Perineurial Cells Coexpress Genes Encoding Interstitial Collagens and Basement Membrane Zone Components

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Abstract. Perineurial cell cultures were established from the sciatic nerves of adult Wistar rats. Highly enriched cultures were studied with respect to the production of extracellular matrix components under conditions free from the influence of Schwann cells, axons, or the extracellular matrix of peripheral nerves. Indirect immunofluorescence staining revealed the presence of collagen type IV epitopes, and electron microscopy demonstrated patches of basement membrane on the perineurial cell surfaces. Collagenous fibrils with a diameter of 15-20 nm were also observed in the intercellular space. SDS-PAGE of radiolabeled medium proteins showed a pattern of bands suggesting the synthesis and secretion of fibronectin, and type I and IV collagens. Northern hybridizations revealed characteristic polymorphic mRNA transcripts corresponding to fibronectin, laminin B2 chain, as well as to the \( \alpha \)-chain subunits of type I, III, and IV collagens. Furthermore, in situ hybridizations suggested expression of these genes by cultured perineurial cells without apparent heterogeneity within the cell populations. In situ hybridizations of sciatic nerve tissue from 2-wk-old rats also suggested that perineurial cells express \( \alpha \)I(1) and \( \alpha \)2(IV) collagen, as well as laminin B2 chain genes in vivo. This profile of matrix gene expression is different from that of Schwann cells, which do not synthesize fibronectin, or that of fibroblastic cells, which do not form a cell surface basement membrane. The capability of perineurial cells to express genes for the basement membrane zone and for interstitial collagens further adds to our understanding of the functional role of perineurial cells in developing and healing peripheral nerve, as well as in certain neoplastic lesions of neural origin, such as von Recklinghausen's neurofibromas.

The Schwann cell-axon units and the endoneurial connective tissue of peripheral nerves are surrounded by the tubular perineurium (14, 16, 49). It isolates the individual nerve fascicles from the epineurial connective tissue, and constitutes the basis for the maintenance of endoneurial homeostasis (24, 34), which is essential for the proper function of normal peripheral nerves. This selective barrier is, in part, a result of the filter function (12) of exceptionally thick basement membranes which surround the perineurial cell layers on both sides (16, 49). The skeleton of these basement membranes is formed by type IV collagen which associates with noncollagenous glycoproteins, such as laminin and fibronectin (30, 54).

In nerve trauma, the perineurial cells play an important role already during the first few days of Wallerian degeneration (33) as well as during the subsequent regeneration (22, 41). The perineurial cells react to trauma by losing their close intercellular contacts, they begin to proliferate, and collagenous fibrils accumulate between the perineurial cell layers (22, 33, 50, 51). The nerve fascicles become compartmentalized by perineurial cells which initially lack basement membrane, but subsequently achieve the characteristics of mature perineurial cells (31, 51). It has been speculated that the typical perineurial cell phenotype is determined by the stimulus of Schwann cells and axons since, in developing and regenerating nerves, the perineurial cells are derived from cells morphologically resembling fibroblasts (15, 50, 51). On the other hand, Radek et al. (41) emphasize the prominent influence of extracellular matrix components in determining the differentiation of the perineurial cells in interchange grafts of rat peripheral nerve and spinal root. However, our recent demonstrations that perineurial cells can be maintained in culture in the absence of other cell types (38), emphasize the independent entity of these cells.

The present study was designed to elucidate the metabolic capacity of perineurial cells to express the genes coding for interstitial collagen types I and III, and the basement membrane zone components, i.e., type IV collagen, laminin, and fibronectin, under conditions free from the influence of Schwann cells and axons. These extracellular matrix proteins are essential components of normal peripheral nerve and undergo profound quantitative changes during the healing process (2, 11, 36, 43-45).
Materials and Methods

Cell Cultures

Perineural cell cultures were established from sciatic nerves of adult Wistar rats as described previously (38). For in situ hybridizations, the nerve pieces were explanted or the cells were plated onto acetylated slides (4, 18). The cultures were maintained in DME, which contained antibiotics (100 U/ml penicillin-G and 50 μg/ml streptomycin sulphate), and 10% fetal calf serum (Flow Laboratories, Inc., McLean, VA).

