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The role of fibroblasts and macrophages of the inner nerve sheath in the healing of diabetic peripheral nerves

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Abstract: Peripheral neuropathy is a serious diabetic complication. It is accompanied by progressive nerve fiber loss, which may be attributed to ongoing axonal degeneration of the Wallerian type or dying back type or secondary to severe demyelination. To study the role of fibroblasts and macrophages of the inner nerve sheath in the healing of diabetic peripheral nerves, sixty-six albino male rats aged between 30 and 40 days (weighing 200 g to 250 g) were used in the present study. The right and left sural nerves of 60 rats were obtained from diabetic animals. The right and left sural nerves of 6 normal rats were used as a control. Three weeks after induction, the endoneurium showed channel-like spaces that were lined by fibroblast-like cells and collagen bundles. These channels contained degenerated myelin and were connected with the perivascular and subperineurial spaces. Some of the flattened fibroblast-like cells were arranged in several layers in the subperineurial and perivascular spaces, forming barrier-like cellular sheets localizing the endoneurial edema in these spaces. Fibroblast-like cells also wrapped the regenerating nerve fibers by their branching cytoplasmic processes. At the end of the third week, the flattened fibroblasts formed nearly continuous sheets in the subperineurial and perivascular spaces. Macrophages were frequently noticed between these cellular barrier-like sheets and in the subperineurial and perivascular spaces. Conclusion: The endoneurial fibroblast-like cells form barrier-like cellular sheets that localize endoneurial edema in the subperineurial and perivascular spaces and create endoneurial channel-like spaces containing degenerated myelin and endoneurial edema, helping resolve such edema.

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Literature review

Diabetic peripheral neuropathy (DPN) shows more severe physiological and anatomical changes in insulin-dependent diabetes than in noninsulin-dependent diabetes in animals and humans (Dyck and Giannini 1996, Summer et al., 2003).

More than 50% of diabetic patients show different types of peripheral nerve impairment, ranging from mononeuropathy, plexopathy, mononeuritis multiplex and distal symmetric sensory-motor polyneuropathy. The most common distal polyneuropathy is associated with a spectrum of functional and structural changes in peripheral nerves, such as a slowing in nerve conduction velocity (NCV), axonal degeneration, paranodal demyelination and loss of myelinated fibers (Sugimoto et al., 2000 (Vinik et al., 2000).

Decreased Na+, K+-ATPase activity in peripheral nerves and reduced intraepidermal nerve fiber density associated with impaired nociceptive threshold are well characterized both in humans and in different experimental models of diabetic neuropathy, such as streptozotocin (STZ)-treated rats (Berry, 1997), (Bianchi et al., 2004), (Biessels et al., 1999), (Eckersley, 2002), (Lauria et al., 2003), (Lauria et al., 2005), (Periquet et al., 1999). Moreover, it has recently been demonstrated that the gene expression of P0 and PMP22 is also affected in this experimental model (Leonelli et al., 2007). STZ-induced diabetes produces several morphological alterations in the myelinated fibers of the peripheral nerves. These alterations include myelin invaginations in the axoplasm (infoldings) and myelin evaginations in the Schwann cell cytoplasm (outfoldings) as well as alterations in myelin compaction, such as abnormally wide incisures and abnormal separation of myelin lamellae. Similar to what was observed in aged rats, the most abundant myelin abnormality observed in STZ rats was the presence of myelin infoldings in the axoplasm (Veiga et al., 2006).

In diabetic neuropathy, many of these facets of nerve function are defective. Hypoxia,

hyperglycemia, and increased oxidative stress contribute directly and indirectly to Schwann cell dysfunction (England and Asbury, 2004). The results include impaired paranodal barrier function, damaged myelin, reduced antioxidative capacity, and decreased neurotrophic support for axons. (Lauria et al., 2005) and (Veiga et al., 2006).

