Extracellular Matrix Allows PC12 Neurite Elongation in The Absence of Microtubules

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Abstract. Several groups have shown that PC12 will extend microtubule-containing neurites on extracellular matrix (ECM) with no lag period in the absence of nerve growth factor. This is in contrast to nerve growth factor (NGF)-induced neurite outgrowth that occurs with a lag period of several days. During this lag period, increased synthesis or activation of assembly-promoting microtubule-associated proteins (MAPs) occurs and is apparently required for neurite extension. We investigated the growth and microtubule (MT) content of PC12 neurites grown on ECM in the presence or absence of inhibitors of neurite outgrowth. On ECM, neurites of cells with or without prior exposure to NGF contain a normal density of MTs, but frequently contain unusual loops of MTs in their termini that may indicate increased MT assembly. On ECM, neurites extend from PC12 cells in the presence of 10 μM LiCl at significantly higher frequency than on polylysine. On other substrates, LiCl inhibits neurite outgrowth, apparently by inhibiting phosphorylation of particular MAPs (Burstein, D. E., P. J. Seeley, and L. A. Greene. 1985. *J. Cell Biol.* 101:862–870). Although 35–45% of 60 Li^+-neurites examined were found to contain a normal array of MTs, 25-30% were found to have a MT density ~15% of normal. The remaining 30% of these neurites were found to be nearly devoid of MTs, containing only occasional, ambiguous, short tubular elements. We also found that neurites would extend on ECM in the presence of the microtubule depolymerizing drug, nocodazole. At 0.1 μg/ml nocodazole, cells on ECM produce neurites that contain a normal density of MTs. This is in contrast to the lack of neurite outgrowth and retraction of extant neurites that this dose produces in cells grown on polylysine. At 0.2 μg/ml nocodazole, neurites again grew out in substantial number and four of five neurites examined ultrastructurally were found to be completely devoid of microtubules. We interpret these results by postulating that growth on ECM relieves the need for MTs to serve as compressive supports for neurite tension (Dennerll, T. J., H. C. Joshi, U. L. Steel, R. E. Buxbaum, and S. R. Heidemann. 1988. *J. Cell Biol.* 107:665). Because compression destabilizes MTs and favors disassembly, this would tend to increase MT assembly relative to other conditions, as we found. Additionally, if MTs are not needed as compressive supports, neurites could grow out in their absence, as we also observed.

MICROTUBULES (MTs)[1] are crucial for the growth and shape of neural axons (Landis 1983; Mitchison and Kirschner, 1988); eg., MT depolymerizing drugs cause neurite collapse of many types of cultured neurons (Yamada et al., 1970; Daniels, 1975; Solomon and Magendantz, 1981; Joshi et al., 1985). An inference drawn from these and related observations, that MTs are compressive supports for neurite tension, is supported by direct measurements of neurite forces (Dennerll et al., 1988). Compression of microtubules must destabilize them, making them more likely to depolymerize compared to microtubules without a load (Hill and Kirschner 1982; Buxbaum and Heidemann, 1988). Consistent with the notion of neurite MT assembly in the face of a destablizing influence, nerve growth factor (NGF)-induced neurite outgrowth of PC 12 cells appears to require transcriptionally-dependent MT stabilization (Greene, 1984). NGF-induced neurite outgrowth of this cell line is strongly correlated with the synthesis of the assembly promoting microtubule associated proteins tau, MAP 1 (Drubin et al., 1985; Greene et al., 1983), MAP 2 (Black et al., 1986; Brugg and Matus, 1988), MAP 5 (Brugg and Matus, 1988), and the phosphorylation of similar proteins called chartins (Black et al., 1986). The need for MT assembly promotion to support neurite outgrowth is further suggested by the inhibition of neurite outgrowth when chartin phosphorylation is inhibited by Li^+ (Burstein et al., 1985). Conversely, removal of NGF from differentiated PC 12 cells causes neurite retraction and degradation of tubulin and microtubule associated proteins within 2 d (Drubin et al., 1988).

1. *Abbreviations used in this paper*: ECM, extracellular matrix; NGF, nerve growth factor; MAP, microtubule-associated protein; MT, microtubule.

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Many laboratories have shown that extracellular matrix (ECM) or its components promote neuronal growth (for review, see Sanes, 1989). Of particular relevance here, growth on ECM from various sources promotes the outgrowth of PC12 neurites in the absence of NGF (Vladovsky et al., 1982; Fujii et al., 1982; Bellot et al., 1985; Wujek and Akeson, 1987). The rapidity of PC12 neurite outgrowth on ECM, and the normal MT array observed in these neurites (Fujii et al., 1982) suggested to us that part of the growth stimulation by ECM is because of an ability to stabilize and/or promote neurite MT assembly. We have reexamined the extension of PC12 neurites on ECM with a focus on the neurite MT arrays. Our data are consistent with the interpretation that growth on ECM relieves neurite MTs of their compressive support function; this has the dual effect of stimulating MT assembly relative to growth on other substrates and of permitting neurite outgrowth in the absence or near absence of MTs.

