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Increased expression of phosphotyrosine after axotomy in the dorsal motor nucleus of the vagus nerve and the hypoglossal nucleus

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Abstract To investigate the role of tyrosine kinase underlying glial cell proliferation after axotomy, the localization of phosphotyrosine was studied immunohistochemically in the dorsal motor nucleus of the vagus nerve and the hypoglossal nucleus after nerve transection in adult rats. An anti-phosphotyrosine antibody weakly stained the cytoplasm of the neurons and some glial cells on the control side of both nuclei, while preferentially staining the plasma membrane of perineuronal microglial cells and neurons weakly on the severed side 2 days after axotomy and intensely between 3 and 7 days. Some of the microglial cells reacted positively with both anti-bromodeoxyuridine and anti-phosphotyrosine antibodies, suggesting that tyrosine kinase is involved in microglial cell proliferation. Proliferation of numerous microglial cells was observed in the severed nuclei between 2 and 4 days after axotomy, while only a few were detected on days 5 and 7. These findings suggest that tyrosine kinase is involved in not only the proliferation of perineuronal microglial cells but also in some retrograde neuronal reactions such as differentiation and regeneration.

Key words Axonal reaction · Proliferation
Phosphotyrosine · Neuron · Microglia

Introduction

The proliferation of perineuronal glial cells has been reported [1, 9] in several nuclei in association with the axonal reaction following nerve transection [2, 14, 27, 29, 30]. In this case neuronal cells must stimulate glial cell proliferation, either directly or indirectly, because only neurons and their axons are affected. Cellular proliferation is stimulated by various growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) [6, 31]. These growth factors stimulate mitogenesis by interaction with cell-surface receptors that possess an intrinsic, ligand-sensitive, protein tyrosine kinase activity [31]. Since tyrosine kinase plays a key role in cellular growth regulation, the first step must include tyrosine phosphorylation of cytoplasmic polypeptides [31].

In the axonal reaction, which growth factor stimulates the proliferation of perineuronal glial cells remains unknown. Since the presence of various growth factors have been reported in neuronal cells [3, 17, 20, 21, 32], tyrosine kinase is expected to be involved in the proliferation process of glial cells in the nervous system. Phosphotyrosine (pTyr), the reaction product of tyrosine kinase, is a sensitive marker for detection of the activity of tyrosine kinases [5, 16, 22, 25].

In the present study we investigated, immunohistochemically, the changes in pTyr in the dorsal motor vagal and hypoglossal nuclei occurring after axotomy, and observed intense immunoreactions along the plasma membranes of neurons and proliferating perineuronal glial cells.

Materials and methods

Adult Wistar rats were subjected to left vagal and right hypoglossal nerve transection at the neck under ether anesthesia, and killed after 1, 2, 3, 4, 5 or 7 days (six animals per group). Under pentobarbital anesthesia, three animals in each group were perfused through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brain stem was removed and immersed in the same fixative for 6 h at 4 °C, after which the tissue was rinsed with 20% sucrose in 0.1 M phosphate buffer (pH 7.2) for 48 h and frozen in liquid nitrogen. Frozen 8-μm sections were

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cut and incubated with mouse monoclonal antibodies raised against pTyr (1G2, 1 μg/ml, Oncogene Science Inc.) and rat Ia antigen (RT1.B, diluted 1:100, Cedarlane Laboratories Ltd.) overnight at 4 °C. Bound immunoglobulins were detected with a Histofine SAB-PO kit (Nichirei Corp.) using 3,3'-diaminobenzidine as a chromogen. For electron microscopy, incubated frozen sections were dehydrated in ethanol, post-fixed in osmium tetroxide, and embedded in epoxy resin. The other animals were injected with bromodeoxyuridine (BrdUrd, 25 mg/kg body weight) intraperitoneally 30 min before sacrifice. The animals were perfused as above and each brain stem was removed and immersed in the same fixative for 24 h at 4 °C. Tissue was dehydrated, embedded in paraffin and 5-μm serial sections were cut. Sections were incubated with mouse monoclonal antibodies against pTyr and BrdUrd (B44, diluted 1:100, Becton Dickinson Immunocytometry Systems) and rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP, diluted 1:800, Dako Japan Co., Ltd.) overnight at 4 °C. *Ricinus communis* agglutinin-1 (RCA-1) lectin conjugated with peroxidase (1 μg/ml, incubated overnight at 4 °C, Seikagaku Corp.) was used to detect microglia [15]. The antibody against pTyr does not cross-react with phosphoserine, phosphothreonine or phosphohistidine, nor with a variety of other phosphorylated molecules including ribose phosphate, IMP, AMP and ATP. The specificity of staining with the anti-pTyr antibody was demonstrated by preincubation with 10 mM phenyl phosphate for 1 h at room temperature. Three non-operated animals served as controls.