Streptozotocin-induced diabetes is the most widely studied experimental model of human diabetic neuropathy. In addition to hyperglycemia and reductions in nerve conduction velocity (Biessels et al., 1999), this model shares a number of disorders, including the accumulation of polyol pathway metabolites (Yamagishi et al., 2003). In streptozotocin models, reduced axonal caliber has been proposed to have a role in the reductions in nerve conduction velocity. Although axonal caliber reductions have been reported, they are observed only after 6–8 weeks of streptozotocin diabetes (Park et al., 2007) despite decreased nerve conduction velocity as early as 7 days (Malik et al., 2005) and (Hotta et al., 2006).

These systematic studies demonstrated that peripheral nerve pathology in diabetic patients is characterized by progressive nerve fiber loss with a panmodal fiber size pattern (Dyck and Giannini 1996) (Dyck et al., 2000) (Polydefkis et al., 2003) (Sumner et al., 2003). At the early stage of diabetes, microangiopathic changes are detected before apparent nerve fiber loss (Thrainsdottir et al., 2003) (Malik et al., 2005).

One particular characteristic of diabetic neuropathy is the presence of endoneurial microangiopathy that parallels nerve fiber loss. There are thickened and multiplicated basement membranes and swollen endothelial cells, similar to those encountered in other sites in diabetic patients, including the skin, eye, kidney, and muscles. A significant correlation of endoneurial vessels basement membrane thickness with reduced myelinated nerve fiber density was also demonstrated, suggesting a close correlation of microangiopathy with the development of diabetic neuropathy (Yagihashi 1995).

In diabetic nerves, all complicated degenerative processes are found depending on the nature of the injury. On the other hand, to prevent progressive nerve fiber loss and inhibit the development of neuropathy, fundamental treatment based on pathogenetic mechanisms is essential (Yagihashi et al., 2007).

2. Materials and Methods

2-1 **The experimental animals**:

Sixty-six albino male Sprague–Dawley rats aged between 30 and 40 days (weighing 200 g to 250 g) were used in the present study. The rats were housed in department animal quarters with controlled temperature and humidity. The light schedule was 14-h light and 10-h dark (lights on at 06:30 h). The animals were handled with the approval of the Institutional Animal Use and Care Committees. Six animals were used as a normal control (Group 1). The right and left sural nerves of 60 rats were obtained from diabetic animals. The right and left sural nerves of the 6 normal rats were used as a control.

2-2 Induction of diabetes:

Diabetes was induced in 60 animals by a single i.v. injection of freshly prepared streptozotocin (STZ) (65 mg/kg; Sigma, Milano, Italy) in 0.09 M citrate buffer at pH 4.8 after overnight fasting (Group 2).

Control animals (6 animals) were injected with 0.09 M citrate buffer at pH 4.8 (Group 1). Hyperglycemia was confirmed 48 h after STZ injection by measuring tail vein blood glucose levels using a Glucomen tester (Ames Glucostix). For all invasive measurements, anesthesia was induced by 2 ml/kg, i.p. of a solution containing pentobarbital 12.5 mg/ml and diazepam 1.25 mg/ml in 0.9% saline. When the toe or tail pinch elicited withdrawal reflexes, anesthetic supplements were given as needed to maintain anesthetic depth. Pulmonary secretions in anesthetized animals were minimized with 1.2 mg/kg atropine, i.p., and core temperature was maintained at 378°C using a heating pad, rectal probe and temperature controller. Only animals with mean plasma glucose levels above 300 mg/dl were classified as diabetic. Glycemia was also confirmed before treatment with steroids (6 months after STZ injection) and tested at scheduled euthanasia 7 months after STZ.

2-3. Tissue removal and preparation:

The sural nerves of the right and left sides of all groups were treated in an identical manner. Following dissection, the samples were immediately immersed in buffered 2.5% glutaraldehyde for a period of 24 h at 10 degrees centigrade. Secondary fixation was performed using 2% osmium tetroxide prior to dehydration in an ascending alcohol series with eventual embedding in epoxy resin. Transverse sections of 1-micrometer thickness that included the entire nerve surface area were cut from the epon blocks using glass knives. Semithin sections were stained with 1% toluidine blue in borax. Transverse ultrathin silver-colored sections of 0.06-micron thickness were cut from the blocks using a diamond knife. The samples were stained with uranyl acetate and lead citrate prior to examination with a transmission electron microscope.

