FIBRONECTIN AND PROCOLLAGEN PRODUCED BY A CLONAL LINE OF SCHWANN CELLS

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1 Introduction

Axons in the peripheral nervous system are surrounded by Schwann cells In case of myelinated nerves, a single axonal process is covered with a cocentric multimembranous tube, myelin sheet, extending from the Schwann cells lining the axon [1] In ganglion cultures, proliferation and migration of Schwann cells along the axons, as well as myelinization, appears to be triggered by utilizing collagenous substrate [2] A clonal cell line (RN-2), obtained from a chemically-induced rat Schwann cell tumor, has been described [3] This cell line produces unusual collagenous polypeptides [4] and has been recently examined as a model for the demyelinization process induced by viruses [5] We report here that this same cell line synthesizes procollagen of an unknown type and fibronectin

Fibronectin is a high molecular weight glycoprotein found in basement membranes and in loose connective tissues [6] In a soluble form, fibronectin is present also in plasma [7] and other body fluids [8,9] In culture conditions many cell types produce and deposit fibronectin in the cell layer [10,11] Recent studies have indicated a role for fibronectin in cell–cell and cell–substratum adhesion [12,13], and in cell migration [14,15]

2 Experimental

Rat Schwann cell-tumor line (RN-2 [3]), kindly provided by R P K Penttinen (University of Turku), was maintained and subcultured biweekly in Dulbecco's modified Eagle's basal medium supplemented with 10% fetal calf serum, 50 μg/ml streptomycin, and 100 units/ml penicillin For metabolic labeling, day 1 cultures were transferred into serum-free medium containing [^{3}H]glycine and [^{3}H]proline (10 μCi/ml each), and 30 μg/ml ascorbate, incubated overnight, and when indicated in the presence of 50 μg/ml β-aminopropionitrile fumarate (β-APN, Sigma) or 0 5 mM 2,2′-bipyridyl (Merck) or in the absence of ascorbate

For immunofluorescence, RN-2 cultures were fixed with 3% paraformaldehyde and acetone, and stained for fibronectin or collagen as detailed in [16] Fixation with paraformaldehyde alone ensures that only the pericellular structures are detected in the subsequent immunofluorescent staining [17] Production and specificity of the antisera against fibronectin [17] and collagen types I–IV [18,19] has been described The collagen antibodies were kindly provided by R Timpl (Max-Planck-Institut, Munchen) through A Vaheri

Media from the labeled RN-2 cultures were precipitated with $(NH_4)_2SO_4$ (176 mg/ml), in the presence of proteinase inhibitors [20] and 50 μg/ml gelatin (Sigma, Type I) Some culture media were digested with 50 μg/ml of bacterial collagenase (form III, Advance Biofacturers) for 60 min at 37°C, or with pepsin as in [20] before ammonium sulfate precipitation Washed gelatin, coupled to agarose beads [24], was added (10 μl/ml) to medium, stirred overnight at 4°C, and collected by centrifugation The supernatant fluid was precipitated with $(NH_4)_2SO_4$ as above Immunoprecipitation of the medium with fibronectin or collagen antibodies was done as in [13] RN-2 cells were collected from cultures treated with 0 5% trypsin–0 02% EDTA