Results

Light microscopy showed that the anti-pTyr antibody stained the cytoplasm of neurons and some glial cells weakly within the neuropil of the control animals and stained ependymal cells intensely (Fig. 1). The anti-pTyr immunoreactivity observed in the neurons and perineuronal glial cells was weak on day 1 after nerve transection, and slightly stronger on day 2 on the severed side of both vagal and hypoglossal nuclei. Immunoreactivity became intense on day 3 (Fig. 2a) and continued to grow stronger until day 7 (Fig. 2b) in these cells. Electron microscopical investigation with anti-pTyr antibody revealed immunoreactivity along the plasma membrane of neurons and perineuronal glial cells as scattered clusters (Fig. 3). Immunoreaction products were also observed around the endoplasmic reticulum in the neuronal cytoplasm. Preincubation with phenyl phosphate abolished the staining reaction.

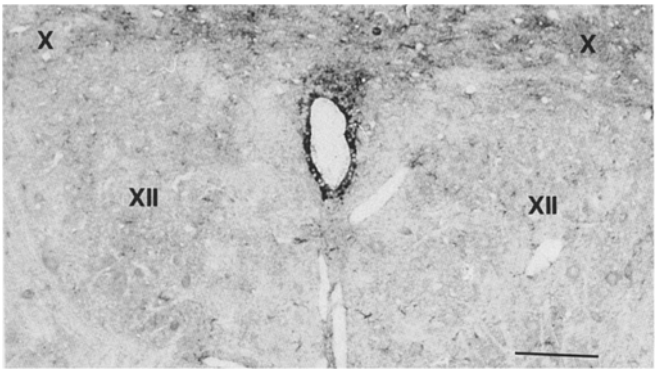

Fig. 1 Anti-phosphotyrosine antibody stained the cytoplasm of neurons and some glial cells weakly in the neuropil of vagal (*X*) and hypoglossal (*XII*) nuclei, and stained ependymal cells intensely in the control animals. *Bar* = 75 μm

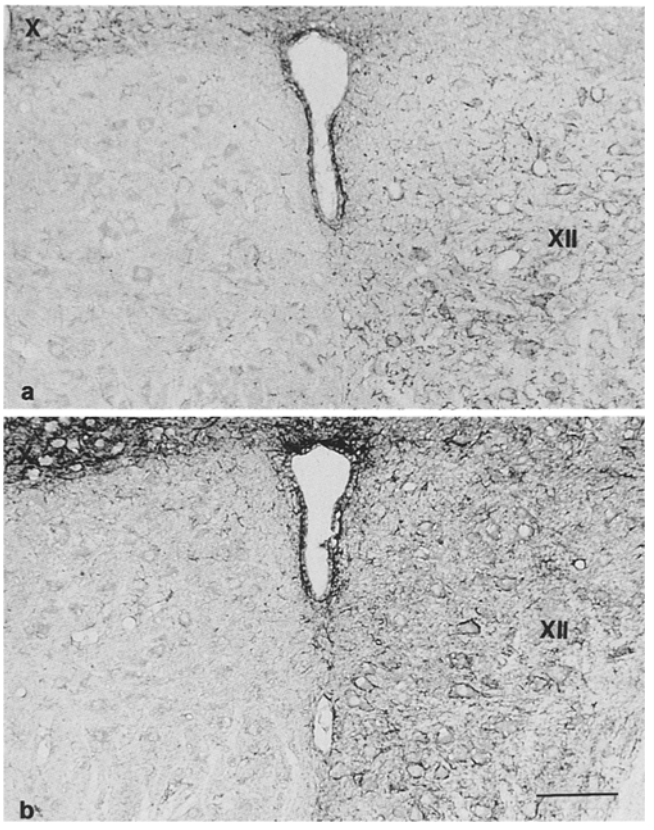

Fig. 2 Anti-phosphotyrosine antibody stained the plasma membrane of neurons and perineuronal glial cells intensely in the severed side of vagal (*X*) and hypoglossal (*XII*) nuclei on days 3 (**a**) and 7 (**b**). *Bar* = 75 μm

