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Regeneration-associated high level expression of apolipoprotein D mRNA in endoneurial fibroblasts of peripheral nerve

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A cDNA clone containing the entire coding region of rat apolipoprotein D (Apo D) was isolated from a cDNA library of regenerating sciatic nerve by differential hybridization. Only small amounts of Apo D mRNA were detected in noninjured mature nerve. Moderately increased levels of Apo D transcripts were found in transected nerves, which were prevented from regeneration by ligation. In contrast, in regenerating crushed nerve, the steady-state level of Apo D mRNA transiently increased at least 40-fold above control levels at the time when axons from the proximal stump grow into the distal nerve segment. Using transverse sections and primary cell cultures from regenerating nerve, Apo D transcripts could be localized by *in situ* hybridization in endoneurial fibroblasts but not in Schwann cells, macrophages or perineurial and epineurial cells. Apo D protein (M_r 32.8 kd) was secreted and accumulated in the endoneurial extracellular space where it could be detected in lipoprotein fractions by immunoblotting using established antibodies to human Apo D. High level expression of Apo D mRNA seems to be a novel regeneration-associated molecular event of endoneurial fibroblasts indicating a function for Apo D and fibroblasts in nerve repair.

Key words: apolipoprotein D / endoneurial fibroblasts / intraneurial lipoprotein/nerve regeneration/rat sciatic nerve

Introduction

Interruption of axons in the mammalian peripheral nervous system by injury or disease leads to the initiation of a stereotyped sequence of histopathological events called Wallerian degeneration (for reviews: see Sunderland, 1978; Lubinska, 1977). The highly predictable and reproducible degenerative as well as subsequent regenerative responses presumably reflect an underlying sequence of specific molecular and cellular reactions.

The importance of the local environment for successful axonal regeneration has long been suspected (Ramon y Cajal, 1928) and later supported by transplantation experiments (Aguayo *et al.*, 1979; Richardson *et al.*, 1980; Weinberg and Raine, 1980; Kromer and Cornbrooks, 1985). Recently, the differential expression of Schwann cell genes encoding myelin proteins, nerve growth factor (NGF), NGF-receptor and glia-derived nexin (protease nexin I) has been investigated in response to nerve injury (Lemke, 1986; Heumann *et al.*, 1987; Lindholm *et al.*, 1987; Lemke and Chao, 1988; Trapp *et al.*, 1988; Meier *et al.*, 1989) and results suggest that Schwann cell gene expression may, at least in part, be regulated by cellular interactions including axons and macrophages.

Following peripheral nerve injury large numbers of hematogenous macrophages enter the distal nerve stump (Stoll and Müller, 1986; Perry *et al.*, 1987), participate in phagocytosis and degradation of myelin membranes (Beuche and Friede, 1984; Stoll *et al.*, 1989) and express large amounts of apolipoprotein E (Skene and Shooter, 1983; Ignatius *et al.*, 1986; Müller *et al.*, 1985, 1986; Snipes *et al.*, 1986).

Endoneurial fibroblasts, a major cell type in peripheral nerve (Schubert and Friede, 1981), have been widely ignored regarding specific regeneration-associated functions. Recently, however, cultured fibroblasts from adult rat sciatic nerve have been stimulated by interleukin I, a lymphokine produced by macrophages in injured nerve, to express NGF mRNA (Lindholm *et al.*, 1988).

In the present paper we describe: (i) the cloning of a cDNA that represents a highly enriched transcript in injured nerve; (ii) the complete nucleotide sequence of the cDNA and deduced amno acid sequence, identifying this transcript as Apo D which had not previously been detected in the peripheral nervous system; (iii) the time-course and spatial distribution of increased Apo D mRNA steady state levels in regenerating and nonregenerating nerve segments using two different lesion paradigms; (iv) the cellular localization of the Apo D transcript by *in situ* hybridization in tissue sections and primary cell cultures from rat sciatic nerve; and (v) the identification of Apo D protein as a component of endoneurial lipoprotein fractions.

Results

Following the procedure of Okayama and Berg (1982, 1983), we constructed a plasmid cDNA library using poly(A)$^+$ RNA isolated from the distal stumps of rat sciatic nerves 7 days after a crush lesion. First strand cDNA was derived from poly(A)$^+$ RNA, isolated either from crushed sciatic nerve or non-injured nerve, labelled with [^{32}P]dCTP and used to screen 2000 colonies of this library by differential hybridization. Twelve individual cDNA clones were yielded, which appear to be differentially expressed in regenerating nerve. The independence of these clones was proven by cross hybridization experiments.

The cDNA clone which showed the most intense hybridization differences between poly(A)$^+$ RNA from crushed versus non-injured sciatic nerve was identified as Apo D by sequence analysis. The nucleotide sequence and the deduced amino acid sequence of rat Apo D are shown in Figure 1.

