CELL-SUBSTRATUM ADHESION OF NEURITE GROWTH CONES, 
AND ITS ROLE IN NEURITE ELONGATION

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SUMMARY

The adhesion of chick embryo sensory neurons to glass coverslips was examined with interference reflection optics. On untreated glass, adhesive contacts are common only beneath growth cones and are small. On polylysine-treated glass growth cones are highly spread, microspikes reach treat lengths and extensive adhesive contacts underlie growth cones, microspikes and nerve fibers. Veils, expanded from the growth cone, are adherent to the substratum either centrally or laterally, while the extending edge of the cell margin is non-adherent. Linear adhesions are frequent beneath microspikes and pass centrally beneath the growth cone margin. The distribution of linear adhesions resembles that of microfilament bundles seen within whole mounts of growth cones. Adhesive contacts stabilize extensions of the growth cone margin and may influence the organization of the microfilamentous network within the growth cone. Regulation of microfilament organization by adhesion may influence microfilament functions in growth cone mobility and the assembly of neurite structures.

Much attention has been focused on understanding cell locomotion and its regulation [18, 23]. Cell-substratum adhesion is one element of the mechanism of cell locomotion, possibly by acting to stabilize protrusions from the cell margin and by providing anchorage during actomyosin-driven cell displacement [2, 10, 11]. Among the approaches to studying cell-substratum adhesion, interference reflection microscopy offers the advantages that living, migratory cells can be examined, the dimensions of cell-substratum contacts can be determined, and the cells are not altered by the process, allowing subsequent procedures [9, 13]. This technique has been profitably used to investigate the progression of adhesive contacts during cell migration, adhesive interactions during cell-cell contact and the relationship of adhesive sites to the distribution of microfilament bundles [1, 11, 13].

Measurements of nerve cell morphogenesis in vitro indicate the importance of cell adhesion in nerve fiber elongation [14]. Proposed mechanisms of nerve fiber growth suggest that the assembly of components into the cell surface and into axonal structures is stabilized or promoted by adhesive contacts [6, 14]. The directions of neurite growth might also be regulated by the adhesive interactions of nerve tips with their environment [3, 15].

I have studied growth cone function with interference reflection optics and transmission electron microscopy (TEM) of whole

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growth cones. The results support the notion that cell-substratum adhesion stabilizes extensions of the growth cone margin, and may promote nerve fiber extension through its influence on the organization of the microfilaments within the growth cone.

MATERIALS AND METHODS

Media
Tissue culture medium was Ham's F12 buffered to pH 7.4 by sodium bicarbonate and supplemented with penicillin and streptomycin plus 10% fetal calf serum (FCS) (all components from GIBCO). Ganglia were rinsed and dissociated in Ca²⁺-, Mg²⁺-free Hanks' salt solution (GIBCO) buffered with sodium bicarbonate. Bactotryptsin (Difco) was used for trypsin dissociation of sensory ganglia. Polylsine-HBr was purchased from Sigma Chemical Co.

Tissue dissociation and cell culture
Dorsal root ganglia were dissected from 8-day chick embryos and dissociated into single cells by incubation in Ca²⁺-, Mg²⁺-free Hanks' salts containing 0.2% Bactotryptsin, as described in Letourneau [14]. After centrifugation the cells were resuspended in tissue culture medium containing 10 ng/ml bNGF (a gift of Dr Eric Shooter) and 4-8×10⁶ cells were plated in each 35 mm plastic bacteriological Petri dish (Falcon Plastics) which contained a 25 mm glass coverslip or polylsine-coated coverslip [14]. The cultures were incubated at 37°C in a 5% CO₂ humidified incubator.

Light microscopy
After 24 h the coverslips with cells were prepared for microscopic observation. Three or four small pieces of broken coverslip were placed on a 13×3 glass slide approximately at the perimeter of a 25 mm circle. A coverslip was removed from a Petri dish with tine forceps, the underside was quickly washed with wet cotton swabs, and then the coverslip was inverted and set on the glass chips on the slide. The space between the coverslip and slide was filled with warm culture medium, and the edge of the coverslip was sealed with silicone grease (Dow-Coming) to prevent evaporation and pH change.