Control fibroblasts were cultured from rat skin and from those nerve pieces which initially gave only a fibroblastic cell outgrowth.

Indirect Immunofluorescence and Transmission Electron Microscopy

For immunofluorescence staining, the following antibodies were used. Rabbit antibodies against human plasma fibronectin (AXL 368) were obtained from Accurate Chemical and Scientific Corporation (Westbury, NY). Rabbit IgG to 7-S fragment of human type IV collagen (42) was kindly provided by Dr. Leila Risteli, University of Oulu (Oulu, Finland). TRITC-conjugated goat anti-rabbit IgG (2212-0081) was purchased from Cappel Laboratories (Cochranville, PA) and Cooper Biomedical, Inc. (Malvern, PA). For transmission electron microscopy, cultured cells were fixed and prepared as described earlier in detail (38).

Isotopic Labeling Procedures

Essentially pure perineural cell cultures or cocultures containing ~20–40% fibroblasts together with perineurial cells were labeled in the second through sixth passage with radioactive amino acids, as follows: culture medium was first supplemented with ascorbate (50 μM/ml) for 24 or 48 h before and during the labeling period. Some cultures were labeled with 5 μCi of L-[U-14C]-amino acid mixture (Amersham International, Amersham, UK) in 1.5 ml of DME. Other cultures were incubated with 100 μCi of L-[2,3-3H]proline (New England Nuclear, Boston, MA) in 1.5 ml of DME without glutamine. After 14 h of labeling, the medium was collected, dialyzed exhaustively against distilled water for 3 d and lyophilized. [3H]Hydroxyproline was assayed by a radiochemical method (23).

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to O'Farrell (32). Lyophilized medium proteins were dissolved in sample buffer containing 2-mercaptoethanol, heated at 100°C for 1 min, and separated on 5–22% or 5–11% polyacrylamide gradient gels.

RNA Isolation and Northern Transfer Analysis

Total RNA was isolated according to the single-step method described by Chomczynski and Sacchi (8). RNA specimens were separated on 0.9% agarose gels under denaturing conditions, and transferred to nitrocellulose filters (53). The following cDNAs were used for hybridizations: mouse 1.8- and 1.1-kb cDNAs, pPE123 and pPE19, coding for amino acid sequences within α(I)IV and α2(IV) collagen chains, respectively (3, 25, 26); rat 1.3- and 0.9-kb cDNAs, p5RI and p2CR2, corresponding to amino acid sequences within α(I) and α2(II) chains of type I collagen (17); a mouse 0.5-kb cDNA, Mpi J1, complementary to the 3' untranslated region of α(I)III of type III procollagen mRNA; a rat 0.5-kb cDNA, prl1, coding for amino acid sequences at the COOH terminus of fibronectin (47); and a mouse 0.675-kb cDNA, pPE9, coding for the COOH-terminal end of the laminin B2 chain and 20 bases of 3' untranslated region of the corresponding mRNA (1, 3).

The probes were labeled radioactive either with [32P]dCTP or [35S]-dATP by nick translation to a specific activity of at least 1 × 108 cpm/μg (29). The filters were prehybridized and hybridized in solution containing 50% formamide, 4 × SSC, 0.1% SDS, 200 μg/ml denatured salmon sperm DNA, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% ficoll at 37°C for 16–24 h. When homologous rat probes were used, the filters were washed at 60°C to the final stringency of 0.1× SSC containing 0.1% SDS. When mouse cDNAs were used, the final stringency was 0.2× SSC plus 0.1% SDS at 52°C.

After washings, the filters were kept moist in sealed plastic bags and exposed to x-ray films (Eastman Kodak Co., Rochester, NY, X-Gma) for 4–10 d at −70°C. The filters were washed in aqueous solution containing 0.1% SDS at 90°C for 10 min. Reexposure of the filters revealed that the radioencoded cDNA had been completely removed. The filters were then prehybridized and hybridized with another cDNA labeled radioactive by nick translation. This procedure was repeated four times with the same filter using different cDNAs.