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CTCGCAGGAT TCTCCTGTGG AAACTGCCTT CATCGTATCT GAGAGGCCTC      50

TCCTGCAGCC GCCAACCCCAAG ATG GCG ACC ATG CTG TTG CTC CTG     96
                        Met Ala Thr Met Leu Leu Leu Leu

GCC ACA CTG GCA GGT CTC TTC ACC ACA ACC GAA GGA CAA AGC    138
Ala Thr Leu Ala Gly Leu Phe Thr Thr Thr Glu Gly Gln Ser

TTC CAT CTT GGG AAA TGC CCA TCT CCT CCG GTG CAA GAG AAT    180
Phe His Leu Gly Lys Cys Pro Ser Pro Pro Val Gln Glu Asn

TTC GAT GTG AAA AAG TAT CTT GGA AGA TGG TAC GAA ATC GAG    222
Phe Asp Val Lys Lys Tyr Leu Gly Arg Trp Tyr Glu Ile Glu

AAG ATC CCG GTG AGC TTT GAG AAA GGA AAC TGC ATT CAA GCC    264
Lys Ile Pro Val Ser Phe Glu Lys Gly Asn Cys Ile Gln Ala

AAC TAC TCG CTG ATG GAG AAC GGA AAC ATC AAA GTG CTA AAC    306
Asn Tyr Ser Leu Met Glu Asn Gly Asn Ile Lys Val Leu Asn

AAG GAG CTG CGT CCT GAC GGA ACC CTG AAC CAA GTT GAG GGT    348
Lys Glu Leu Arg Pro Asp Gly Thr Leu Asn Gln Val Glu Gly

GAA GCC AAA CAG AGC AAC ATG TCA GAG CCA GCC AAG CTG GAA    390
Glu Ala Lys Gln Ser Asn Met Ser Glu Pro Ala Lys Leu Glu

GTC CAG TTC TTC TCG TTG ATG CCA CCG GCA CCC TAC TGG ATC    432
Val Gln Phe Phe Ser Leu Met Pro Pro Ala Pro Tyr Trp Ile

CTG GCC ACG GAT TAC GAG AGC TAT GCC CTC GTG TAT TCC TGC    474
Leu Ala Thr Asp Tyr Glu Ser Tyr Ala Leu Val Tyr Ser Cys

ACC ACC TTC TTC TGG TTC TTC CAC GTG GAC TAT GTT TGG ATC    516
Thr Thr Phe Phe Trp Phe Phe His Val Asp Tyr Val Trp Ile

CTG GGA AGA AAT CCT TAC CTC CCT CCA GAA ACA ATA ACC TAC    558
Leu Gly Arg Asn Pro Tyr Leu Pro Pro Glu Thr Ile Thr Tyr

CTA AAA TAT ATC CTT ACT TCT AAT GAC ATC GAC ATC GCC AAA    600
Leu Lys Tyr Ile Leu Thr Ser Asn Asp Ile Asp Ile Ala Lys

ATA ACA ACA AAA GAC CAA GCA AAC TGC CCG GAC TTC CTG TAA    642
Ile Thr Thr Lys Asp Gln Ala Asn Cys Pro Asp Phe Leu ---

AGGGGGGTGG GCAACCGCTC CAGGTTATTT CTTCGCTTTG GCTCCCTGGC    692

CCCACCCCCA CTCCTCATCA GGACCGAGCA ACCCCGCCAG CACTAGAGGG    742

AAAGTATTGC TATAGAAGCC AATGGAGGGG ACTGATGGGA AGGTGGCCCA    792

AACCCAAGAC CCCACATTGT TACTCGCCAG CCCAATAATA AACATTTTGC    842

TGATC poly(A)                                             847
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Fig. 1. Nucleotide sequence of rat Apo D cDNA and deduced amino acid sequence. The coding region of the cDNA which comprises 189 amino acid residues is preceded by a 72 nucleotide 5′-noncoding region and followed by a 208 nucleotide 3′-untranslated region. The polyadenylation signal (AATAAA) occurs 19 bases upstream to the poly(A) stretch of the Apo D mRNA. The first 20 amino acids represent the sequence of the leucine-rich signal peptide of Apo D. The mature protein starts with glutamine 21 and comprises 169 amino acids. Underlined amino acids are exchanged in human Apo D. Boxed areas represent the highly conserved sequences found in the recently suggested new protein superfamily of hydrophobic molecule transporters.