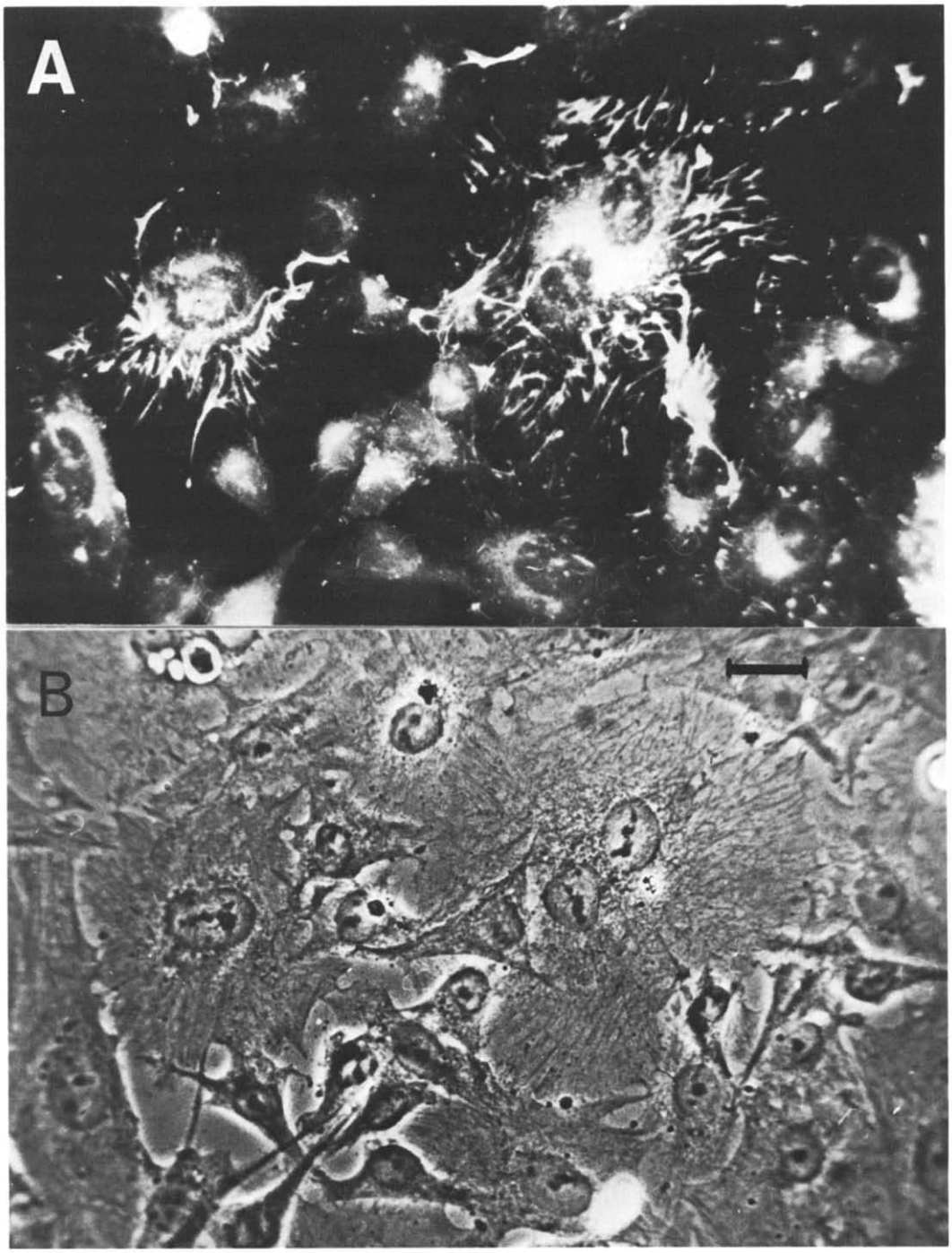

Fig.1. Fibronectin immunofluorescence (A) and phase contrast (B) of the same field in the RN-2 culture. Note the abundant pericellular fibronectin fibrils in the well spread cell containing two nuclei. Bar denotes 10 μm.

SDS–polyacrylamide gel electrophoresis of the samples reduced with 2-mercaptoethanol was carried out in 5% slab gels according to the Laemmli discontinuous gel system [21] Polypeptides were visualized by scintillation autoradiography using Kodak RP Royal X-Omat films [22] [^{14}C]Formaldehyde labeled [23] molecular weight markers included reduced α_2-macroglobulin (165 000), phosphorylase A (98 000), human serum albumin (68 000), and ovalbumin (43 000)

3 Results

RN-2 cultures were fixed and stained for fibronectin by the indirect immunofluorescent method As shown in fig 1A, intracellular fibronectin was visualized in many cells as a distinct perinuclear staining Short branching fibronectin fibrils were also present, especially in the well spread RN-2 cells (fig 1A,B) Staining of the fibronectin fibrils, but not of the intracellular fibronectin, was also seen in cultures fixed with paraformaldehyde only, suggesting that the fibronectin fibrils are localized pericellularly outside the cells (not shown) By focusing the immunofluorescence microscope at different levels, the fibronectin fibrils were predominantly seen underneath the cells No immunofluorescence was detected in the RN-2 cultures stained with anti-fibronectin antiserum preabsorbed with purified fibronectin, or with antibodies against collagen types I, II, III, or IV (not shown)