2-4. The ultrastructural study:

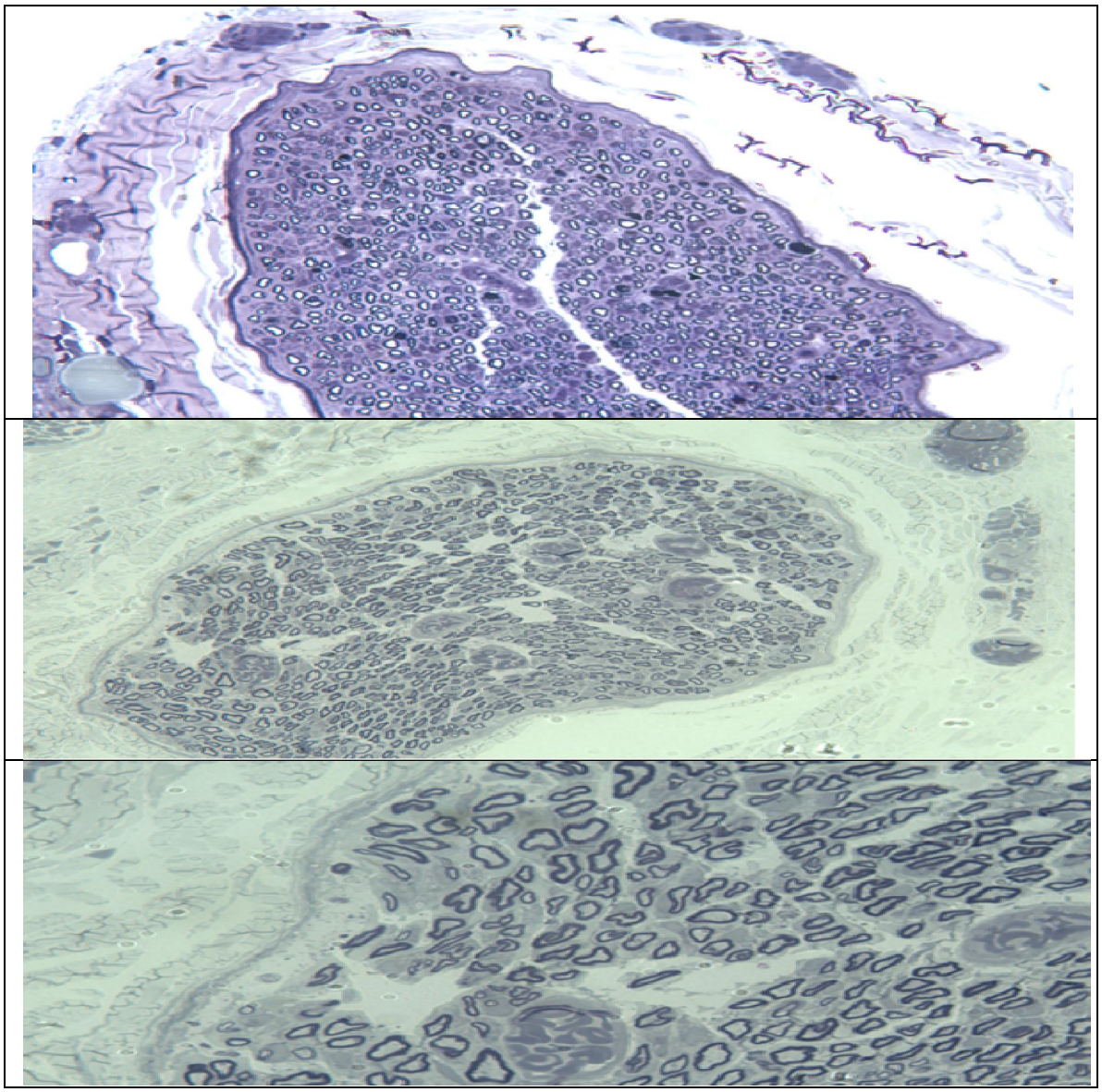
Ultrathin sections of all sural nerve specimens were assessed under scanning electron microscopy for ultrastructural changes in Schwann cells, myelinated axons and myelin sheaths.

