Hiroshima Journal of Medical Sciences
Vol.35, No.2, 109~115, June, 1986
HIJM 35-18

Immunohistochemical Studies of Experimental Diabetic Neuropathy

Shozo KITO, Takenobu KISHIDA, Hiroaki MATSUBAYASHI and Rie MIYOSHI

The Third Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan

(Received March 2, 1986)

Key words: Streptozotocin-diabetic rats, Ischiadic nerve, Immunohistochemistry, Aldose reductase inhibitor

ABSTRACT

Accumulations of substance P, somatostatin, vasoactive intestinal polypeptide (VIP) and dopamine-β-hydroxylase (DBH) after ligation of the ischiadic nerve of streptozotocin-induced diabetic rats were observed by an immunohistochemical technique and the results were examined both qualitatively and quantitatively. Besides, effects of administration of an aldose reductase inhibitor (ONO 2235), a key enzyme for polyol synthesis, on these accumulation patterns were studied. Immunofluorescent positiveness of these biological active substances was intensely observed along the axon in nerves of control animals. In the ischiadic nerves of diabetic rats, it was weakly observed in irregular shapes. On the other hand, in the ischiadic nerve of the diabetic rat treated with the aldose reductase inhibitor, substance P-, DBH- and VIP- immunoreactive positiveness was recovered in both distribution pattern and accumulation length. Our findings show that there are abnormalities of the axonal flow of the peripheral nerve in diabetic animals. These results also suggest that polyol pathway activity may contribute to the pathogenesis of diabetic neuropathy and ONO-2235 may be useful in preventing diabetic complications.

Pathophysiology of diabetic neuropathy has been so far examined mainly from viewpoints of clinical signs and symptoms, clinical electrophysiology and morphological pathology. Nevertheless, factors involved in development of diabetic neuropathy have not yet been clarified. The authors studied on the axonal flow of the peripheral nerve of experimental diabetic rats whose abnormalities might be one of factors associated with etiology of diabetic neuropathy. Ischiadic nerves of alloxan- or streptozotocin-induced diabetic rats were used in this experiment. Accumulations of neuropeptides or neurotransmitter synthesizing enzymes after ligation of the ischiadic nerve were observed by an immunohistochemical technique and the results were examined both qualitatively and quantitatively. Besides, how much effects an aldose reductase inhibitor (ONO 2235)[11], a new therapeutic drug against diabetic neuropathy when orally administered, had on these accumulation patterns was studied.

MATERIALS AND METHODS

Wistar strain male rats were used in this experiment.

1. alloxan treated rats

Two rats weighing 150 g were administrated 40 mg/kg body weight of alloxan intravenously. After administration, the blood sugar level was checked with glucose analyzer YSI 23 A. Mean fasting blood sugar levels of rats were 304 mg/dl and 267 mg/dl after 42 and 95 days of alloxan administration, respectively. One hundred days after administration, the ischiadic nerves were ligated under pentobarbital anesthesia. The animals were perfused with 4% paraformaldehyde buffered in 0.1 M phosphate solution with

pH 7.4.

2. streptozotocin treated rats

Thirty six rats weighing 100 g were administered 100 mg/kg body weight of streptozotocin intravenously. Two weeks after, fasting blood sugar levels of all rats were higher than 250 mg/dl. Fifteen of these rats were feeded by the fodder that was prepared to include 50 mg/kg/day of the aldose reductase inhibitor. The ischiadic nerves were ligated at various times from 28 to 100 days after streptozotocin administration. The animals were sacrificed 2 days after ligation.

3. control rats

Five rats weighing 400—450 g and 3 rats weighing 150 g were used as control. The animals were perfused 2 days after ligation of the ischiadic nerves.

After perfusion, the ischiadic nerves were removed, post-fixed in the same fixative for 3 hr and embedded in OCT compound. Cryostat sections of the longitudinal direction of 6 μm thickness were prepared. As for immunohistochemical technique, the indirect fluorescent antibody technique was applied. Sections were incubated with an antibody against substance P, somatostatin, vasoactive intestinal polypeptide (VIP) or dopamine-β-hydroxylase (DBH) at 37°C for 30 min. As the next step, the sections were incubated with FITC-labeled anti-rabbit IgG goat IgG at 37°C for 30 min. Sections were mounted in glycerine-PBS. A Nikon fluorescence microscope was used for observation and photographing. The length, intensity and pattern of immunofluorescence positiveness in the ischiadic nerves were observed. Careful comparison among control, drug induced diabetic animals and diabetic models treated with the aldose reductase inhibitor was done.