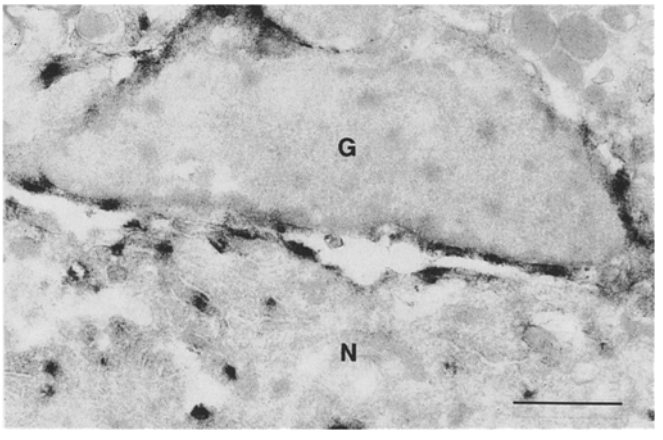

Fig. 3 Anti-phosphotyrosine immunoreaction products were observed along the plasma membrane of microglia (*G*) and neurons (*N*) on day 7 after axotomy. *Bar* = 1 μm

The anti-BrdUrd antibody stained numerous perineuronal glial cells between days 2 and 4 after axotomy, while only a few were stained on days 5 and 7. Few BrdUrd-positive cells were observed on day 1. Some of the perineuronal glial cells reacted positively in the serial sections with both anti-BrdUrd (Fig. 4b) and anti-pTyr (Fig. 4a) antibodies. RCA-1 lectin preferentially stained the perineuronal glial cells, while these cells showed no reaction with anti-GFAP antibody in the present study. Perineuronal glial cells also reacted with the anti-Ia antibody on day 7 (Fig. 4c).

Discussion

In the present study, weak immunoreactivity with the anti-pTyr antibody was observed in the cytoplasm of neurons and some glial cells in the neuropil of the vagal and hypoglossal nuclei of control animals. In operated animals, on the severed side of both nuclei, anti-pTyr antibody showed intense staining in the plasma membranes of neurons and perineuronal glial cells. The increased immunoreactivity for pTyr points to two possibilities; the activation of protein-tyrosine kinase [6, 31], or the inactivation of protein-tyrosine phosphatase [8]. In both cases, the elevated levels of phosphotyrosine would eventually elicit signal transduction identical to that with activation of tyrosine kinase [8].

Numerous BrdUrd-positive perineuronal glial cells were observed between days 2 and 4 after axotomy, while only a few were detected on days 5 and 7, and scarcely any on day 1. Some of the perineuronal glial cells showed positive reactions with both anti-pTyr and anti-BrdUrd antibodies. These findings suggest that tyrosine kinase is related to perineuronal glial cell proliferation. RCA-1 lectin and anti-rat Ia antibody preferentially stained perineuronal glial cells, while these cells showed no reaction with the anti-GFAP antibody in the present study, indicating that the proliferating glial cells are microglia [9, 15, 24–26]. Electron microscopy showed immunoreaction products with the anti-pTyr antibody localized along the plasma membranes as scattered clusters, suggesting that receptor tyrosine kinases contribute to this pTyr immunoreactivity. Various growth factors such as FGF [3, 17] and PDGF [10, 20, 21, 32] have been reported in neuronal cells. One of these factors may cause the activation of tyrosine kinase in microglial cells resulting in their proliferation.

Affected neurons also showed intense pTyr immunoreactivity in both the vagal and hypoglossal nuclei. Localization of pTyr-containing protein was reported in rat brain to correspond to that of the insulin receptor [16]. In addition, several growth factor receptors, such as the nerve growth factor (NGF), PDGF, and FGF receptors have also been reported in neuronal cells [7, 10, 23] and injured nerves [18, 19]. Hypoglossal neurons have been reported to express NGF receptor protein after axotomy [4]. Synthesis of basic FGF (bFGF) has been reported in neurons and glial cells [3, 12, 17], and both bFGF receptor protein and mRNA have also been observed in various neurons, including those in the dorsal motor nucleus of vagus nerve and hypoglossal nuc-