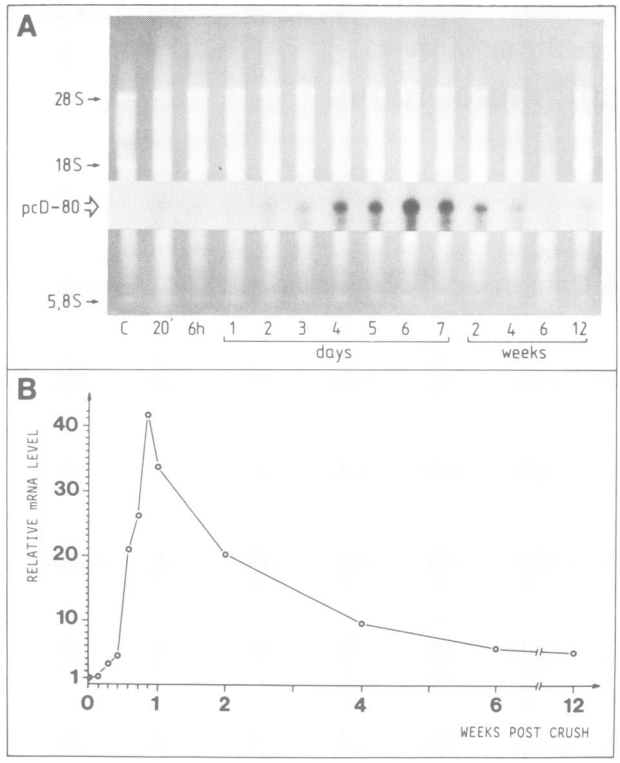

Fig. 2. **(A)** Steady state levels of Apo D transcripts in distal segments of rat sciatic nerve after crush lesion. Equal amounts (8 μg) of total RNA extracted from normal mature sciatic nerves and from distal stumps were dissected at various times after crush lesion and were fractionated in 1.5% citrate−urea agarose gels, stained with ethidium bromide to evaluate the quality and quantity of the RNA preparation in each lane, transferred to Nytran NY 13 membranes and hybridized to a [^{32}P]dCTP-labelled *Hin*dIII−*Bam*HI fragment (290 bp) derived from the rat Apo D cDNA clone pCD80. Part of the autoradiograph is shown at the appropriate position superimposed on the ethidium bromide stained gel. Arrows indicate the positions of the 28S, 18S, and 5.8S ribosomal RNAs. Abbreviations: C, total RNA derived from non-injured control nerves; 20′, 20 min; h, hours after crush lesion. **(B)** Densitometric evaluation of hybridization signals shown in (A, inset) plotted versus time after crush injury. Note: the RNA prepared at 6 weeks post crush was degraded in this experiment (see A). Therefore the level of Apo D transcript for this time point shown in (B) was obtained from another independent time-course experiment.

Computer-assisted nucleotide and amino acid sequence comparison demonstrated a 73.4% homology to human Apo D at the amino acid level (Drayna *et al.*, 1986). Two amino acid stretches which are highly conserved in the recently described superfamily of proteins involved in the transport of small hydrophobic molecules (Godovac-Zimmermann, 1988) are located in positions 24−27 and 103−105, respectively, of the mature rat Apo D molecule (boxed areas in Figure 1).

In a temporal analysis to monitor changes in Apo D mRNA levels, distal stumps of sciatic nerves were obtained at various times after crush injury and analysed by Northern blotting (Figure 2). Starting at day 2, an increase in the steady-state level of Apo D mRNA was observed. Rising rapidly it reached a maximum level of at least 40-fold above control by day 6 and slowly declined thereafter to a steady-state level of approximately 5-fold above control at 12 weeks post-injury. The transcript was estimated to be 1 kb in length.

In order to test whether the dramatic increase in Apo D mRNA observed in peripheral nerve within 1 week after crush injury is related to either nerve fibre regeneration, degeneration or nonspecific lesion effects we have compared the relative steady-state levels of Apo D transcripts in distinct portions of regenerating and nonregenerating nerve following two different lesion paradigms. A comparison of relative Apo D mRNA levels in proximal versus distal segments of crushed (regenerating) versus transected and ligated (nonregenerating) sciatic nerves is shown in Figure 3 for 1 and 4 week periods after injury. In the proximal part of crushed sciatic nerves as well as in both the proximal and distal stumps of transected nerves, moderate levels of Apo D mRNA between 3- and 10-fold above control were detectable. However, the steady-state level of the transcript was further increased 4- to 13-fold above these levels in the distal segment of crushed nerves at 1 week after the lesion.