RESULTS

Fig. 1 shows immunofluorescence microphotographs of DBH immunoreactivities in the ligated ischiadic nerves of a control rat and an experimental model of alloxan-induced diabetes. DBH immunoreactivities in the ischiadic nerves of control rats as shown in Fig. 1-c were intensely observed along the axon. In contrast, DBH immunoreactivities of alloxan-induced diabetic models were shortened in an accumulation length from a ligation point to a distal end of a series of fluorescence and did not come into line along the axon. They were markedly irregular in shape, when compared with those of control nerves (Fig. 1-DM).

Fig. 2 shows substance P immunoreactivities in the ligated ischiadic nerves of control, streptozotocin-induced diabetic animals and diabetic models treated with the aldose reductase inhibitor. Substance P positive immunofluorescence was observed along the axon in control nerves as shown in Fig. 2-C. In the ischiadic nerves of diabetic rats 59 days after streptozotocin administration, substance P immunoreactivities did not get into line along the axon and an accumulation state was abnormal appearing as irregularly shaped masses (Fig. 2-DM). On the contrary, in the diabetic rat feeded with the aldose reductase inhibitor-containing fodder for 76 days, an accumulation of substance P positiveness was observed as serial spots along the axon. Abnormal immunofluorescence of irregularly shaped accumulative substance within the nerve had gone (Fig. 2- DM-T). Accumulation lengths of substance P immunoreactivities in the ischiadic nerves of both diabetic rats and disease models treated with the aldose reductase inhibitor were about half of those of control rats. Fig. 3 shows DBH immunoreactivities in the ischiadic nerves of control and experimental model rats. An accumulation state of a control nerve is shown in Fig. 3-C. DBH immunoreactivities in the ischiadic nerve of a diabetic rat 59 days after streptozotocin administration are shown in Fig. 3-DM. They do not bring into line along the axon and show abnormal fragmentary distribution. The ischiadic nerve of a rat that survived for 38 days after streptozotocin administration, followed by treatment by the aldose reductase inhibitor for 24 days, is shown in Fig. 3- DM-T. The abnormal immunofluorescent pattern which was found in Fig. 3-DM was improved. The lengths of DBH accumulation of diabetic rats and those treated with the aldose reductase inhibitor were shorter than control rats. Fig. 4 shows VIP immunoreactivities in the ligated ischiadic nerve. VIP immunofluorescence of control rats was intensely observed along the axon (Fig. 4-C). In the ischiadic nerve of a diabetic rat 77 days after streptozotocin injection, VIP immunoreactivities were weakly observed in irregular shapes (Fig. 4-DM). In the ischiadic

nerve of a diabetic rat that survived for 41 days after streptozotocin administration and was feeded by the aldose reductase inhibitor-containing food for 35 days, fine spots of VIP immunofluorescence were observed. An accumulation length of diabetic rats with the treatment was longer than that of non-treatment diabetic rat (Fig. 4- DM-T). Immunofluorescence microphotographs of somatostatin immunoreactivities in the ligated ischiadic nerve are shown in Fig. 5. Somatostatin positiveness of a control rat was observed along the axon (Fig. 5-C). In the ischiadic nerves of the rat 100 days after streptozotocin administration, however, somatostatin immunoreactivities were sparsely observed (Fig. 5-DM). After treatment with the aldose reductase inhibitor, somatostatin immunoreactivities did not show much difference from those of non-treatment diabetic rats (Fig. 5- DM-T).