When the culture medium from metabolically labeled RN-2 cells was precipitated with $(NH_4)_2SO_4$, and analyzed by SDS–polyacrylamide gel electrophoresis, one major and three minor polypeptide bands were visualized in the SDS–gel (fig 2, track A) The major broad band around the mol wt 220 000 was identified as fibronectin by the following experiments Immunoprecipitation of the medium with anti-fibronectin antibodies yielded one polypeptide band of 220 000 as seen in SDS–gel electrophoresis (fig 2, track E) Fibronectin has been shown to specifically bind to collagen, especially to denatured collagen, gelatin [24,25]

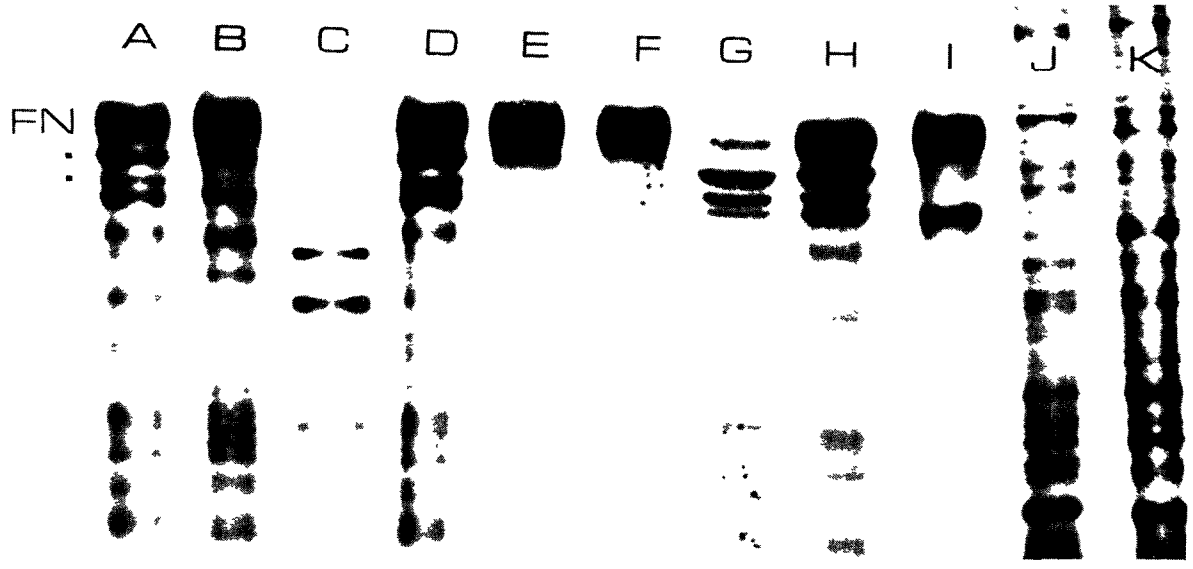

Fig 2 SDS–polyacrylamide gel electrophoresis of the following samples of RN-2 cultures control (A), collagenase-digested (B), pepsin-digested (C) culture media of untreated cells Media from cultures incubated in the presence of βAPN (D), of 2,2′-bipyridyl (I), or in the absence of ascorbate (H) Immunoprecipitation of the medium with anti-fibronectin antibodies (E) Gelatin-bound (F) and unbound (G) fractions of the medium Trypsinized cells of untreated (J) and 2,2′-bipyridyl-treated (K) cultures FN denotes fibronectin and dots the two collagenase-sensitive procollagen chains

Accordingly, the culture medium of RN-2 cells was first treated with gelatin covalently linked to agarose beads, and after collecting the gelatin–agarose beads by centrifugation, the 'post-gelatin' medium was precipitated with $(NH_4)_2SO_4$. Analysis by SDS–gel electrophoresis showed that the 220 000 polypeptide was effectively removed from the medium and was absorbed to gelatin (fig.2, track F), whereas the three other prominent polypeptides remained in the culture medium after absorption with gelatin–agarose beads (fig.2, track G).