Results:

Ultrastructure of the Peripheral Nerve

The external epineurium, which constitutes the most superficial layer of the peripheral nerves, is a dense connective tissue, the surface of which is nourished by a thin vascular plexus known as the vasa nervorum. Normal peripheral nerves freely glide in the adjacent soft tissues during movement; this plane is a potential space for disease infiltration.

Deep within the epineurium, the internal epineurium provides loose connective tissue for the individual fascicular bundles surrounded by the perineurium. Deep within the perineurium, the endoneurium surrounds the individual nerve fibers or axons. Despite the term *perineural,* the spread of disease does not always follow the perineurium. Although somewhat controversial, perineural spread of malignancy is thought to occur on the surface of the nerve in the low resistance plane between the external epineurium and the adjacent soft tissues or throughout the substance of the nerve with breach of the external epineurium. (Fig 1).



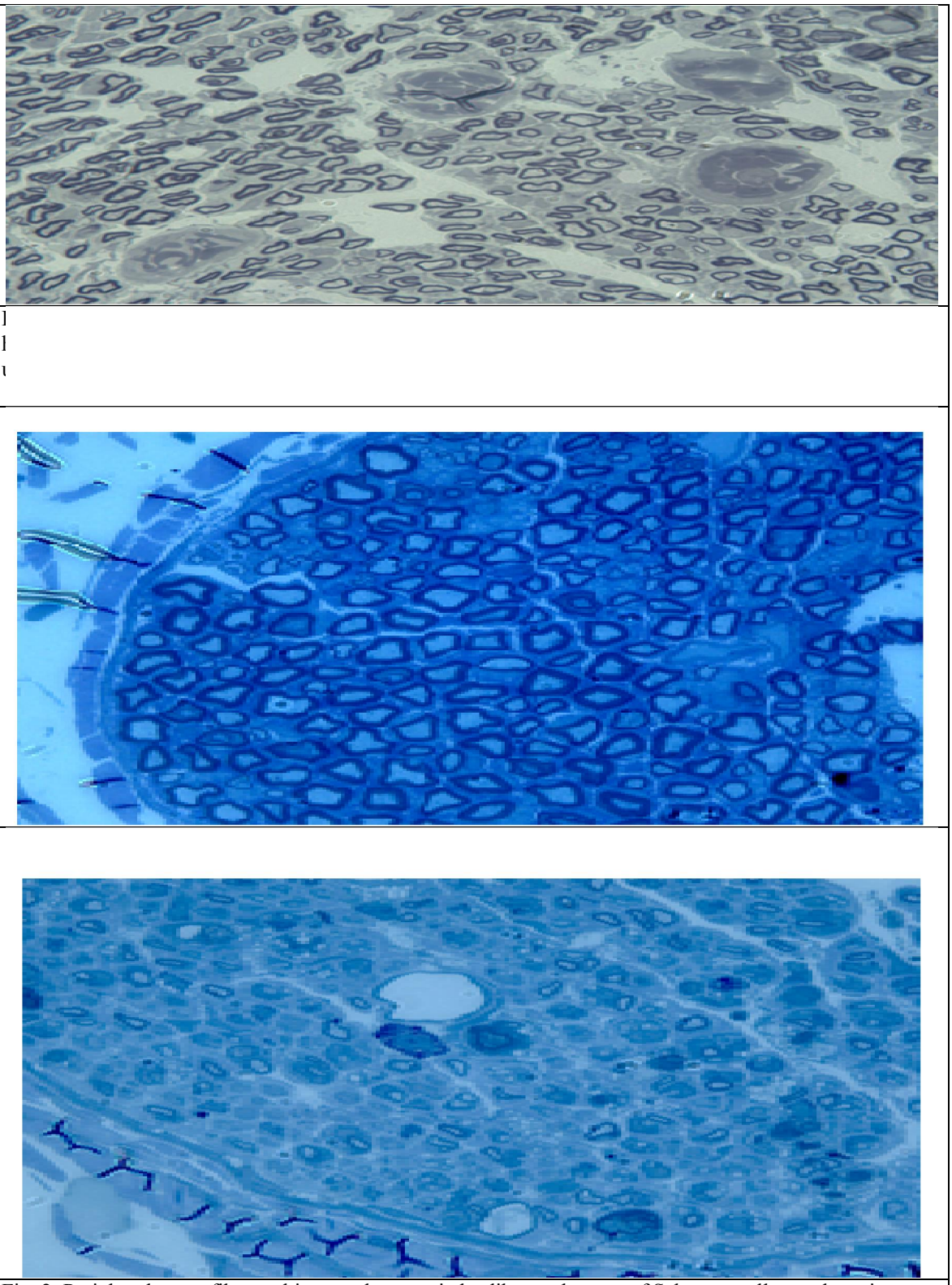


Fig. 2. Peripheral nerve fiber architecture have varied caliber and extent of Schwann cell cytoplasm in normal rats in (A) but macrophages and lymphocytes are seen in the nerves in diabetic rats in (B).

Ultrastructural observations of the connective tissue sheaths of peripheral nerves and ganglia showed that the epineurial collagen fibrils are densely packed, oriented in different directions and associated with irregularly distributed fibroblasts. The endoneurium displays loosely packed, longitudinally disposed bundles of collagen fibrils located mainly in the triangular spaces limited by the nerve fibers; they are associated with very few fibroblasts (Fig. 3).

Discussion

The new findings of the current study are as follows:

(26) Fibroblasts have a potential therapeutic effect in ameliorating myelin pathology and increasing the expression of myelination in a diabetic T2DM model, and (27) this beneficial effect is probably due to the fibroblast-mediated increase in apoptosis . These findings highlight that fibroblasts, as an available therapeutic strategy, ameliorate the pathological demyelination changes during the pathogenesis of T2DM.