Accumulated lengths of immunoreactive positiveness against substance P, somatostatin, VIP and DBH in ligated ischiadic nerves were compared among control rats, diabetic model animals and diabetic ones with treatment by the aldose reductase inhibitor (Fig. 6). Eight control rats, 21 diabetic ones and 15 diabetic ones treated with the aldose reductase inhibitor were used. Immunofluorescence microphotographs of the ligated ischiadic nerves were taken and 50 times enlarged. The distance in a straight line from a ligation point to a distal end of a series of fluorescence observed was measured. As for the length of substance P immunofluorescence, those of diabetic rats were decreased with significant difference ($p<0.05$). In addition, the length of somatostatin immunoreactivities also had a tendency to be decreased in diabetic animals. The difference between the accumulation length of non-treated diabetic rats and that of diabetic rats treated with the aldose reductase inhibitor was not significant. Nevertheless, in the ischiadic nerves of the rat given the aldose reductase inhibitor, abnormal irregular distribution pattern of accumulated immunofluorescence within the nerve had gone and immunoreactivities in line along the axon were observed. These changes were found in 6 rats among 15 rats which were treated with the aldose reductase inhibitor.

DISCUSSION

It is known that the axonal flow of the nerve cell is altered in various neurological disorders. On the other hand, the rate of the axonal flow is dependent on the flowing substance and the axonal flow are generally divided into 3 groups, that is, slow-, intermediate- and fast axoplasmic transports[6]. The authors studied the axonal flow of the ischiadic nerve of diabetic model animals in order to understand one aspect of the pathologic state of diabetic neuropathy through a simple experimental system. The axonal flow of peripheral nerves in diabetic neuropathy was reported by Schmidt et al[9]. They produced streptozotocin-induced diabetic rats, ligated the ischiadic nerve after three or four weeks and measured the accumulation of acetylcholine esterase and choline acetylase with use of enzyme-fluorescence technique. These enzymes flow within the axon at an intermediate rate. In the ischiadic nerve of diabetic rats, the flow of acetylcholine esterase was 20% reduced and that of choline acetylase was 40% reduced. The authors measured the length of accumulation from ligation point using immunohistochemistry. Measured substances were substance P, somatostatin, VIP and DBH. In the ischiadic nerve of diabetic rats, the accumulation length of substance P immunoreactive positiveness was significantly reduced. From this result, it is considered that the axonal flow of substance P is slowed down or substance P itself is reduced in the ischiadic nerve of diabetic rats. It is confirmed by many investigators that substance P is neurotransmitter of a small-calibered primary sensory neuron[7]. The results obtained from these experiments are consistent with Bischoff's result that the primary change of diabetic neuropathy occurs in the small-calibered nerve fiber[1].

There have been several reports that imply the importance of polyol accumulation in peripheral nerve tissue in the pathogenesis of diabetic neuropathy[3,10]. Furthermore, inhibitors of aldose reductase, a key enzyme for polyol synthesis, have been reported to improve the conduction abnormalities of peripheral nerves in animal experiments[2,8] and clinical trials[4,5]. In our experiments, in the ischiadic nerve of the diabetic rat treated with the aldose reductase inhibitor, substance P- and DBH- immunoreactive

positiveness was improved in distribution pattern and accumulation length in 6 out of 15 rats. It is assumed that an accumulation of sorbitol in the ischiadic nerve of diabetic animals causes such abnormalities of accumulated biological active substances as above-mentioned after ligation of the nerve. We conclude that the results offer an evidence of an effect of the aldose reductase inhibitor on the peripheral nerve of diabetic animals.

