence the response to NGF and is therefore crucial in the analysis of the pathogenesis of autoimmune demyelinating diseases (2, 3, 10). However, studies on the expression on glial cells of NGFR\textsuperscript{p75} and TrkA have provided conflicting results. Most information on NGF receptors on glial cells has come from studies in vitro, where oligodendrocytes display a TrkA-negative/NGFR\textsuperscript{p75}-positive phenotype (4, 5, 9), while expression of these molecules on microglia and astrocytes depends upon culture conditions (16). Regarding the presence of NGF receptors in vivo, Frisen et al (17) reported that both receptors were not present on glial cells in the normal CNS, while other investigators described NGFR\textsuperscript{p75} reactivity on microglia (18). In the course of CNS autoimmune diseases, NGFR\textsuperscript{p75} has been reported on oligodendrocytes (18, 19), on their precursors (20), and on astrocytes (15).

In view of such discrepancies on the presence of NGFR\textsuperscript{p75} in normal CNS white matter and in chronic MS lesions, we decided to investigate its expression using different antibodies, some of which have been used in...
All tissues were obtained under a protocol approved by the and used as positive control for Western blotting experiments. Sciatic nerve isolated from limb amputation was homogenized served, either on glial or inflammatory cells.

NGFRp75 mAbs clone 8211 (Chemicon, Temecula, CA; 1:100) served, either on glial or inflammatory cells. Interestingly, both NGFRp75 and TrkA were present on a subset of inflammatory cells and no association between presence of NGFRp75 and DNA fragmentation was observed, either on glial or inflammatory cells.

**MATERIALS AND METHODS**

**Tissue Samples**

Postmortem (between 4 and 15 h) CNS tissue was studied from 15 subjects (mean age 46 yr), with a clinical diagnosis of chronic progressive MS. A total of 22 blocks containing lesions and normal appearing white matter were examined. Histopathologically, 5 cases displayed a predominance of chronic active lesions with hypercellular margins, ongoing demyelination with macrophage and lymphocytic infiltration, and a hypocellular demyelinated center displaying fibrous astrogliosis and oligodendrocyte depletion. In the remaining 10 cases the majority of lesions were defined as chronic silent based on the absence of inflammation and the presence of gliotic, demyelinated centers. Brain tissue from 5 subjects (mean age 62.3 yr), with other neurological diseases (OND), was available for control purposes (1 case each of Alzheimer disease, amyotrophic lateral sclerosis, and olivopontocerebellar atrophy and 2 cases of stroke). Normal CNS tissue with no evident lesions came from 5 subjects (mean age 62 yr), succumbing to non-neurologic conditions. Sections of sural nerve biopsies from 2 patients with chronic inflammatory demyelinating polyradiculoneuropathy were used as positive controls for NGFRp75 reactivity. All tissues were snap-frozen and stored at −80°C until use. A fragment of sciatic nerve isolated from limb amputation was homogenized and used as positive control for Western blotting experiments. All tissues were obtained under a protocol approved by the Institutional Review Board (IRB) for human subject research.

**Immunohistochemistry**

Immunofluorescence was performed on frozen sections according to a previously described procedure (21). Briefly, sections were fixed in acetone, blocked with normal serum, and incubated overnight with primary antibodies at 4°C. The primary antibodies and their cellular targets were as follows: anti-NGFRp75 mAbs clone 8211 (Chemicon, Temecula, CA; 1:100) (22) and clone ME20.4 (Sigma, St. Louis, MO; 1:100); rabbit antiserum against the cytoplasmic domain of NGFRp75 (Promega, Madison, WI; 1:100); anti-TrkA antiserum (sc-763; Santa Cruz Biotechnology; Santa Cruz, CA; 1:50, respectively); antisera to myelin basic protein (MBP), GFAP, CD68, and CD3 for the identification of oligodendrocytes, astrocytes, microglia, and T lymphocytes, respectively (all from Dakopatts, Glostrup, Denmark; 1:200, 1:400, 1:100 and 1:50); anti-HLA-DR LN3 mAb for microglia/macrophages (Biotest, Dreieich, Germany; 1:5); and anti-NG2 antiserum (Chemicon; 1:100) for oligodendrocyte precursors. Anti-NGFRp75 antibodies were visualized with appropriate biotinylated Ig followed by streptavidin-Texas Red (Vector Laboratories, Burlingame, CA), while for phenotypic markers, fluorescein-conjugated Ig were employed either for single and double staining. Double staining with anti-NGFRp75 mAbs and the microglial markers, CD68 or HLA-DR, was performed by using fluorescein-conjugated KP1 or CR3/43 mAbs (Dako, 1:10), respectively. After washing, slides were mounted and viewed under a Zeiss MC80 fluorescence microscope. Negative controls included the use of non-immune serum or isotype-specific, irrelevant antibody; for TrkA, pre-absorption with the generating peptide (Santa Cruz Biotechnology) abolished the signal.