In Situ Hybridizations

5-μm cryosections were cut from snap-frozen sciatic nerves of 2-wk- or 10-mo-old Wistar rats, and fixed immediately with fresh 4% paraformaldehyde in phosphate-buffered saline for 20 min. Cultured cells were fixed and permeabilized in 100% ethanol for 15 min at −20°C, followed by fixation in 4% paraformaldehyde at room temperature. The samples were then pre- treated as described previously (39). Just before hybridizations the samples were heated at 90°C for 5 min and cooled rapidly on ice.

The samples were hybridized for 16 h at 42°C in solution containing 0.1 μg/ml 32P-labeled cDNA probe (see above), 50% formamide, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.6 M NaCl, 10% (wt/vol) dextran sulphate, 200 μg/ml denatured and sheared salmon sperm DNA, 0.5 mM EDTA, 0.02% (wt/vol) ficoll, 0.02% (wt/vol) polyvinyl pyrrolidone, and 10 mM Tris-HCl, pH 7.4. After the hybridizations, the samples were washed as described (39), the final stringency being 0.2× SSC at 42°C.

The [35S]cDNA-mRNA hybrids were detected by dipping the samples into Kodak NTB-3 autoradiography emulsion diluted with an equal volume of 0.6 M ammonium acetate, and exposing them in a desiccant-containing box for 3–5 d at 4°C. The samples were developed with Kodak D-19 developer, stained with hematoxylin, and dehydrated with ethanol.

Results

Characterization of the Extracellular Matrix of Cultured Perineural Cells

Cell cultures consisting predominantly (70–99% of the total cell population) of perineural cells were established from adult rat sciatic nerves, and the cells were identified by criteria recently described by us (38). Cultured perineural cells were then examined with particular reference to the extracellular matrix. Indirect immunofluorescence with antibodies to the 7-S fragment of type IV collagen revealed a patchy distribution of the epitopes on the perineurial cells (Fig. 1 a). Ultrastructural analysis by transmission electron microscopy demonstrated the presence of cells with basement membrane structures on their surface (Fig. 1 b). Distinct fibrils with apparent diameters of 15–20 nm were also noted between the cell layers (Fig. 1 b); these may be representative of the fibrillar extracellular collagen.

Synthesis of Collagenous Proteins

The biosynthetic capacity of the perineural cells was examined in cultures which consisted of essentially pure (~99%) perineural cell population. To examine the synthesis of collagenous proteins, the cells were labeled with [3H]proline, and [3H]hydroxyproline was assayed by a radiochemical method (23). Clearly detectable amounts of radioactive hydroxyproline were present in the medium proteins. The ratio of [3H]hydroxyproline to total [3H]-incorporation in two separate incubations varied from 0.28 to 0.36, suggesting that a significant fraction of the proteins synthesized and secreted by perineural cells were collagenous.

To examine the newly synthesized proteins in further detail, 3H-labeled medium proteins subjected to limited pepsin proteolysis were examined by SDS-PAGE, followed by autoradiography. The results revealed distinct bands of 3H-labeled proteins with migration positions corresponding to
\( \alpha_1(1) \) and \( \alpha_2(1) \) chains of type I collagen (Fig. 2, lane 2). Additionally, two minor bands with slightly higher apparent molecular masses, corresponding to the migration positions of \( \alpha_1(5) \) and \( \alpha_2(5) \) chains of type V collagen could be visualized in some cultures (37) (Fig. 2, lane 2). A distinct band with an apparent molecular mass of 190 kD was observed in the media of perineurial cell but not fibroblast cultures (Fig. 2, lane 2). This migration position is consistent with the presence of type IV collagen polypeptides (7). These polypeptides were clearly pepsin resistant, since they were quantitatively present in samples subjected to extended (18 h) digestion with high concentration of pepsin (1 mg/ml). In samples which were not subjected to pepsin digestion, a distinct band in the migration position of fibronectin (\( \sim \)240 kD) was observed (Fig. 2, lane 1). Similar results were noted when the cells were labeled with a mixture of \( ^{14} \)C-amino acids. These findings suggest that perineurial cells are capa-
ble of synthesizing and secreting collagenous proteins, among them type I, and probably type IV and V collagens, as well as fibronectin.