Materials and Methods

PC12 cells were cultured as previously described (Heidemann et al., 1985). "Primed" cells (Burstein and Greene, 1978) were grown for 6 d in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% horse serum, 5% FBS and 50 ng/ml 7s NGF. Cultures were maintained at 37°C in a humidified atmosphere containing 10% CO_2. "Unprimed" cells were grown under the same conditions excluding NGF. At the beginning of an experiment, 5×10^4 "primed" or "unprimed" cells were replated onto a 60-mm tissue culture dish; either on Extracell dishes (ECM surface from corneal endothelial cells; Accurate Chemical & Scientific Corp., Westbury, NY) or on polylysine treated tissue cutlure plates as previously described (Joshi et al., 1985). The above plating density maximized neurite outgrowth on ECM; we confirmed the report of Vlodavsky et al. (1982) that the response of PC12 to ECM was density dependent. All cells were maintained in RPMI-1640 containing horse and fetal bovine sera as above in the presence or absence of additional experimental variables; 50 ng/ml NGF and 10 mM LiCl, as described in Results.

Neurite outgrowth was assayed quantitatively by a method similar to that previously described (Heidemann et al., 1985; Joshi et al. 1985). On day 1 of the experiment, cultures were examined using an inverted phase microscope and two 1-mm circles were marked on the bottom of culture dishes. On days 1–5, the total number of neurites and cell bodies for a given experimental treatment were counted from photographs of the circled regions. In some later experiments, cell and neurite counts were made only on days 3–5. Data is reported as a fraction: the number of neurites counted (cell processes longer than 2-cell diameters) over the number of cell bodies. This figure is called the "neurite frequency."

Electron microscopic examination of cells was carried out by standard methods previously described (Joshi et al., 1986). The two-dimensional microtubule length per unit area (μm of MTs/μm^2 of neurite area) was measured in several neurites from electron micrographs. Prints of micrographs at a final magnification of 36,000 were used to create a composite of the entire neurite from base to terminal at a single plane of sectioning. The MT length and cytoplasmic area were measured by tracing with a calibrated electromagnetic platten and handheld viewer coupled to an IBM XT computer for file storage. Integrations were made with a Sigma Scan (Jandel Sci. Inc., Sausalito, CA) program.

Results

We confirmed the results of Vlodavsky et al. (1982) and Fujii et al. (1982) that ECM stimulates a rapid, but transient, outgrowth of PC12 neurites in the absence of NGF. Table I summarizes experiments comparing neurite outgrowth of PC12 cells treated with nerve growth factor plated on polylysine with the neurite outgrowth on ECM without NGF. These transient cell processes were similar to neurites grown in the presence of nerve growth factor in having small growth cones at their distal tips, the frequency of process branching and their length, given the short time of growth. An outgrowth extending from the cell body was called a neurite if it was more than two cell bodies long; this is a more selective criterion than used in past studies (Heidemann et al., 1985; Joshi et al., 1986). On ECM, the peak of neurite outgrowth appeared on day 3 when there were nearly as many neurites as cell bodies, compared to a 20% ratio of neurites/cell bodies on the third day for NGF-stimulated cells. On day 3, most neurites on ECM were between 120–160 μm long, some as long as 200 μm. We were surprised by the high, but transient, number of neurites for NGF-treated cells on day 2. Although this outgrowth on day 2 was somewhat variable, it was observed in all four experimental repetitions.

Using transmission electron microscopy, we examined five neurites grown on ECM for 3 or 4 d. Consistent with the results of Fujii et al. (1982), 4/5 of these had microtubule array densities that we judged were within the normal range (Table II; Fig. 1), based on our previous ultrastructural observations of NGF-induced neurites (Joshi et al., 1985; Joshi et al., 1986). Because some neurites were branched, we were able to observe seven terminals in the five neurites and found that two of these contained microtubule "loops" (Fig. 2), similar to those observed by Tsui et al. (1984) and by Letourneau and Ressler (1984). As shown in fig. 1, PC12 neurites grown on ECM contain an unusually large number of ribosomes, as reported by Fujii et al. (1982).

Growth on Extracellular Matrix Permits Neurite Outgrowth from Primed PC12 Cells in the Absence of Nerve Growth Factor

PC12 cells that are primed by previous exposure to NGF for several days regrow substantial numbers of neurites within a day of replating (Burstein and Greene, 1978). This regrowth of primed PC12 cells on collagen or polylysine-treated surfaces requires the presence of NGF after replating (Burstein and Greene, 1978). Tomaselli et al. (1987) previously reported that laminin-treated substrates supported regrowth of neurites from primed cells in the absence of added NGF. We wished to determine whether ECM would also support neurite outgrowth from primed cells in the absence of NGF. Indeed, replating primed PC12 cells onto ECM in the absence of NGF produced substantial neurite outgrowth, in marked contrast to primed cells plated on polylysine without NGF (Table III). In many experiments, the neurites stimulated by ECM were unusually long after only a day of growth and were among the most rapidly grow-

Table I. Neurite Outgrowth from PC12 Cells not Previously Exposed to NGF

	Culture conditions*	
Days of culture	Polylysine surface + NGF	ECM − NGF
1	34/474	232/673
2	201/445	333/660
3	170/1091	751/769
4	74/608	345/568
5	226/1276	232/316

* Neurite outgrowth is expressed as the number of neurites (numerator) over the number of cell bodies (denominator) counted in circled regions of culture dishes (see Materials and Methods). These data represent between 2 and 6 experimental repetitions.