In situ hybridization of transverse sections derived from the distal part of crushed sciatic nerves 7 days after lesion with [^{35}S]UTP labelled antisense RNA transcribed *in vitro* from an Apo D cDNA template showed intense foci of specific hybridization signals within the endoneurium (Figure 4a,c). In contrast, these signals were significantly reduced

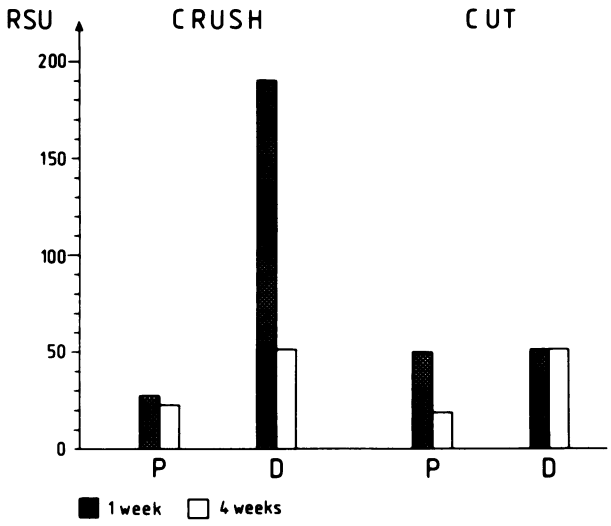

Fig. 3. Steady state levels of Apo D mRNA at 1 week and 4 weeks after crush or transection (cut) in the proximal (P) and distal (D) stumps of sciatic nerves. Each column represents the densitometric evaluation of hybridization signals (RSU, relative scan units). Equal amounts (8 μg) of total RNA from the indicated segments of crushed or transected rat sciatic nerves were separated by agarose gel electrophoresis, transferred to Nytran NY 13 membranes and hybridized with [^{32}P]dCTP labelled rat Apo D cDNA.

in transverse sections from intact sciatic nerves hybridized with the same probe (Figure 4b,d). Both perineurium and epineurium were lacking specific hybridization signals. In order to identify the cells expressing the Apo D transcript, cell cultures of dissociated sciatic nerves (distal stump) were prepared for *in situ* hybridization at day 6 after crush lesion and maintained for 1 day *in vitro*. As shown in Figure 5, hybridization signals with [^{35}S]UTP labelled Apo D antisense RNA were exclusively found in fibroblasts, whereas Apo D transcripts could not be detected in spindle-shaped Schwann cells and macrophages. It should be noted that Apo D mRNA is not constitutively expressed at high levels in cultured fibroblasts since (i) the hybridization signal markedly declined when the cells were maintained in culture for several days and (ii) no hybridization signal could be detected in fibroblasts cultured from normal rat sciatic nerve or meninges (unpublished data).

Apo D protein was detected by immunoblotting in the lipoprotein fraction of conditioned medium obtained from cultured explants (distal segments) of crushed sciatic nerve at 2–3 weeks after injury (Figure 6). The apparent molecular weight of Apo D in regenerating nerve is approximately 33 kd (Figure 6a) corresponding well with Apo D of the high-density lipoprotein complex (HDL) in human serum (Figure 6b). In contrast to human serum we could not detect Apo D by immunoblotting in rat serum using three different