**Western Blotting and Immunoprecipitation**

Western blotting and immunoprecipitation analyses were performed according to previously described procedures (21). Homogenized samples from normal human brain and sciatic nerve were dissolved in lysis buffer (DTT 0.5%, Nonidet P-40 1%, EDTA 10 mM in Tris-buffered saline) with protease inhibitor Complete™ (Roche, Mannheim, Germany). Samples were clarified by centrifugation and supernatants used for Western blotting and immunoprecipitation. Protein concentration was determined by bicinchoninic acid and bovine serum albumin as standard. Prior to immunoprecipitation, samples were pre-cleared with protein A-agarose (Roche) for 3 h and subsequently incubated with the anti-NGFRp75 mAbs or antiserum. Immunocomplexes were retrieved with protein A-agarose beads after overnight incubation. Total cell lysates, immunoprecipitates, and the respective supernatants were boiled for 5 min in Laemmli buffer; proteins were separated in 8% SDS-PAGE and transferred to PVDF membranes Immobilon P (Millipore, Bedford, MA). After saturation, membranes were probed with anti-TrkA anti-serum and monoclonal or polyclonal antibodies to NGFR. Immunoprecipitates obtained with the mAbs were probed with the anti-NGFRp75 antiserum and, vice versa, immunoprecipitates with the antiserum were visualized with anti-NGFRp75 mAb. Immunoreactive bands were detected with the appropriate Ig conjugated to peroxidase and visualized on autoradiographic film by enhanced chemiluminescence (Amer sham, Buckinghamshire, England).

**Apoptosis Assay**

Apoptotic figures in MS sections were assessed by the terminal deoxynucleotidyl transferase-mediated fluorescein-conjugated dUTP nick end-labeling (TUNEL) technique (Roche), as previously described (23, 24). After fixation and permeabilization, reaction mixture was added and the sections incubated at 37°C. In negative controls the enzyme was omitted. The phenotype of TUNEL-positive cells was assessed by double immunostaining, as described above.

**RESULTS**

In normal CNS, immunohistochemistry with anti-NGFRp75 antiserum revealed constitutive expression on microglia, oligodendrocytes, axons, and some perivascular cells (Fig. 1A). On the other hand, immunoreactivity with anti-TrkA antiserum and anti-NGFRp75 mAbs was...
observed on cells around meningeal and parenchymal vessels, but not on glial elements (Fig. 1B, C); positive cells had an elongated morphology, were GFAP-negative and corresponded to the leptomeningeal cells described by Frisen et al (17). High levels of reactivity for NGFR\(^{p75}\) was detected on Schwann cells in nerve biopsy sections from chronic polyradiculoneuritis, while staining for TrkA was present on perineurial structures (data not shown), as previously reported (25).

The different patterns of NGFR\(^{p75}\) reactivity observed on CNS glial cells using the mAbs and the antiserum prompted us to investigate their fine specificity by Western blotting and immunoprecipitation techniques. By Western blotting, mAbs 8211 and ME20.4 yielded a single immuno-reactive band of 75 kDa in PNS homogenate (Fig. 1D, lane 3), and bands migrating at 50-35 and 15 kDa in the CNS homogenate (Fig. 1D, lane 4), the latter likely representing truncated and/or deglycosylated forms of the receptor, respectively; peptide preabsorption abolished the staining of these bands (data not shown). Thus, of the mAbs and ME20.4 were employed in subsequent analyses of NGFR\(^{p75}\) expression in MS lesions.