Table II. Summary of Ultrastructural Observation of Microtubule Density in PC12 Neurites Grown on ECM in the Absence of NGF

Culture conditions	Categories for MT density		
	Normal	Low	Absent
Unprimed (n = 5)	80%	20%	0%
Primed (n = 48)	77%	23%	0%
Unprimed, + LiCl (n = 29)	45%	24%	31%
Primed, + LiCl (n = 31)	35%	32%	32%

ing PC12 neurites we have seen under any conditions (Fig. 3). 37 of 48 of these neurites examined (77%) contained normal microtubule arrays, similar to those of unprimed PC12 cells grown on ECM (Table II). We were able to observe 39 neurite terminals from these primed cells on ECM and found 17 of them contained "loops" of microtubules similar to the loops observed in unprimed cells.

Growth on Extracellular Matrix Permits Substantial Neurite Outgrowth in the Presence of Lithium Ions, Many with a Near Absence of Microtubules

LiCl has been shown to block neurite outgrowth from PC12, apparently by selectively inhibiting phosphorylation of three MAPs identified as the "chartins," which appear to stimulate MT assembly when phosphorylated (Burstein et al., 1985). We confirmed this observation finding that <10% of cell bodies produced neurites on polylysine-treated surfaces in NGF-containing medium with 10 μM LiCl during 5 d of observation (data not shown). On ECM, however, both primed and unprimed PC12 cells plated in the presence of this Li^+ concentration showed significant neurite outgrowth, between 25 and 50% of cells had neurites between days 2 and 4 (Table IV; Fig. 4). However, there was less neurite outgrowth than in Li^+-free conditions (Tables I and III; Fig. 3).

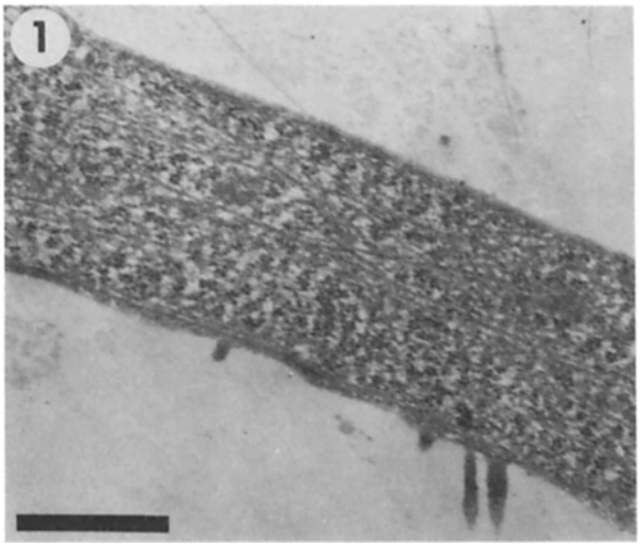

Figure 1. Neurites of PC12 cells grown on ECM in the absence of NGF generally have a normal density of MTs. Transmission electron micrograph of a "normal" microtubule array in a PC12 neurite extended on ECM. The cell had never been exposed to NGF. Bar, 1 μm.

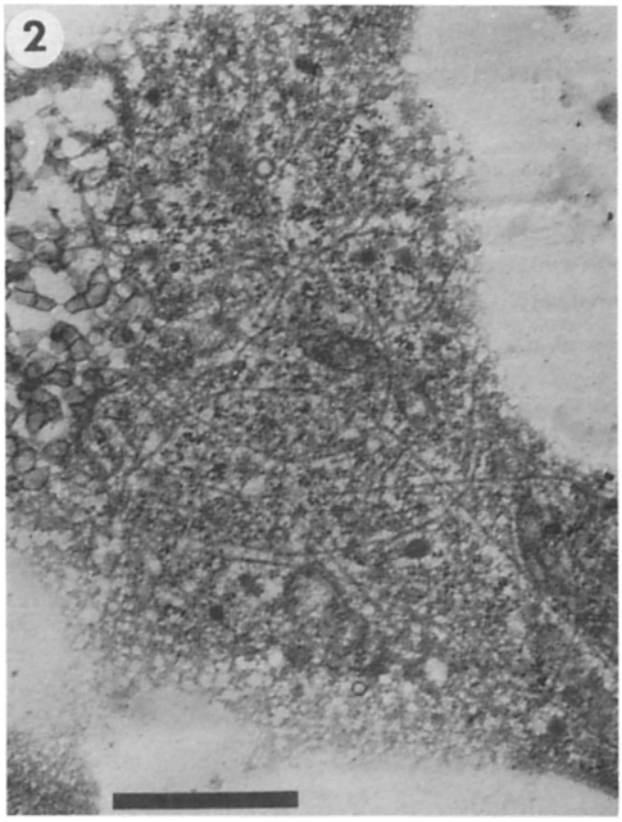

Figure 2. Neurite terminals of PC12 cells grown on ECM frequently contain MT "loops." Transmission electron micrograph of the terminal of a PC12 cell that had never been exposed to NGF after 4 d of growth on ECM. Loops of microtubules, as seen here, were observed in ~25% of such neurites and in ~45% of neurites from cells previously primed by exposure to NGF then replated on ECM in the absence of NGF. Bar, 1 μm.