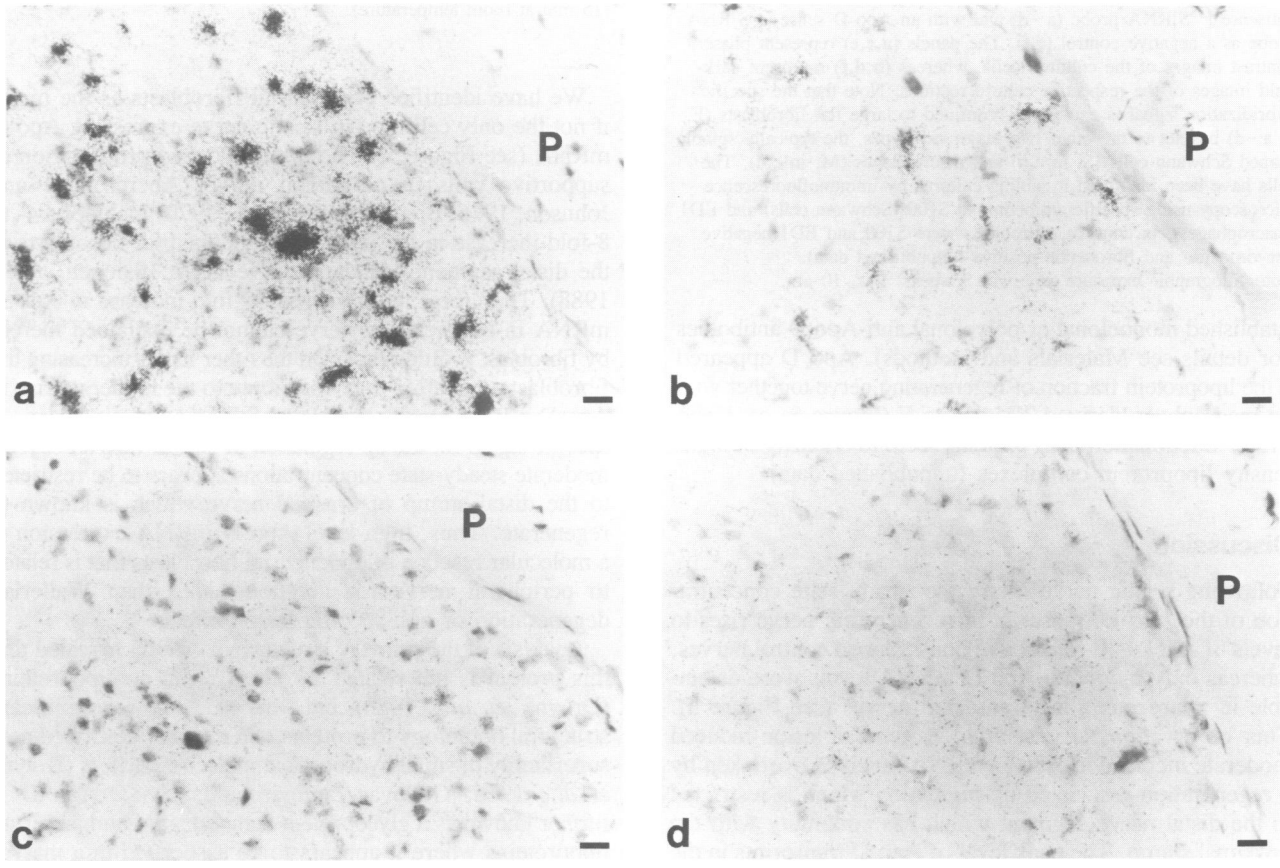

Fig. 4. Localization of Apo D mRNA in paraffin sections of rat sciatic nerve by hybridization with a [^{35}S]UTP labelled Apo D antisense RNA probe. Intense signals of specific hybridization can be detected in sections from the distal part of regenerating nerve at day 7 after crush (**a**), whereas sections derived from mature intact nerve show specific Apo D signals of rather low intensity (**b**). On adjacent sections an Apo D sense [^{35}S]RNA probe was used as a negative control for regenerating (**c**) and intact (**d**) sciatic nerve. Note the absence of specific hybridization signals within the perineurium and epineurium (P). Autoradiographic exposure time was 4 weeks. Bar, 10 μm.

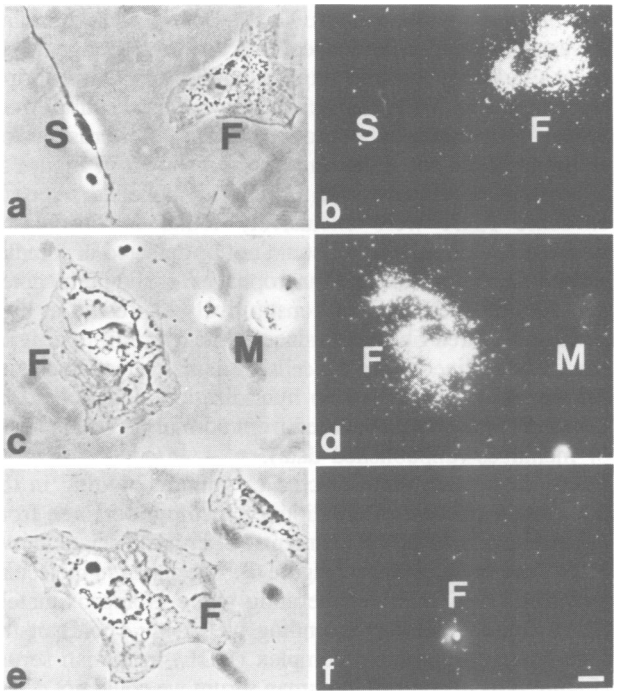

Fig. 5. Localization of Apo D mRNA in primary cell culture prepared from the distal stump of sciatic nerve 6 days after crush lesion. Prior to cell dissociation the epineurial sheath was removed. Cells were maintained in culture for 1 day and then hybridized with an Apo D antisense [^{35}S]RNA probe (a–d) and with an Apo D sense [^{35}S]RNA probe as a negative control (e,f). The panels (**a,c,e**) represent phase contrast images of the cultured cells, whereas (**b,d,f**) represent dark-field images of the respective culture sections. Note that the specific hybridization signal is exclusively confined to large flat fibroblasts (F, in a–d) but not to the other two major cell types, the typically spindle shaped Schwann cells (S, in a,b) and macrophages (M, in c,d). The cells have been identified in sibling cultures by immunofluorescence microscopy using specific antibodies to S100 (Schwann cells) and ED1 (macrophages). In contrast, fibroblasts were S100 and ED1 negative but vimentin- and fibronectin-positive (unpublished data). Autoradiographic exposure time was 3 weeks. Bar, 10 μm.

established monoclonal or polyclonal anti-Apo D antibodies (for details see Materials and methods). Apo D appeared in the lipoprotein fraction of regenerating nerve together with the macrophage-derived 37 kd Apo E (Figure 6a,c). Upon further fractionation both proteins seem to copurify in high-density lipoprotein complexes (unpublished data).