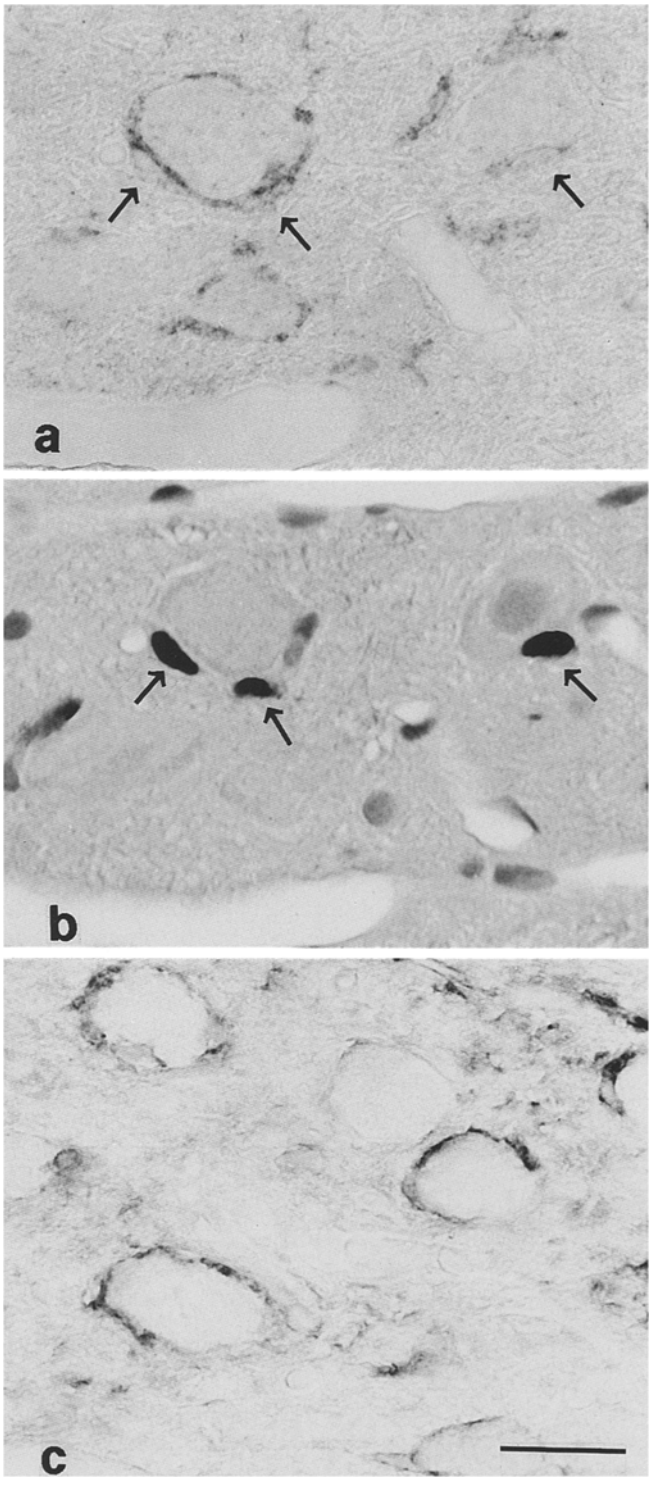

Fig. 4 **a** Anti-phosphotyrosine immunoreaction products were observed between the neurons and microglia (*arrows*) on day 2 post-operatively. **b** These glial cells also showed BrdUrd immunoreactivity (*arrows*) in the serial sections. **c** Perineuronal glial cells were labeled with anti-Ia antibody on day 7. *Bar* = 10 μm

leus [28]. The mechanisms of secretion of some of these growth factors are still controversial. They are considered to work in paracrine and/or autocrine modes [12]. A certain growth factor synthesized in the nervous system may bind to the receptor of affected neurons and glial cells, resulting in the pTyr immunoreactivity observed in the present study along the plasma membrane after axotomy.

Microglial cell proliferation was mainly observed from 2 to 4 days after axotomy, while pTyr immunoreactivity remained intense until day 7. This prolonged immunoreactivity for pTyr in the microglial cells and neurons suggests that tyrosine kinase is involved in not only glial cell proliferation but also in some retrograde reactions in injured neurons. Increased synthesis of tyrosine kinase, $pp60^{c\text{-}src}$, has been reported during the regeneration process in the severed sciatic nerve, and the activity of the enzyme increased between 7 and 90 days in regenerating axons [13]. Immunoreactivity for $pp60^{c\text{-}src}$ was observed in neuronal cell bodies and some glial cells in the spinal cord and dorsal root ganglion after crushing injury of the sciatic nerve [11]. In addition, hypoglossal neurons have been shown to express NGF receptor protein, a member of the *trk* family of tyrosine kinases, between 1 and 90 days after axotomy [4]. Thus, $pp60^{c\text{-}src}$ or NGF receptor tyrosine kinase may have contributed to the increase in levels of pTyr observed in the affected neurons and glial cells in the present study.

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