Electron microscopic examination of Li^+-treated neurites of unprimed PC12 cells grown on ECM revealed that only 45% of the neurites examined contained an array of microtubules we judged as being of normal density, as compared with 80% of control ECM neurites (Table II). Additionally, neurites from 9 of the 29 Li^+-treated, unprimed cells examined were substantially devoid of microtubules (Table II; Fig. 5). No neurites similarly devoid of microtubules were observed in Li^+-free conditions (Table II). Similarly, neurites from cells initially primed by growth in NGF and then placed

Table III. Neurite Outgrowth from Primed PC12 Cells in the Absence of NGF

Days of culture	Substrate*	
	Polylysine	ECM
1	0/73	53/109
2	0/76	47/152
3	0/123	91/215
4	0/129	54/208
5	0/105	47/204

* As in Table I, neurite outgrowth is expressed as the number of neurites (numerator) over the number of cell bodies (denominator) counted in circled regions of culture dishes (see Materials and Methods). Each datum represents between 1 and 3 experimental repetitions.

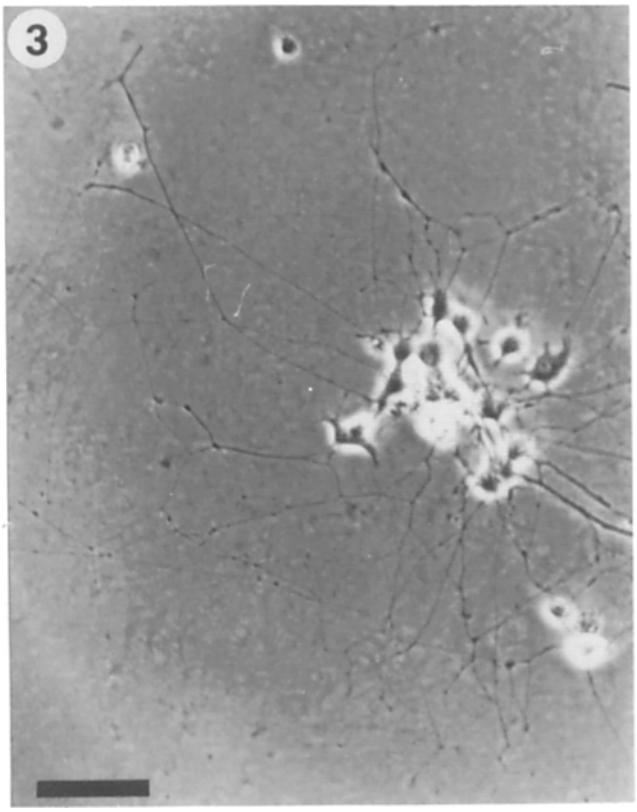

Figure 3. "Primed" PC12 cells extend robust neurites on ECM in the absence of NGF. Phase micrograph of cells after 6 d in the presence of NGF on untreated tissue culture plastic and 1 d on ECM in the absence of NGF. Bar, 60 μm.

into Li^+-containing medium on ECM also exhibited a significant decrease in microtubules. One third of these neurites had a MT array whose density was ~10% of normal (see below). An additional 10:31 neurites examined had an even lower MT density, being essentially devoid of microtubules (Table II). As in the unprimed cells scored in this "absent" category, only short, ambiguous tubular elements were observed in these neurites, possibly MT fragments or sections through endoplasmic reticulum (Fig. 6).

Our judgement for scoring neurites as containing "normal," "low," or "absent" MT arrays (Table II) was based on substantial previous work on neurite ultrastructure in PC12 and chick sensory neurons (Heidemann et al., 1985; Joshi et al. 1985; Joshi et al., 1986; Baas et al., 1987; White et al., 1987; Sinclair et al., 1988). "Normal" arrays typically had four or five microtubules arrayed transversely across the diameter of the neurite; low arrays clearly had a substantially diminished MT array with no adjacent microtubules; the absent category contained only occasional short, ambiguous tubular elements. However, we wished to provide more quantitative data concerning our scoring of neurites as containing normal, reduced or absent MT arrays as in Table II. We assessed the two-dimensional microtubule density (MT length/μm^2 of neurite area) of 5–7 neurites randomly chosen from each of the three categories (normal, low, and absent). In all cases, complete neurites from the cell body to the growth cone were analyzed at a single plane of sectioning; at least 200 μm^2 of neurite shaft was assessed for each category. Neurite area and MT length were measured as described in Materials and Methods. Table V confirms what we had noted visually; the three categories differed in MT density by factors of ~7–10. The MT density in six normal neurites ranged from 1.31 to 3.76 $\mu m/\mu m^2$ with a mean of 2.12 $\mu m/\mu m^2$; the seven low category neurites ranged in density from 0.04 to 0.63 $\mu m/\mu m^2$ with a mean of 0.31 μm MTs/μm^2; and the five absent category neurites ranged in density from 0 to 0.08 $\mu m/\mu m^2$ with a mean of 0.03 $\mu m/\mu m^2$.