Discussion

Following sciatic nerve injury the steady state concentration of the Apo D transcript in regenerating nerve rises to levels of at least 40-fold above non-lesioned control nerves, whereas only moderate Apo D mRNA levels were detectable in nonregenerating transected nerves (see Figure 3). This observation suggests that a general lesion-induced moderate increase in Apo D mRNA levels is overtaken by a regeneration-associated up-regulation which is restricted to the distal nerve segment which has continuity with the proximal stump. The peak level of Apo D transcripts in the distal stump at day 6 after crush coincides with a period of nerve repair when large numbers of regenerating axons from the proximal stump have normally grown into the distal segment.

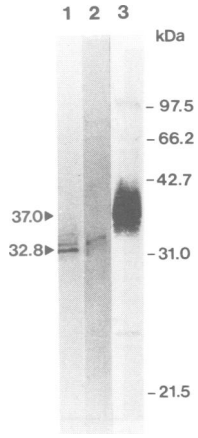

Fig. 6. Immunblot analysis of lipoprotein fraction from media conditioned by cultured rat sciatic nerve explants. Nitrocellulose transfers of 10 μg protein/lane separated by electrophoresis in a 12% SDS–polyacrylamide gel (for details see Materials and methods). **Lane 1**: separation of the top fraction (d < 1.21 g/ml) from KBr density-gradient centrifugation and incubation with a specific monoclonal antibody against Apo D (clone 5G10; dilution 1:1000; incubation for 2 h at room temperature). **Lane 2**: separation of 10 μg HDL from human serum and incubation with the 5G10 antibody (dilution 1:1000; incubation 2 h). **Lane 3**: separation of the same lipoprotein fraction as in lane 1 but incubation with a specific antiserum to Apo E (dilution 1:10 000; incubation 2 h). The primary antibodies were followed by an alkaline phosphatase conjugated second antibody (dilution 1:20 000; incubation 2 h) prior to enzyme reaction (15 min at room temperature).

We have identified endoneurial fibroblasts as the major if not the only cell type in sciatic nerve expressing Apo D mRNA (see Figure 5). The injury-induced proliferation of supportive cells in peripheral nerve (Abercrombie and Johnson, 1946; Bradley and Asbury, 1970) includes a 4- to 8-fold increase in the number of endoneurial fibroblasts in the distal segment of sciatic nerve in rat (Salonen *et al.*, 1988). Therefore, the observed 40-fold increase in Apo D mRNA in regenerating nerve cannot be explained merely by fibroblast proliferation. On the other hand, increasing the fibroblast population may contribute to the moderate rise in Apo D mRNA steady state level following nerve injury. A specific signal for the up-regulation of Apo D mRNA beyond moderate steady-state concentrations appears to be restricted to the distal stump of crushed nerve which is known to regenerate. Thus, high level Apo D mRNA expression is a molecular reaction of endoneurial fibroblasts that is related to peripheral nerve regeneration rather than Wallerian degeneration or non-specific lesion effects.

Analysis of the primary structure of Apo D revealed that this protein is not related to other serum apolipoproteins (Drayna *et al.*, 1986) but, instead, shows significant structural homology to proteins of a recently described new superfamily of small hydrophobic molecule carriers (Drayna *et al.*, 1986; Godovac-Zimmermann, 1988). Apo D is further known as a glycoprotein component of human serum lipoproteins where it appears to be associated in a macromolecular complex with the enzyme lecithin:cholesterol-acyltransferase (Fielding and Fielding, 1980), suggesting a putative role in cholesterol binding or transfer. An increased level of a similar cholesterol-esterifying enzyme activity was

previously detected in rat sciatic nerve following crush lesion (Yao and Dyck, 1981).