Long-term exposure to elevated levels of glucose gives rise to chronic demyelination, a notable characteristic of neuropathy that seriously damages myelin, axons, and neuron cell bodies. In the PNS, axonal demyelination is closely associated with Schwann cell abnormalities because myelin formation depends on Schwann cell development and maturation to wrap around axons. In addition, proliferating Schwann cells are capable of providing abundant neurotrophins and oxygen to support axonal regeneration. Under diabetic conditions, Schwann cells are vulnerable to glucotoxicity-induced damage or apoptosis, which deteriorates myelin splitting and myelin shedding, leading to axonal dysfunction and demyelination. Previous reports have shown that hyperglycemia-induced dysfunction and toxicity are associated with metabolic alterations and/or inflammatory aggravation (29)

Moreover, SC proliferation and dedifferentiation are requisites for myelin generation and maintenance (30). Therefore, we attempted to restore myelin disorganization and maintain normal myelin morphology by improving and activating intrinsic reprogramming. (31) Fibroblasts play multiple regulatory roles in the biological processes of cellular survival, proliferation and migration. Previous studies by our group and others have confirmed that fibroblasts possess striking neuroprotective and neuroregenerative effects in the nervous system. (32-33).

Conclusion

Based on the results, we speculate that fibroblasts play a positive role in improving myelin pathology in diabetic hyperglycemia. The role of macrophages warrants further investigation by measuring oxidation and immunity markers.

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Disclosure and Conflicts Statement

The authors declare no conflicts of interest.

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