Table IV. Neurite Outgrowth from PC12 Cells on ECM in the Absence of NGF and the Presence of 10 μM LiCl

Days of culture	Previous culture conditions*	
	Unprimed	Primed
1	127/567	10/67
2	239/560	31/111
3	200/598	28/105
4	162/300	27/69
5	39/208	15/66

* As in Table I, neurite outgrowth is expressed as the number of neurites (numerator) over the number of cell bodies (denominator) counted in circled regions of culture dishes (see Materials and Methods). Each datum in the unprimed category is the result of four experimental repetitions, and in the primed category data for all but the first day (n = 1) is the result of two experimental repetitions. Cell number declines after day 3, apparently from Li^+ toxicity.

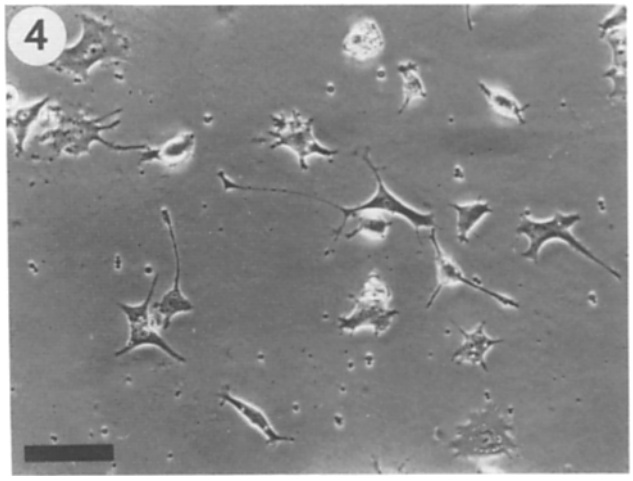

Figure 4. Neurites extend from PC12 in the presence of Li^+ on ECM. Phase micrograph of cells that had never been exposed to NGF after 3 d of growth on ECM in the presence of 10 μM LiCl. Bar, 90 μm.

Growth on ECM Permits Neurite Outgrowth in the Presence of Nocodazole

The finding that neurites were elaborated on ECM in the near absence of microtubules suggested that neurites also might be produced in the presence of microtubule depolymerizing drugs. Nocodazole is an antimicrotubule drug that has little effect on protein, RNA and DNA synthesis (Zieve et al., 1980). Previous results in our lab indicated that doses as low as 0.05 μg/ml effectively depolymerize neurite microtubules and cause neurite retraction in primed and replated PC12

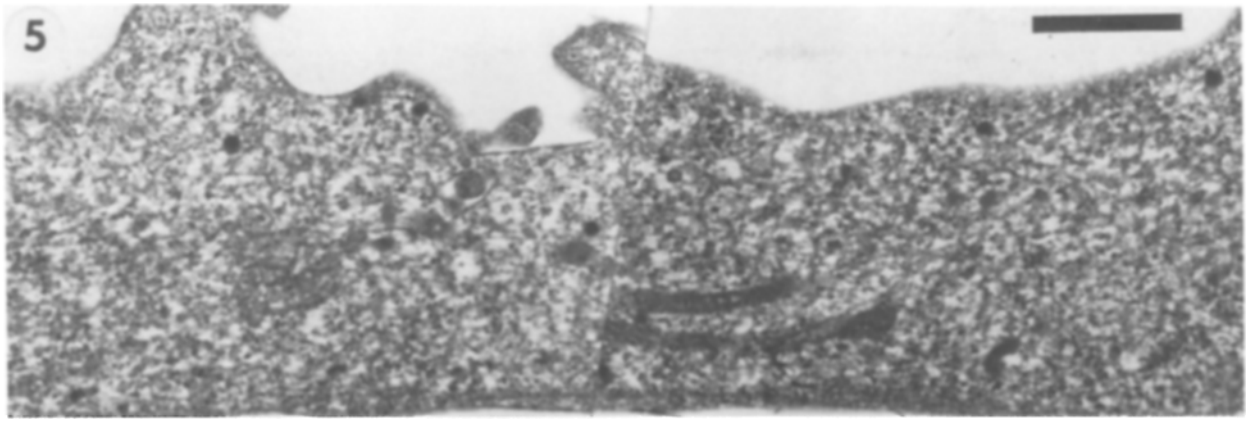

Figure 5. Lack of MTs in a neurite from an "unprimed" cell in the presence of 10 μm Li^+. Transmission electron micrograph of a region from an "absent category" neurite (see Table II) of a cell cultured as in Fig. 4. Bar, 1 μm.