During Wallerian degeneration of peripheral nerve infiltrating hematogenous macrophages synthesize large amounts of apolipoprotein E (Apo E, M_r 37 kd) and release this protein into the endoneurial space where it accumulates associated with high-density lipoprotein complexes (Skene and Shooter, 1983; Müller *et al.*, 1985; Stoll and Müller, 1986; Ignatius *et al.*, 1987; Boyles *et al.*, 1989). Recently the uptake of Apo E-containing lipoprotein complexes from regenerating nerve into neuronal growth cones (Ignatius *et al.*, 1987; Rothe and Müller, 1989) and cultured Schwann cells (Müller and Rothe, 1988; Rothe and Müller, 1989) could be demonstrated. Since Apo D is released into the extracellular environment where it can be detected together with macrophage-delivered Apo E in lipoprotein fractions (see Figure 6) we propose a function for fibroblast-derived Apo D in endoneurial lipoprotein formation, intraneural lipid transport and/or re-utilization for the biosynthesis of membranes in regenerating nerve. Further investigation is required to identify the putative signal which could stimulate endoneurial fibroblasts to express high levels of Apo D mRNA. It is possible that Schwann cells committed to myelination when receiving axons or regenerating axons themselves release such a signal. Since hematogenous Apo E-expressing macrophages infiltrate both the distal stumps of crushed and transected nerve, it is rather unlikely that these cells release a signal triggering high level expression of Apo D mRNA (Müller *et al.*, 1985; Stoll and Müller, 1986).

Materials and methods

Animals and surgery

Adult male Wistar rats (200–250 g) were anaesthetized with Rompun/Ketanest (150–350 mg/kg body weight) administered intramuscularly. Sciatic nerves were exposed by a skin incision and blunt dissection through the thigh muscle layers. The nerves were either crushed with jeweler's forceps at upper thigh level or transected with a pair of scissors. To prevent regenerating axons of the proximal stump from entering the distal segment of transected nerves, both stumps were ligated. For comparative studies of crushed versus transected nerves both types of injury were carried out each at midthigh level of one of the sciatic nerves in the same animal. After dissection of the nerve stumps, the lesion zone (2–3 mm from each segment adjacent to the site of injury) was discarded.

Isolation of RNA

Total RNA was isolated from sciatic nerves by the guanidinium thiocyanate method (Kaplan *et al.*, 1979). Ultracentrifugation was performed at 36 000 r.p.m. and 20°C in a Beckman 70Ti rotor for 22 h. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972).

cDNA cloning

Following the cloning procedure of Okayama and Berg (1982, 1983), a cDNA library was constructed from 2 μg poly(A)$^+$ RNA derived from the distal segment of crushed sciatic nerves at 7 days after lesion. Competent DH5 cells (BRL) were used for transformation of the resulting cDNA constructs. The complexity of independent transformants was estimated to be 0.5×10^6 by plating of small aliquots.

Differential colony hybridization

Two filter replicas of 2000 single colonies were hybridized to [^{32}P]dCTP-labelled cDNA derived from poly(A)$^+$ RNA extracted from (i) the distal part of crushed sciatic nerves (7 days after lesion) and (ii) noninjured sciatic nerves. Synthesis of the first cDNA strand was accomplished following the protocol of Okayama and Berg (1983) except that the vector primer was replaced by 100 ng/μl oligo(dT). Filter replicas (Whatman 541; Whatman Ltd) were prepared according to the method of Taub and Thompson (1982), except that randomly sheared *Escherichia coli* genomic DNA was added to the hybridization mixture at a concentration of 80 μg/ml. Out of the original 2000 colonies differentially hybridizing clones were identified by autoradiography on Kodak XAR films.

Northern blotting

Two μg poly(A)$^+$ RNA or 8 μg total RNA were fractionated on 1.5% citrate–urea agarose gels (Lehrach *et al.*, 1977), stained with ethidium bromide, transferred to Nytran NY13 membranes (Schleicher and Schuell) and hybridized according to the manufacturer's protocol. Prior to exposure, the filters were washed in 0.3 M NaCl, 0.1 M Na-citrate, 1% $NaDodSO_4$ at 20°C for 30 min, followed by two 30 min washing steps in 15 mM NaCl, 10 mM Na-citrate, 1% $NaDodSO_4$ at 65°C. DNA probes were labelled by nick-translation or random priming in the presence of [^{32}P]dCTP (Nick Translation Kit, BRL; Random Priming Kit, Boehringer Mannheim). Autoradiographs were analysed by densitometric scanning (Quick Scan, Helena Laboratories) in order to obtain relative scan unit data representing the levels of hybridization intensities.

DNA sequencing and computer analysis

DNA sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977) after subcloning cDNA inserts into pSP72/73 vectors (Melton *et al.*, 1984). The sequences were compared with known nucleotide sequences provided by the gene data libraries of the University of Wisconsin Gene Computer Group and the European Molecular Biology Laboratory using the VAX/VMS system with 'Word Search', 'Bestfit' and 'Translate' programs, respectively (Devereux *et al.*, 1984; Wilbur and Lipman, 1983).