REFERENCES

1. **Bischoff, A.** 1973. Ultrastructural pathology of peripheral nervous system in early diabetes. Advanc. Metab. Dis. **2 (Suppl.2)**:441—449.
2. **Dvornik, D., Simard-Duquesne, N., Krami, M., Sestanj, K., Gabbay, K.H., Kinoshita, J.H., Varma, S.D. and Merola, O.** 1973. Polyol accumulation in galactosemic and diabetic rats: control by an aldose reductase inhibitor. Science **182**:1146—1148.
3. **Gabbay, K.H.** 1973. The sorbitol pathway and the complications of diabetes. N. Engl. J. Med. **228**:831—836.
4. **Handelsman, D.J. and Turtle, J.R.** 1981. Clinical trial of an aldose reductase inhibitor in diabetic neuropathy. Diabetes. **30**:459—464.
5. **Judzewitsch, R., Jaspan, J.B., Pfeifer, M.A., Polonsky, K.S., Halar, E., Vukadinovic, C., Richton, S., Gabbay, K., Rubenstein, A.H. and Porte, D., Jr.** 1981. Inhibition of aldose reductase improved motor nerve conduction velocity in diabetics. Diabetes. **30 (Suppl.1)**:118.
6. **Ochs, S.** 1982. Axoplasmic transport and its relation to other nerve functions. John Wiley and Sons.
7. **Otsuka, M., Konishi, S. and Takahashi, T.** 1972. The presence of a motoneuron - depolarizing peptide in bovine dorsal roots of spinal nerves. Proc. Jpn. Acad. **48**:342—346.
8. **Peterson, M.J., Sarges, R., Aldinger, C.E. and MacDonald, D.P.** 1979. A novel aldose reductase inhibitor that inhibits polyol pathway activity in diabetic and galactosemic rats. Metabolism. **28**:456—461.
9. **Schmidt, R.E., Matschinsky, F.M., Godfrey, D.A., Williams, A.D. and McDougal, J.D.B.** 1975. Fast and slow axoplasmic flow in sciatic nerve of diabetic rats. Diabetes. **24**:1081—1985.
10. **Stewart, M.A., Sherman, W.R. and Anthony, S.** 1966. Free sugars in alloxan diabetic rat nerve. Biochem. Biophys. Res. Commun. **22**:488—491.
11. **Terashima, H., Hama, K., Yamamoto, R., Tsuboshima, M., Kikkawa, R., Hatanaka, I. and Shigeta, Y.** 1984. Effects of a new aldose reductase inhibitor on various tissues in vitro. J. Pharmacol. Exper. Ther. **229**:226—230.

Fig. 1. Immunofluorescent microphotographs of DBH immunoreactivities in the ligated ischiadic nerves. The left plate shows the ischiadic nerve of a control rat and the ligation point is on the left side. DBH immunoreactivity in the ischiadic nerve of a control rat is intensely observed along the axon. The right plate shows the ischiadic nerve of an experimental model of alloxan-induced diabetes. DBH immunoreactivity is shortened in an accumulation length from a ligation point to a distal end of a series of fluorescence and do not come into line along the axon.

Fig. 2. Immunofluorescent microphotographs of substance P immunoreactivities in the ligated ischiadic nerves. The ligation point is on the right side. The upper plate shows the ischiadic nerve of a control rat and substance P immunoreactivity is observed along the axon. The middle plate shows the ischiadic nerve of a streptozotocin-induced diabetic rat. Substance P immunoreactivity do not get into line along the axon and an accumulation pattern is abnormal appearing as irregularly shaped masses. The lower plate represents the ischiadic nerve of a disease model treated with the aldose reductase inhibitor. An abnormal distribution pattern as shown in Fig. 2-DM has gone.

Fig. 3. Immunofluorescent photographs of DBH immunoreactivities of the ligated ischiadic nerves. The upper plate shows that of a control rat, the middle one shows that of a diabetic rat and the lower one shows that of a diabetic model treated by the aldose reductase inhibitor. In the ischiadic nerve of a diabetic rat, an accumulation length of DBH immunoreactivities is short and its pattern is irregular when compared with that of a control rat. This abnormal immunoreactive pattern is improved in a diabetic rat treated with the aldose reductase inhibitor.

Fig. 4. Immunofluorescent microphotographs of VIP immunoreactivities in the ligated ischiadic nerves. The ligation point is on the right side. The upper plate shows the ischiadic nerve of a control rat and VIP immunoreactivity is intensely observed along the axon. The middle plate shows the ischiadic nerve of a streptozotocin-induced diabetic rat. VIP immunoreactivity is weakly observed in irregular shapes. The lower one shows that of a diabetic rat treated with the aldose reductase inhibitor. Fine sports of VIP immunofluorescence are observed and an accumulation length of a diabetic rat with the treatment is longer than that of non-treatment diabetic rat.

Fig. 5. Immunofluorescent microphotographs of somatostatin immunoreactivities in the ligated ischiadic nerves. The left plate shows the ischiadic nerve of a control rat, the middle one shows that of a diabetic rat and the right one shows that of a diabetic rat with treatment by the aldose reductase inhibitor. Somatostatin immunoreactivity of diabetic rat with treatment do not show much difference from that of non-treatment diabetic rat.

Fig. 6. Accumulation lengths of immunoreactive positiveness in the ligated ischiadic nerves. SP: substance P, ST: somatostatin.

Fig. 1.