cells grown on polylysine (Heidemann et al., 1985; Joshi et al., 1985). Primed PC12 neurites were plated onto Extracell dishes in medium with or without NGF. After allowing 3 h for the cells to settle and attach, nocodazole was added to give a final concentration of 0.1 or 0.2 μg/ml. Although many cells detach from the substratum by day 1, a substantial number of cells at both nocodazole concentrations were found to grow neurites (Fig. 7) that persisted until $\sim$ day 4. On day 5, neurites were rare and the cells did not appear healthy. These experiments were repeated 7 times and in all nocodazole-poisoned dishes neurite outgrowth was noted. Neurite outgrowth was slightly better in poisoned medium containing NGF than in medium without NGF. No neurite outgrowth of any kind was noted in control experiments that, as in previous experiments (Heidemann et al., 1985; Joshi et al., 1985), used polylysine-treated dishes instead of Extracell dishes. Ultrastructural examination of 5 cells grown in 0.1 μg/ml nocodazole showed essentially normal arrays of MTs (Fig. 8 *a*). In contrast, four of five neurites grown in the presence of 0.2 μg/ml nocodazole and examined ultrastructurally were found to be devoid of microtubules (Fig. 8 *b*); 1/5 neurites had two short lengths of MT. As shown in Fig. 8 *b*, neurites that extended in the presence of 0.2 μg/ml nocodazole were found to have a high density of 10-nm filaments. Such filaments are not typically a prominent feature of PC12 cytoplasm (Luckenbill-Edds et al., 1979; Heidemann et al., 1985; Jacobs and Stevens, 1986).

Discussion

The experiments reported here were undertaken to test our hypothesis that microtubules in PC12 neurites serve as compressive supports for neurite outgrowth (Joshi et al., 1985; Dennerll et al., 1988; Buxbaum and Heidemann, 1988). The introduction outlines the evidence that NGF-induced neurite outgrowth from PC12 is accompanied by an increase in MT stability/assembly promotion, possibly to enable the MTs to bear compression. The rapidity of neurite outgrowth on ECM suggested that to us that this substrate either induces much more rapid changes in MT stability or that such stabilization is not required because neurite outgrowth is occurring without a compressive load on MTs. Indeed, we can account for our data, both the relative stimulation of MT assembly accompanying neurite outgrowth on ECM and neurite outgrowth in the absence of MTs, by hypothesizing that ECM relieves the compression that MTs otherwise support. Although this interpretation is somewhat incautious, the relationships between cell substrate adhesion and the mechanical interactions of the cytoskeleton are very poorly understood, it is not only consistent with the observations, but predictions based on this view anticipated most of the results reported here. A limitation of the work reported here is that no effort was made to determine which component(s) of the ECM is responsible for the effects of PC12 neurite outgrowth. However, Rogelj et al. (1989) recently reported that ECM must contain basic fibroblast growth factor to support PC12 differentiation.

Using electron microscopy, we found that 80% of the neurites of NGF or ECM grown cells, both primed and unprimed, contained normally extensive arrays of microtubules. We take this to indicate that microtubule assembly on ECM is at least as active as with NGF. Additionally, a significant fraction of neurite terminals on ECM contained microtubule loops. Such loops were not observed in NGF-induced neurites in this or in previous ultrastructural studies of PC12 (Luckenbill-Edds et al., 1979; Joshi et al., 1985; Heidemann et al., 1985; Joshi et al. 1986; Jacobs and Stevens, 1986). Similar loops are present however, in embryonic chick retinal neurons (Tsui et al., 1984) and in chick sensory neurons after treatment with taxol, a MT-assembly promoter (Letourneau and Ressler, 1984). Following these workers, we suggest that the microtubule loops indicate conditions of increased microtubule polymerization. The robust neurite outgrowth from primed PC12 cells on ECM after withdrawing NGF also argues that MT assembly was stimulated relative to other substrates: Drubin et al. (1988) showed that PC12 cells from which NGF had been withdrawn for 2 d retracted their neurites, depolymerized their microtubules, and degraded both tubulin and MAPs. In contrast, 80% of neurites from primed cell grown on ECM in the absence of NGF contained a normal array of MTs on day 3 or 4. The extensive outgrowth of neurites from primed PC12 cells on ECM in the absence of NGF was one of the results we anticipated: the period of NGF priming would allow for the synthesis of assembly/stability-promoting associated proteins such as tau and phosphorylated chartins. Presumably, these

Figure 6. Lack of MTs in a neurite from a "primed" cell in the presence of 10 μm Li^+. Transmission electron micrograph of a region from an "absent category" neurite (Table II) of a cell that had been exposed to NGF for 6 d, replated onto ECM in the presence of Li^+ and the absence of NGF and cultured for 4 d before fixation. The arrow shows an ambiguous tubular element of the kind seen in absent category neurites that were measured as contributing to MT length in Table V. Bar, 1 μm.