Preparation of tissue sections

Seven days after crush lesion nerves were fixed by cardiac perfusion with Bouin's solution. Samples from crushed nerves ~5 mm distal from the lesion site and nonlesioned control nerves were dissected, dehydrated, embedded in paraffin and cut in 8 μm sections.

Preparation of primary cultures

Sciatic nerves were dissected seven days after crush lesion and collected in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin (50 U/μl) and streptomycin (50 U/μl). After removing epineurium and connective tissue 5–6 nerves were cut into 3 mm pieces and treated with 0.1% Collagenase/Dispase (Boehringer Mannheim) for 1 h at 37°C followed by trituration of the tissue. Cells were passed through a 230 μm gauge mesh and plated at a density of 40 cells/mm^2 on laminin-coated (5 μg/ml) glass slides with Flexiperm culture chambers (Heraeus). The cells were cultured overnight in DMEM containing 10% FCS, penicillin and streptomycin and then fixed in 4% paraformaldehyde and PBS for 1 h. The three major cell types present in regenerating nerve were identified by immunofluorescence microscopy in culture. Spindle-shaped Schwann cells and macrophages were specifically labelled by monoclonal anti-S100 antibody (Dakopatts) or ED1 antibody (Serotec), respectively. Fibronectin- and vimentin-positive large flat fibroblasts were both S100- and ED1-negative.

Preparation of single-stranded RNA probes

For *in situ* hybridization a 290 bp *Hin*dIII–*Bam*HI fragment, derived from Apo D cDNA, was inserted into pSP64 and pSP65 vectors (Melton *et al.*, 1984). Run-off transcripts of both template orientations were produced from linearized template DNA using [^{35}S]UTP (1400 Ci/mmol; NEN) and SP6 polymerase kit (Boehringer Mannheim) according to the protocol of the manufacturer.

Hybridization of sections and cultured cells

After immersion in 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na-citrate, pH 7) slides were incubated in Proteinase K solution (1 μg/ml in 0.1 M Tris–HCl, pH 8, 0.05 M EDTA) at 37°C for 30 min (15 min for cultured cells). The protease reaction was terminated by 2 mg/ml glycine in PBS, followed by a wash in PBS. The slides were postfixed in 4% paraformaldehyde and PBS for 15 min and washed in PBS. Acetylation was performed in fresh 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8. After dehydration slides were prehybridized with 50% formamide, 10% dextran sulphate, 1 × Denhardt's solution, 0.02 M Tris–HCl, pH 8, 5 mM EDTA, 0.3 M NaCl, 0.1 M dithiothreitol (DTT), 0.5 mg/ml tRNA at 50°C overnight and again dehydrated. Hybridization was performed at 50°C overnight. RNase A-treatment (10 μg/ml in 0.5 M NaCl, 10 mM Tris–HCl, pH 8, 1 mM EDTA) was carried out at 37°C for 30 min (15 min for cultured cells) to reduce nonspecific signals. The slides were washed twice in 2 × SSC at 55°C for 20 min, then twice in 0.1 × SSC at 55°C for 30 min

and finally in 0.1 × SSC at 20°C for 5 min and dehydrated using an ascending alcohol series. Autoradiography was carried out for 3–4 weeks using Ilford K-5 photoemulsion. Tissue sections were counterstained with Mayer's hematoxylin.

Isolation and characterization of lipoprotein complexes

Distal segments of crushed sciatic nerves were removed 2–3 weeks after injury, cut into small segments (2 mm) and incubated for 2 h in MEM (1 ml/nerve). The conditioned medium was dialysed overnight against 0.15 M NaCl, 0.01% EDTA, pH 7.5 and adjusted to a density of 1.25 g/ml with KBr. Four ml of this medium were overlaid with 7 ml of a KBr-solution (1.21 g/ml containing 2 mM EDTA) and centrifuged for 48 h at 250 000 *g*, 4°C in a Beckman SW41Ti rotor. The floating fraction of lipoprotein complexes was collected with the top ml of fluid. In some experiments these lipoproteins were further separated into subfractions by density-gradient centrifugation using a KBr-step gradient as described in Ignatius *et al.* (1987). After dialysis against 0.15 M NaCl, 0.01% EDTA, pH 7.5, aliquots of fractions were separated on a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane by semi-dry blotting. Filters were stained with monoclonal antibodies 5G10 and 4E11 (a gift from C.Weech, Montreal) or a polyclonal goat antiserum (provided by P.Alaupovic, Oklahoma City) directed to human Apo D as well as a specific rabbit antiserum against rat Apo E (Ignatius *et al.*, 1986). Primary antibodies were detected with the Vectastain-alkaline phosphatase kit.

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