In OND cases and in chronic silent MS lesions, immunostaining for NGFR\(^{p75}\) and TrkA was similar to that observed in normal CNS; in particular, neither mature glial cells (Fig. 1G–L) nor NG2-positive precursors (Fig. 1M, N), displayed NGFR\(^{p75}\). In contrast to non-inflammatory conditions, high levels of NGFR\(^{p75}\) expression were detected within, but not outside chronic active MS lesions and the pattern of reactivity was similar using mAbs 8211 or ME20.4. Immunostaining for NGFR\(^{p75}\) was observed on distinct glial cell types depending on the area and degree of inflammatory activity in the plaque. At the edge of all chronic active MS lesions examined, the majority of cells reactive for NGFR\(^{p75}\)-positive were GFAP-positive astrocytes (Figs. 2A–C); proceeding deeper into the lesion, the proportion of NGFR\(^{p75}\)-positive astrocytes gradually decreased. In addition, immunostaining for NGFR\(^{p75}\) was also observed on different glial elements throughout the lesion area. Based on cell shape and double staining with phenotypic markers, these cells were identified as microglia/macrophages. In fact, immunoreactivity for NGFR\(^{p75}\) was detected on many ramified HLA-DR-positive or CD68-positive microglia in the plaque center (Fig. 2D–F); NGFR\(^{p75}\) was also observed on some foamy macrophages, particularly around inflammatory infiltrates. In general, a positive correlation was observed between expression of NGFR\(^{p75}\) on microglia/macrophages and degree of inflammation. Examination of sections double stained with MBP or NG2 antisera and anti-NGFR\(^{p75}\) mAbs revealed that the majority of oligodendrocytes (Fig. 2G–I) and their precursors (Fig. 2M–O) showed no reactivity for NGFR\(^{p75}\); only scattered MBP-positive (Fig. 2J–L) and NG2-positive cells located at the plaque edge were found to express NGFR\(^{p75}\). Noteworthy was that TrkA was never observed on glial cells (Fig. 3D). With regard to the expression of NGF receptors on inflammatory cells, we found that within the same lesion, some perivascular cuffs showed co-expression of NGFR\(^{p75}\) and TrkA on the majority of inflammatory cells (Fig. 3A, B), while other infiltrates displayed no reactivity for both NGF receptors (Fig. 3C, D).

Since selective expression of NGFR\(^{p75}\) (i.e. in the absence of TrkA) has been shown to trigger oligodendroglial cell death in vitro (4, 5, 7), we assessed whether the presence of NGFR\(^{p75}\) correlated with apoptosis by combining TUNEL and immunostaining for NGFR\(^{p75}\). As previously reported by our group and others (20, 23, 24), TUNEL-reactive profiles were confined to inflammatory elements and scattered microglial cells. We found no correlation between expression of NGFR\(^{p75}\) and TUNEL reactivity both in glial and inflammatory cell. In fact, glial cells undergoing apoptosis showed no expression of NGFR\(^{p75}\) (Fig. 2P, Q) and, within perivascular infiltrates, TUNEL-positive images were also detectable on inflammatory cells not expressing NGF receptors (data not shown).