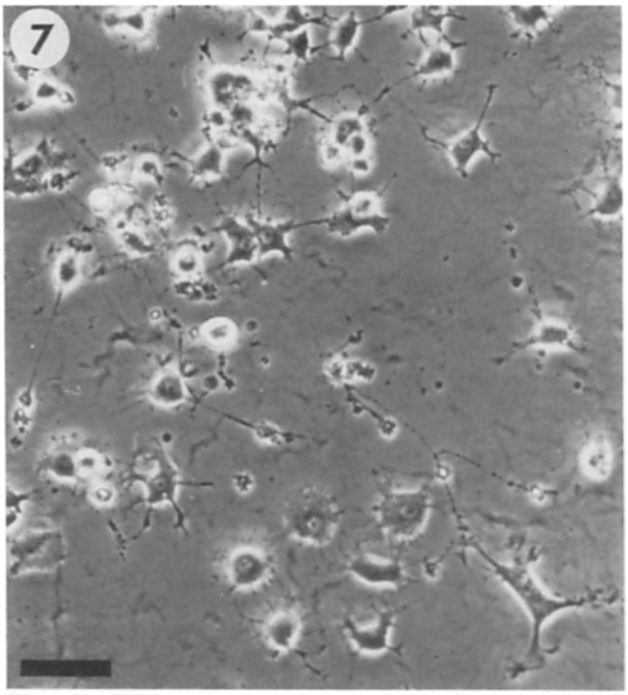

Figure 7. Neurite outgrowth in the presence of 0.2 μg/ml nocodazole. Phase micrograph of PC12 cells that were primed for 6 d in NGF, recultured for 2 d on ECM in the presence of 0.2 μg/ml nocodazole. Bar, 60 μm.

proteins would persist for some hours in the absence of NGF. Given the ability of neurites to extend and MTs to assemble without a period of priming, neurite extension, and MT assembly should initially be even more active with the remnants of priming still available. Similarly, we anticipated the growth of neurites on ECM in the presence of LiCl; the MT stabilization afforded by ECM should permit neurite outgrowth and MT assembly in the absence of phosphorylated chartins. Fig. 4 and Tables II and IV show that substantial numbers of neurites grew on ECM in the presence of LiCl and 35–45% of these neurites had normal MT arrays. This is in contrast to the negligible neurite outgrowth on polylysine-treated plastic with NGF and LiCl as previously reported (Burstein et al., 1985). The strongest evidence that growth on ECM promotes MT assembly is the extension of MT-containing neurites from cells grown in the presence of 0.1 μg/ml nocodazole. This contrasts sharply with the similarly treated cells grown on polylysine-treated plastic that extended no neurites in the presence of the poison. The extension of MT-containing neurites in the presence of nocodazole is particularly unusual because this dose of nocodazole causes MT disassembly and retraction of 50% (Dennerll et al., 1988) to 95% (Joshi et al., 1985) of extant, NGF-induced neurites from primed and replated PC12 cells grown on polylysine-treated surfaces.

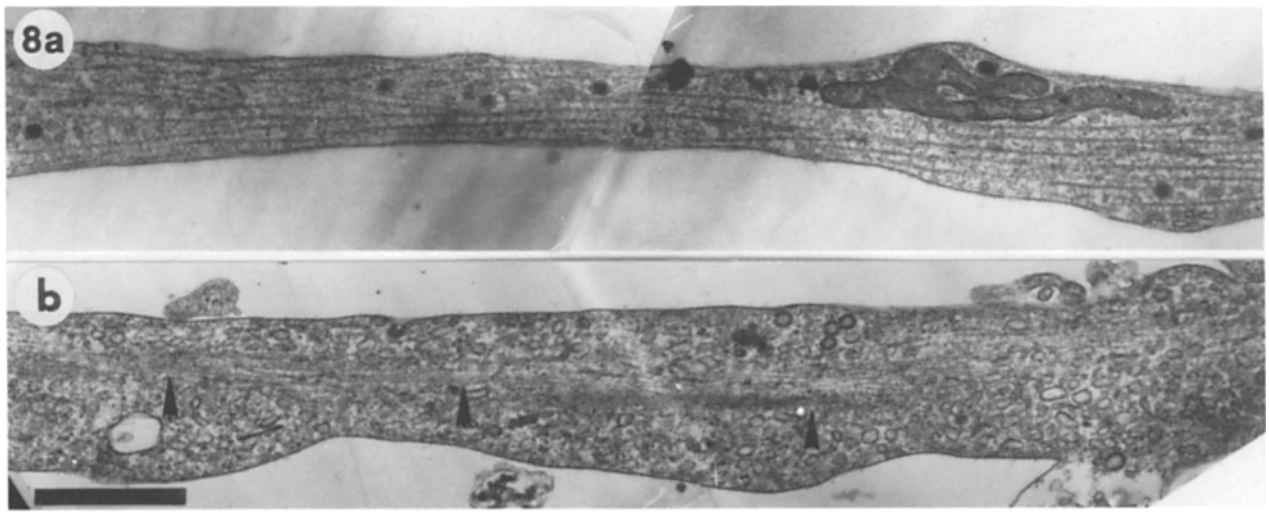

Figure 8. Ultrastructure of neurites grown in the presence of nocodazole. (*a*) Transmission electron micrograph from a typical neurite extended in the presence of 0.1 μg/ml nocodazole on ECM after 2 d of culture. All five neurites examined had a similar MT density. (*b*) Transmission electron micrograph of a typical neurite extended in the presence of 0.2 μg/ml nocodazole on ECM after 2 d of culture. The arrows point to a bundle of intermediate filaments of the type not seen under other culture conditions. Four of five neurites examined were similarly devoid of MTs. Bar, 1 μm.