**Extracellular Matrix Gene Expression**

To examine the expression of extracellular matrix genes at mRNA level, total RNA was isolated from cultured perineurial cells and subjected to Northern hybridizations with cDNAs corresponding to the α-chain subunits of type I, III, and IV collagens, as well as fibronectin and laminin B2 chain sequences (Fig. 3). Hybridizations with these cDNAs revealed characteristic patterns of polymorphic mRNA transcripts (Fig. 3). Specifically, hybridization with a cDNA corresponding to α2(I) collagen revealed two bands (4.2 and 4.5 kb) characteristic of rat α2(I) collagen mRNA transcripts (17); the cDNA corresponding to α1(IV) collagen hybridized to a 6.2- and 6.8-kb mRNA species, previously shown to be specific for α1(IV) collagen (3, 25, 26); hybridization with the cDNA for fibronectin revealed the presence of a single transcript of ~8 kb (46); the cDNA for laminin B2 chain hybridized to a 8.0-kb mRNA (1).

In situ hybridizations with the same cDNA probes were used to address the issue of whether an individual perineurial cell is capable of coexpressing genes for collagen types I, III, and IV, and fibronectin, or whether evidence of heterogeneity with respect to matrix production could be observed within these cultures. The results indicated that essentially all cells that had the phenotypic characteristics of perineurial cells contained mRNA transcripts specifically hybridizing with type I, III, and IV collagens, as well as fibronectin cDNAs (Fig. 4). Thus, on a statistical basis, it is clear that a single perineurial cell can coexpress the genes for these collagens and for fibronectin.

**Expression of Type I and IV Collagens, and Laminin B2 Chain Genes in the Sciatic Nerves**

The expression of the interstitial collagen type I and the basement membrane collagen type IV was also studied in the sciatic nerves of normal young (2-wk-old) and adult (10-mo-old) Wistar rats using in situ hybridizations. In young rats, the mRNA transcripts of α1(I) chain were clearly detectable on the perineurial cells, as well as on some of the endoneurial cells (Fig. 5, a and b). The mRNAs for α2(IV) chain of type IV collagen were localized essentially to all cells of the perineurium (Fig. 5, c and d) and to the majority of the endoneurial cells. The latter observation suggested that type IV collagen genes are actively expressed by the perineurial cells, as well as by the Schwann cells of 2-wk-old rats in vivo (Fig. 5, c and d). Similarly, evidence for laminin B2 chain gene expression in the perineurium of the same rats was obtained by in situ hybridization (Fig. 5 e). However, we were unable to demonstrate the presence of these mRNAs in specimens from 10-mo-old rats, suggesting that the expression of these genes is considerably more active during nerve development than in mature peripheral nerve.

**Discussion**

The function of perineurium as a perifascicular diffusion barrier is well established (24, 28, 34, 35, 52). The barrier function is based on tight junctions between the cells and on the prominent basement membranes which cover these cells on both sides (12, 49). In spite of the essential role of perineurium in nerve homeostasis, the exact composition, origin, and significance of the extracellular matrix between the perineurial cell layers has gained little attention.

During the early nerve development, cells forming peri-
Figure 5. In situ hybridizations of 2-wk-old rat sciatic nerves with cDNAs for \( \alpha_1(I) \) (a and b) and \( \alpha_2(IV) \) (c and d) collagen, and laminin B2 chain (e). (a) The presence of \( \alpha_1(I) \) collagen mRNA is demonstrated in the perineurial cells (arrows). (b) In endoneurium, \( \alpha_1(I) \) collagen mRNA-cDNA hybrids are detected in scattered cells some of which are in close relationship to axons (arrows) and most likely represent Schwann cells (asterisk). (c) The expression of \( \alpha_2(IV) \) chain of basement membrane type IV collagen is evident in the perineurium (arrows), and (d) in the endoneurium of a partly longitudinal section of the nerve. (e) In situ hybridization of rat sciatic nerve with cDNA corresponding to laminin B2 chain localizes the corresponding mRNAs primarily to the perineurium. en, endoneurium. Bars: (a) 20 \( \mu \)m; (b-e) 10 \( \mu \)m.