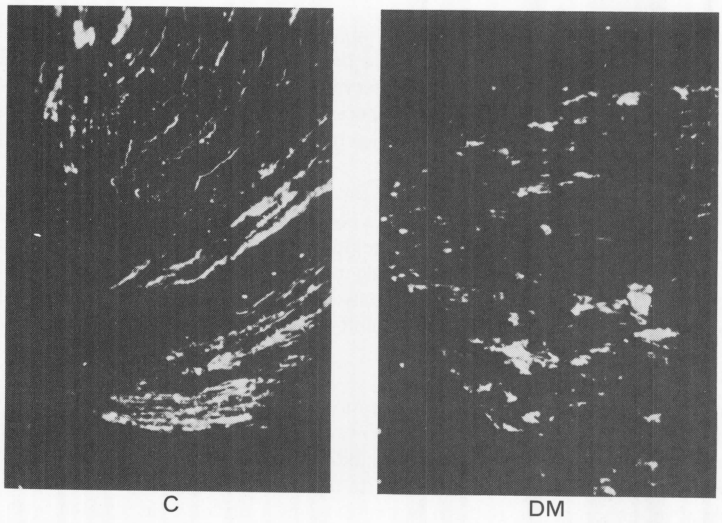


Fig. 2.

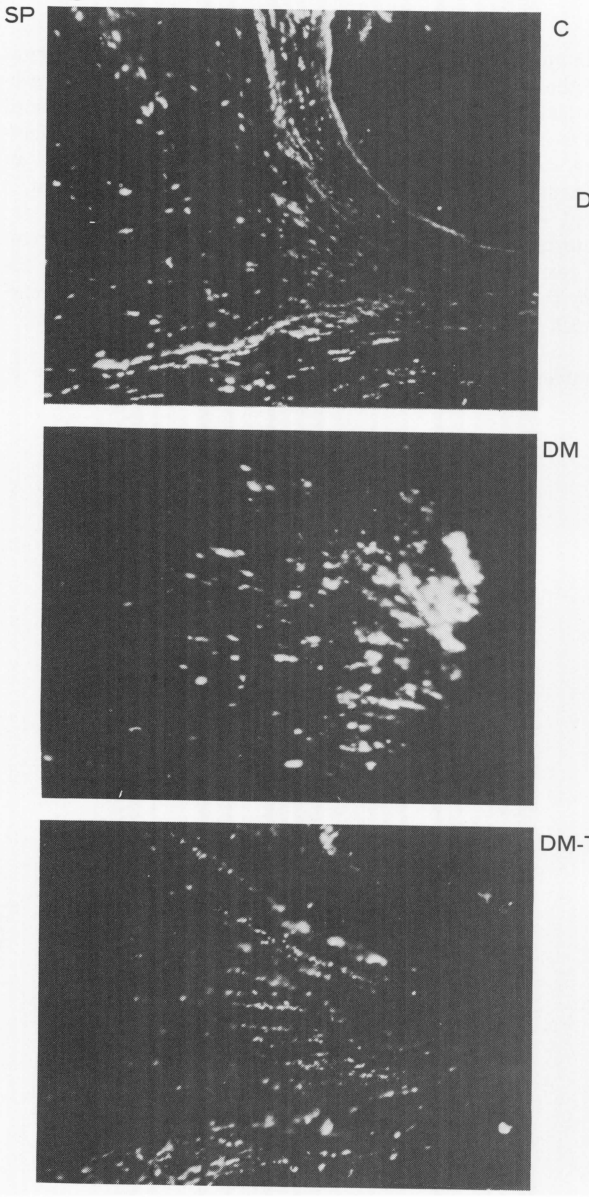
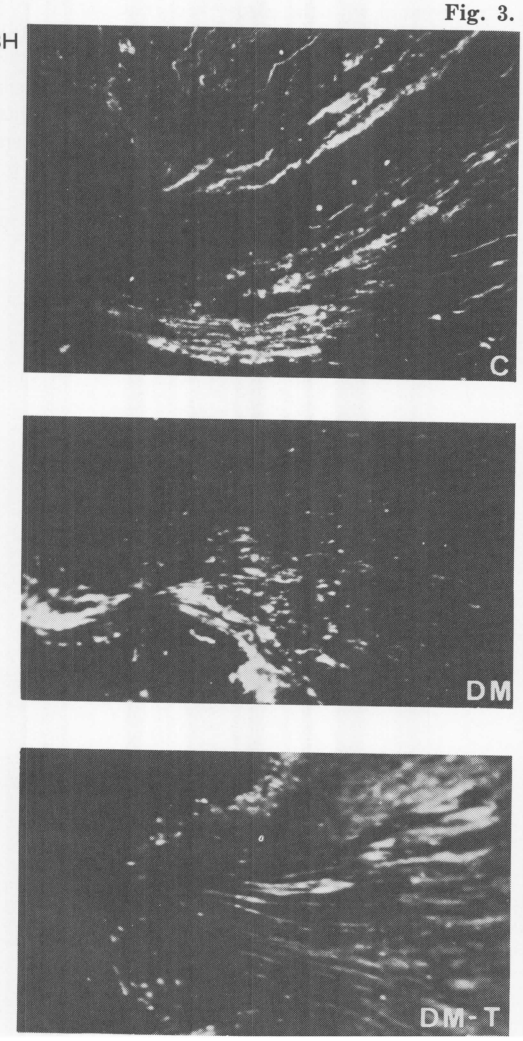