DISCUSSION

The aim of this study was to evaluate the expression and cellular distribution of NGFR\(^{p75}\) and TrkA in normal human CNS and in chronic MS lesions, since conflicting results have been reported regarding expression of NGFR\(^{p75}\) on human glial cells (15–20). To investigate whether this discrepancy was related to technical differences, we assessed the pattern of recognition of a battery of anti-NGFR\(^{p75}\) antibodies, some of which were used in
Fig. 1. Expression of NGF receptors in normal CNS and chronic silent MS lesions. A: Diffuse reactivity was visible with the polyclonal anti-NGFR-p75 antiserum in normal human CNS on oligodendrocytes, microglia, and axonal structures; conversely, perivascular elements but not glial cells were immunostained with the anti-NGFR-p75 mAb 8211 (B), and anti-TrkA antiserum (C). D: The anti-NGFR-p75 antiserum recognized numerous PNS (lane 1) and CNS (lane 2) proteins, whereas mAb 8211 reacted with a 75-kDa band in PNS homogenate (lane 3) and with bands of lower size in the CNS (lane 4). E: Anti-TrkA antibody yielded a 140-kDa-immunoreactive band both in PNS (lane 1) and CNS (lane 2) homogenates; anti-TrkA antibody also specifically recognized a 110-kDa band in the CNS. F: The polyclonal anti-NGFR-p75 antiserum failed to immunoprecipitate any antigen in PNS (lane 1) and CNS (lane 2), while immunoprecipitation with the mAb 8211 resulted in a 75-kDa band both from PNS (lane 3) and CNS (lane 4) lysates, although with different intensities. G–L: In white matter adjacent to chronic silent MS lesions, double
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the above studies. Although all 3 antibodies employed recognized a 75-kDa-immunoreactive band by immunoblotting in PNS homogenates, only NGFR\(^{p75}\) mAbs 8211 and ME20.4 selectively reacted with their antigen, while the polyclonal antibody showed a broader reactivity with numerous bands immunostained in PNS and CNS samples. In CNS homogenates, mAb 8211 showed prominent bands at 50-35 and 15 kDa, probably representing truncated and/or deglycosylated forms. After immunoprecipitation of PNS and CNS proteins, selective recognition of NGFR\(^{p75}\) was accomplished by mAb 8211; in contrast, the polyclonal antiserum failed to immunoprecipitate NGFR\(^{p75}\), which was detectable in the supernates instead.

The differing degrees of reactivity of these anti-NGFR\(^{p75}\) antibodies may help to explain the conflicting results on the distribution of NGFR\(^{p75}\) in normal CNS (17, 18). In line with this, the distinct immunohistochemical patterns observed with anti-NGFR\(^{p75}\) polyclonal antiserum and mAbs reflected the different degree of specificity evidenced by Western blotting and immunoprecipitation. In fact, diffuse glial cell reactivity has been reported in normal CNS using the polyclonal antiserum, has been reported on oligodendrocytes (18) or on their precursors (20), within and around chronic MS lesions. The present immunohistochemical study for TrkA and NGFR\(^{p75}\) with the mAbs 8211 and ME20.4 showed no expression of either NGF receptor on glial cells in non-inflammatory conditions (i.e. normal CNS, OND, and chronic silent MS lesions), and in white matter adjacent to chronic active MS lesions. On the other hand, glial and inflammatory cells within chronic active MS lesions were found to display one or both receptors. Double staining with glial cell markers revealed NGFR\(^{p75}\), but not TrkA, to be on reactive astrocytes at the lesion edge and on microglia/macrophages throughout the plaque. At variance with previous observations (18, 20), we found that only scattered MBP-positive and NG2-positive cells displayed NGFR\(^{p75}\), the majority of oligodendrocytes and their precursors being NGFR\(^{p75}\) negative. In addition to glial cells, reactivity for both NGF receptors was seen on some inflammatory infiltrates, but not on others. On the whole, our results strongly suggest that the discrepancy between observations on NGFR\(^{p75}\) expression (15, 17, 18, 20) is mainly related to the different antibodies employed and, perhaps, to a different sampling or preparation of tissues. In fact, our study focused on frozen sections of chronic MS lesions and does not exclude the possibility that in more acute lesions or in tissue otherwise prepared, NGF receptors may be differently expressed on glial cells.

We also investigated whether NGFR\(^{p75}\) expression on glial elements in MS lesions was associated with programmed cell death. In this regard, we confirmed previous observations that TUNEL-reactive elements displayed no reactivity for NGFR\(^{p75}\) (20), thus ruling out a major role for NGF in vivo in the induction of glial cell death in chronic MS. As an alternative to a cytotoxic effect, one may hypothesize that NGF triggers activation of the transcription factor NF\(_{κB}\) with potential anti-apoptotic effects on NGFR\(^{p75}\) glial cells in MS lesions. In keeping with this, we and others have previously shown that NF\(_{κB}\) nuclear translocation occurred in microglia/macrophages at the edge of chronic active MS lesions (26, 27). Regarding astrocytes, activation of NGFRs by NGF resulted in morphologic changes upon these glial cells in vitro (28), whereas we are unaware of any literature showing astrocyte death by this neurotrophin.