It the present study, in situ hybridizations of rat sciatic nerve with cDNA probes corresponding to \( \alpha_1(I) \) chain of type I collagen, \( \alpha_2(IV) \) chain of type IV collagen, and laminin B2 chain revealed the presence of corresponding mRNAs in perineurial cells, thus indicating active transcription of these genes in the perineuri um of a 2-wk-old rat sciatic nerve. These observations provide direct evidence for the role of perineurial cells in the synthesis of basement membrane components and fibrous extracellular matrix in a developing peripheral nerve. Thus, it appears that the presence of fibroblasts, occasionally seen in the perineurium (49), is not essential for the production of fibrous extracellular matrix between the perineurial cell layers. Type IV collagen mRNA was also present in endoneurial cells, which were most probably Schwann cells.

The rapid and profound changes observed in the perineurium after nerve trauma (22, 33, 51) have provided some evidence on the regulatory factors influencing the behavior of perineurial cells. It has thus been speculated that the perineurial cell phenotype would be, at least in part, influenced by Schwann cells and axons (51), as well as by the extracellular matrix (41).

In the present study, cultures consisting of essentially pure populations of perineurial cells were examined with respect to extracellular matrix production. The molecular hybridizations with mouse or rat cDNAs were shown to be specific in Northern transfer analysis, and the distribution of mRNAs in these cultures was detected by in situ hybridizations. The results indicated that cultured perineurial cells expressed the genes coding for fibrillar collagen types I and III, fibronectin, and the B2 chain of laminin, a noncollagenous protein of basement membranes. It is of interest that laminin has been shown to display neurite growth-promoting activity (10). This evidence clearly attests to the capacity of perineurial cells to synthesize the interlamellar matrix. In addition to the genes for fibrillar collagens, cultured perineurial cells expressed the gene for the \( \alpha_1(IV) \) chain of type IV collagen (54). Perineurial cells are thus capable of synthesizing and secreting basement membrane components, as well as assembling basement membrane structures on their surface in pure cultures. The regulatory signal may be autocrine in nature or could be influenced by cell–cell contacts between adjacent perineurial cells.

Our results, taken together with earlier studies on neural cells (5, 6, 7, 9, 13), indicate that perineurial cells differ from Schwann cells and fibroblasts with respect to their profile of extracellular matrix gene expression. A major common feature of perineurial and Schwann cells is their capability to express basement membrane protein genes and to form an organized basement membrane structure on their surface (6, 38). This feature distinguishes them from fibroblastic cells. On the other hand, Schwann cells and perineurial cells can produce fibrillar collagen types I and III (5, 6). This characteristic is shared with fibroblasts and is thus a common feature for all three types of connective tissue cells in the peripheral nerve. The capacity to synthesize fibronectin, however, distinguishes perineurial cells from Schwann cells which produce very low levels, if any, of fibronectin (13).
Several pathologic situations attest to the importance of perineurial cells in the maintenance of proper neural structure and function. An example of altered barrier function may be presented by diabetic neuropathy. Even though the thickness of the perineural basement membranes increases, there is a local loss and destruction of the perineural cells (19–21). These findings may explain the increased permeability of the perineurium (48), and may in part contribute to the poorly understood sequence of events leading to the development of diabetic neuropathy. The role of perineurial cells in the growth and development of neurofibromas, the cutaneous hallmark of type 1 (von Recklinghausen’s) neurofibromatosis is also of considerable interest. Specifically, neurofibromas contain an abundant extracellular matrix composed of both the fibrillar collagen, types I and III, and the basement membrane collagen, type IV (27, 37, 39, 40, 55).

Previous ultrastructural analyses (27) and our recent immunocytochemical observations (39) have suggested that perineurial cells play an important role in the formation of neurofibromas. Thus, based on the results of the present study demonstrating the expression of genes coding for various collagen, fibronectin, and laminin, it is conceivable that perineurial cells contribute, together with fibroblasts and Schwann cells, to the excessive accumulation of the extracellular matrix in neurofibromas.

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