The ECM-induced stimulation of MT assembly concomitant with neurite extension could result from any of several effects; eg., ECM-induced chartin phosphorylation. However, that ECM allows the extension of neurites without internal MTs argues strongly for the structural interpretation we propose. We interpret both effects as the result of compression relief not only for reasons of parsimony, but also because both effects occurred simultaneously in cultures treated with LiCl and both effects were noted in nocodazole-treated cultures. For example, ~40% of neurites grown in the presence of LiCl had normal MT arrays, but ~30% of these neurites were observed to be virtually devoid of microtubules (Table II). Neurite outgrowth in the absence of MTs was also noted in PC12 cells treated with 0.2 ug/ml nocodazole, no MTs whatever were observed 4 of 5 these neurites. As in previous studies (Joshi et al., 1986; Baas et al., 1987), neurites lacking MTs had much higher concentrations of mitochondria and of membranous vesicles. The absence of microtubules is hypothesized to permit the entry into the neurite of membrane-bound organelles normally excluded by the polarity of their transport on MTs (Black and Baas, 1989). To our knowledge, these are the first instances reported of apparent neurite outgrowth in the absence of MTs. Although we cannot strictly eliminate the possibility that neurite outgrowth occurred in the presence of MTs that were subsequently eliminated, this seems unlikely. If this were occurring, we would expect to find fewer MTs in Li^+-treated cells on day 4 than on day 3, which was not observed. Further, nocodazole was added 3 h after plating, long before any neurite outgrowth. That MTs generally provide required compressive support for neurite outgrowth is argued strongly by neurite retraction and the inability to grow neurites from a variety of cultured neurons under MT-depolymerizing conditions (Yamada et al., 1970; Daniels, 1975; Shaw and Bray, 1978; Solomon and Magendantz, 1981; Joshi et la., 1985); and by direct mechanical measurements (Dennerll et al., 1988). It is difficult for us to avoid the interpretation that neurite outgrowth in the absence of MTs means that their role as compressive members is no longer essential.

It is not clear how ECM relieves the need for MT support. This could arise by shifting compressive support entirely to the substratum; ie., neurites adhere more strongly to ECM than to polylysine allowing the substratum to bear the compressive load. Indeed, this interpretation is supported by the observation that neurites on ECM are attached to the substratum all along their length, while neurites on polylysine are attached only at the growth cone and cell body (Joshi et al., 1985). Although this may play a role, other effects of ECM are also involved. Vlodavsky et al. (1982) found that ECM with altered carbohydrate moieties retained the adhesive properties of untreated ECM but failed to promote neurite outgrowth. We attempted to mimic the effect of ECM with Cell-Tak, a commericially available cell adhesive to which neurons adhere strongly (Notter, 1988). We found PC12 cells had a flattened morphology on Cell-Tak–treated surfaces, similar to that on ECM, but no neurite outgrowth was

Table V. Microtubule Length per Unit Area in Neurites Randomly Chosen from the Three Microtubule Content Categories

Category	No. neurites analyzed	Total area (μm^2)	Total MT length (μm)	MT length/μm^2	
				Range	Mean
Normal	6	268	517	1.31–3.76	2.12
Low	7	201	52	0.04–0.63	0.31
Absent	5	247	13	0–0.08	0.03

noted from either primed or unprimed cells in the absence of NGF. It is possible that the unusually high concentration of intermediate filaments observed in nocodazole-poisoned neurites (Fig. 8 *b*) indicates that these filaments have taken over the role of compressive supports. Alternatively, or perhaps in addition, neurite outgrowth on ECM might occur with less tension development in the actin network, diminishing the need for compressive support; neurites growing on other substrates develop substantial tension (Dennerll et al., 1988; Lamoureux et al., 1989). By whatever mechanism ECM relieves the need for compressive MTs, we wish to emphasize that our structural interpretation is not meant to include all the effects whereby ECM stimulates neurite growth. The available data indicates a complex role for ECM components in neuronal growth (Sanes, 1989).

Our finding that neurites can extend in the absence of MTs echoes recent demonstrations that neurites can extend in the absence of growth cone activity (Marsh and Letourneau, 1984; Spero and Roisen, 1985; Letourneau et al., 1987). We speculate that both growth behaviors reflect a single underlying complementary structural function of actin and microtubules in neurite outgrowth (Buxbaum and Heidemann, 1988). In the "pinch" of poisoned conditions, either the compressive MTs or the tensile actin can play an exaggerated role in supporting elongation without the complementary function. Neurite outgrowth in the presence of cytochalasin (Marsh and Letourneau, 1984) and in the combined presence of cytochalasin and taxol (Joshi et al., 1985; Spero and Roisen, 1985; Letourneau et al., 1987) seems likely to be an exaggeration of the normal compressive role of MTs; they are pushing out the neurite (Buxbaum and Heidemann, 1988). In the present case, neurite elongation without MTs, we imagine ECM stimulates the actin-based activity of the growth cone which pulls out the neurite (Lamoureux et al., 1989). Smalheiser (1989) has shown that laminin, a component of ECM, specifically stimulates growth cone-like behavior of NG108-15 cells. Under normal culture conditions, the interaction of the two cytoskeletal elements provides for higher growth rates; elongation in the presence of cytochalasin, nocodazole or Li^+ is slower and less frequent than without these poisons. We speculate that the cell emphasizes either the compressive or tensile roles under different conditions to regulate growth.

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