With regard to possible mechanisms responsible for NGFR\(^{p75}\) up-regulation on glial cells in MS lesions, although the observed correlation with the degree of inflammation may herald a role for inflammatory mediators, their effect on NGFR\(^{p75}\) expression remains to be clarified. On the other hand, several lines of evidence suggest that autocrine/paracrine mechanisms may be involved in NGFR\(^{p75}\) up-regulation. In support of this, increased levels of NGF have been detected in MS and EAE (12, 14), and astrocytes and T lymphocytes in vitro are capable of producing NGF (29, 30), which in turn is known to up-regulate NGFR\(^{p75}\) on glial cells (31).

Taken in concert, the present findings show that NGF receptors are up-regulated on both glial and inflammatory cells in chronic MS lesions, but only during active inflammation, and that their expression is not associated with the induction of apoptosis. Whether NGF displays a down-regulatory effect on the immune response within the MS lesion, as shown in primate EAE (15), remains a possibility. In this context, ongoing studies on glial cell cultures to investigate the modulation of cytokine profile exerted by NGF will contribute to the understanding of the proposed neuroprotective role of this neurotrophin in human autoimmune diseases.

staining with GFAP (G), MBP (I), HLA-DR (K), and NG2 (M) markers showed that both mature glial cells and oligodendrocyte precursors expressed neither NGFR\(^{p75}\) (H, J, N) nor TrkA (L). A–C, G, I, K, M: Fluorescein; H, J, L, N: Texas red; D–F: Peroxidase. A–C, G–L: ×250; M, N: ×325.
Fig. 2. Expression of NGF receptors in chronic active MS lesions. A–C: Double immunofluorescence for GFAP (A, green) and NGFR<sup>p75</sup> (B, red) showed that the majority of reactive astrocytes at the lesion edge expressed high levels of NGFR<sup>p75</sup> reactivity, resulting in a yellow-orange signal (C). D–F: Double immunostaining with fluorescein-conjugated anti-CD68 mAb (D, green) and anti-NGFR<sup>p75</sup> mAb ME20.4 (E, red) showing that microglia/macrophages expressed NGFR<sup>p75</sup> within chronic active MS lesions; the merged image (F) showed cytoplasmic staining for CD68 on microglia/macrophages, which displayed on their surface the low-affinity receptor of NGF. G–L: Double immunofluorescence for MBP (G and J, green) and NGFR<sup>p75</sup> (H and K, red); the majority of oligodendrocytes in periplaque white matter did not express NGFR<sup>p75</sup> (I); only scattered MBP-positive oligodendrocytes showed expression of NGFR<sup>p75</sup> (L, arrowhead). M–O: Double immunofluorescence for NG2 (M, green) and NGFR<sup>p75</sup> (N, red) showed that most oligodendrocyte precursors displayed no reactivity for NGFR<sup>p75</sup> (O). P, Q: Double staining for TUNEL (P, green) and NGFR<sup>p75</sup> (Q, red); reactivity for TUNEL is visible on the nuclei of glial cells, which showed no expression of NGFR<sup>p75</sup>. Green: fluorescein; red: Texas red. A, B: ×150; C: ×400; D–F, M–O: ×625; G–L: ×475; P, Q: ×200.
Expression of NGF receptors on inflammatory cells in chronic active MS lesions. In some perivascular infiltrates, most inflammatory cells (A: HLA-DR, fluorescein) displayed NGF receptors (B: TrkA, Texas red), while other perivascular cuffs (C: CD3, fluorescein) did not express both receptors (D: NGFRp75, Texas red). ×250.

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REFERENCES

31. Kumar S, Pena LA, de Vellis J. CNS glial cells express neurotrophin receptors whose levels are elevated by NGF. Mol Brain Res 1993;17:163–68

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