The two minor bands with app. mol. wt 191 000 and 185 000, seen next to the fibronectin region in fig.2 (track A), are the collagenous polypeptides produced by RN-2 cultures. Digestion of the culture medium with bacterial collagenase specifically abolished these two bands (fig.2, track B). After pepsin digestion of the medium, four polypeptides with mol. wt 145 000, 130 000, 108 000 and 98 000 were seen in SDS–gels (fig.2, track C). This suggests that a portion in the collagenous molecules secreted by RN-2 cultures is in a pepsin-resistant triple helical form. The two collagenase-sensitive polypeptides (191 000 and 185 000) are probably the precursor chains of collagen, procollagens, and not, e.g., crosslinked products of collagen chains of smaller molecular weight. Culturing the RN-2 cells in the presence of β-aminopropionitrile (βAPN), an agent shown to inhibit the covalent crosslinking between collagen chains [26], had no effect on these polypeptides (fig.2, track D). Furthermore, these two polypeptides could not be immunoprecipitated with antibodies against the collagen types I, II, III, or IV (not shown). This is in line with our failure to detect any immunofluorescence in RN-2 cultures stained with the above collagen antibodies, as mentioned in the previous section.

Lack of ascorbate or addition of 2,2′-bipyridyl to the medium has been shown to inhibit the secretion of procollagens by normal fibroblast cultures [27]. In the presence of 0.5 mM 2,2′-bipyridyl secretion of the collagenous polypeptides by RN-2 cultures was also seen to be completely and selectively inhibited, secretion of fibronectin remained apparently the same as in untreated cultures (fig.2, track I compared to track A). Lack of ascorbate in the medium appeared to have no effect on the secretion of either fibronectin or procollagen (fig.2, track H compared to track A). Although the secretion of procollagen by RN-2 cultures could be effectively inhibited with 2,2′-bipyridyl, no concomitant intracellular accumulation of procollagen chains was seen in the treated cultures (fig.2, track K cf. track J).

4. Discussion

Here we have shown that a clonal line (RN-2) derived from a rat Schwann cell-tumor produces fibronectin and procollagen as the major secretory proteins. By immunofluorescence, few pericellularly deposited fibronectin fibrils were seen in the cell layer. In this respect the RN-2 cells appear to follow the generally observed phenomenon that malignant cell cultures have reduced amount of pericellular fibronectin when compared to the respective normal cultures [10,11].

The type of procollagen produced by RN-2 cultures, and apparently composed of the two collagenase sensitive polypeptides of 191 000 and 185 000, could not be identified with any of the antibodies against collagen types I–IV, either by immunofluorescent staining of RN-2 cultures or by immunoprecipitation of the culture medium. We did not detect any cleavage products of procollagen in the serum-free medium of RN-2 cultures. This is in line with our observations indicating a lack of processing of procollagens in malignant cell cultures [28].

In [4], a total of 5 collagenase sensitive polypeptides were detected in the culture medium of RN-2 cells. However, after subsequent serial culturing, the cells appeared to produce only the largest (155 000) collagenase-sensitive polypeptide [4]. Although not directly shown, it seems reasonable to assume that this polypeptide band contains the two collagenase-sensitive polypeptides identified in the present study (it is to be noted that the mol. wt 191 000 and 185 000 were approximated using non-collagenous protein standards, and are thus overestimates of the true molecular weights).

The findings that the same fibroblastic cell produces fibronectin and procollagen, their codistribution in the pericellular matrix, and concomitant loss in transformation [16], and a similar drop in synthesis in virus-transformed chick fibroblast cultures [29], would suggest a common regulatory mechanism for the biosynthesis and secretion of fibronectin and

procollagen However, this is not the case at least in the RN-2 cultures studied here, since secretion of procollagen, but not of fibronectin, could be inhibited with 2,2′-bipyridyl Moreover, whereas treatment with 2,2′-bipyridyl leads to intracellular accumulation of procollagen in normal fibroblast cultures [27], no such an effect was observed in RN-2 cultures

In vivo Schwann cells are localized in the peripheral nervous system In peripheral nerves, fibronectin has been detected in the basement membrane around the Schwann cells, and is especially abundant in the nodes of Ranvier (A Paetau, M Haltia and A Vaheri, personal communication) The present study suggests that fibronectin detected in these regions is produced by Schwann cells

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