Fig. 3.



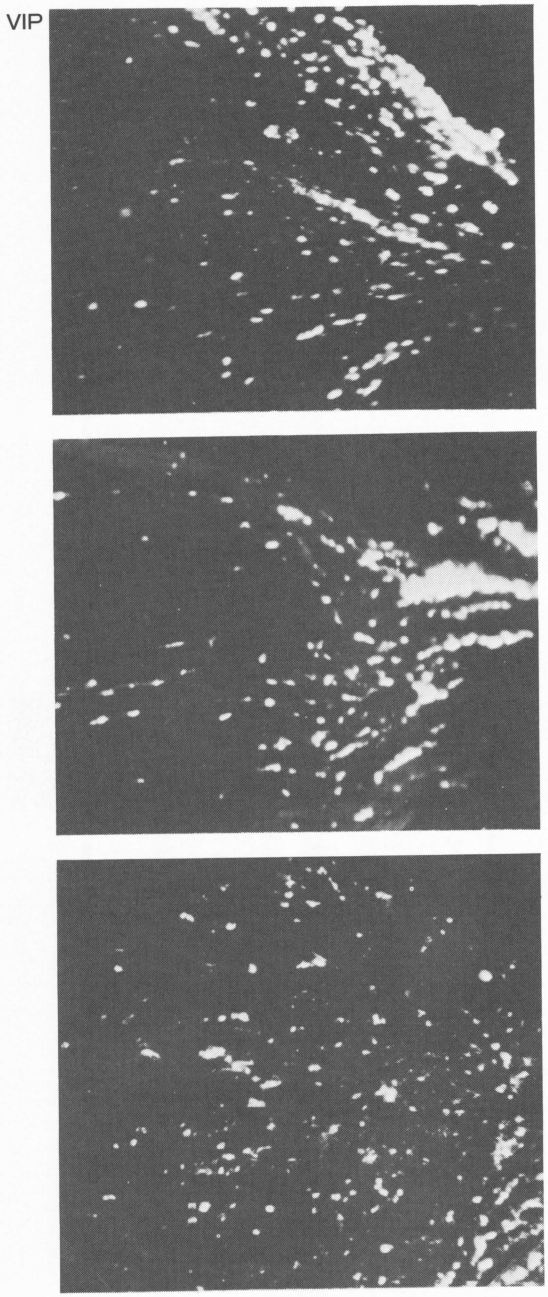


Fig. 4.

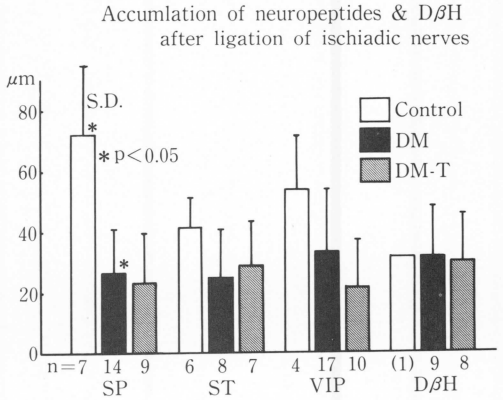


Fig. 6.

Fig. 5.