All microscopy was done with a Zeiss IM inverted microscope. All micrographs were taken on Plus X Pan film (Kodak) using a 63× oil immersion planapochromat objective for phase contrast. Interference reflection microscopy was done using an HBO 100 W mercury bulb, Zeiss H-D reflector, 2 FL housing, 546.1 nm precision filter, and a 100× oil immersion epiplan pol objective [13]. The microscope stage was warmed to 37°C with an air stream incubator (Nicholson Precision Instruments, Inc.). Two minutes or less elapsed between phase contrast and interference reflection photographs of individual growth cones.

Electron microscopy
Sensory ganglion cells were cultured on polylysine-treated, carbon-shadowed, formvar-coated, 100 mesh gold grids (Pelco). After 24 h the cells were fixed for 1 h at 37°C with 2% glutaraldehyde as in [21]. Secondary fixation was for 10 min at 4°C with 1% osmium tetroxide in 50 mM KCl and 5 mM MgCl₂ buffered to pH 7.4 with 50 mM sodium phosphate [22]. Dehydration in acetone, staining with 2% uranyl acetate and critical point drying (Sorvall apparatus) were carried out as in [7]. The grids were examined in a Philips 400 electron microscope and stereo pairs were taken.

RESULTS

Growth cone morphology
In addition to the enhanced formation of nerve fibers obtained when cells are cultured on a polylysine-coated surface, there are differences in nerve cell morphology associated with increased adhesion to the substratum [14]. Under conditions of high adhesivity, nerve fibers are crooked, tracing the pathway taken by the extending nerve tip, and the growth cones are flattened and expanded [14, 20]. Figs 1-4 illustrate the pathways taken by the extending nerve fibers. Figs 1 and 2, phase contrast (7) and interference reflection (6) images of neurons cultured on untreated glass. Figs 3 and 4, phase contrast (5) and interference reflection (6) images of neurons cultured on polylysine-treated glass. Note long microspikes extending from polylysine-treated surface (arrowhead). Figs 5 and 6, phase contrast (5) and interference reflection (6) images of neurons cultured on untreated glass. Figs 7 and 8, phase contrast (7) and interference reflection (8) images of neurons cultured on untreated glass. Contact sites at left in fig. 8 (arrows) are beneath edge of a non-neuronal cell. Small adhesion sites are beneath growth cones (arrowheads) but nerve fibers have little or no reflection image. Figs 9 and 10, neuron cultured on untreated glass. Nerve cell soma makes large adhesion (arrow) to glass and small adhesions are evident beneath tips of nerve fibers (arrowheads).

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Table 1. Mean number of microspikes/growth cone and frequency distribution of microspike lengths

These data represent analysis of 57 photographs of growth cones cultured on untreated glass and 50 growth cones cultured on polylysine-treated glass.

<table>
<thead>
<tr>
<th>No. of microspikes/growth cone</th>
<th>Untreated glass</th>
<th>Polylysine-treated glass</th>
</tr>
</thead>
<tbody>
<tr>
<td>% microspikes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–10 μm long</td>
<td>5 ± 2.9 (S.D.)</td>
<td>11.5 ± 6 (S.D.)</td>
</tr>
<tr>
<td>11–20 μm long</td>
<td>38</td>
<td>33</td>
</tr>
<tr>
<td>21–30 μm long</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>&gt;30 μm long</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

pronounced difference in typical growth cone shape on polylysine-coated glass compared to untreated glass. In addition to the spreading of growth cones the mean number of microspikes per growth cone for cells cultured on polylysine-coated glass is over double that of neurons cultured on untreated glass (table 1). Very long microspikes were projected from growth cones cultured on polylysine-coated glass, as microspikes longer than 30 or 40 μm were regularly observed. If such long microspikes are extended on untreated glass, they must be extended for very brief periods only, as they are rarely seen. A frequency distribution of microspike lengths shows that 14% of the microspikes on polylysine-coated glass were longer than 20 μm while only 6% of microspikes exceeded 20 μm for neurons cultured on untreated glass (table 1). In summary, compared to neurons cultured on untreated glass, the growth cones of neurons cultured on polylysine-coated glass are more spread, have more microspikes, and although many microspikes are short, possess microspikes of greater length.

Interference reflection

According to Izzard & Lochner [13], the varying intensities of the interference reflection images represent a range of cell-substratum separations. The closest contacts, approx. 100 Å separation, are discrete dark grey or black areas in monochromatic light. These focal contacts are often surrounded by gray areas which represent approx. 300 Å separation. These two regions are both thought to function as cell-substratum adhesions. Areas which are white or lighter than the surrounding background represent separations of 1000–1500 Å and are not adhesive sites. The adhesive and non-adhesive nature of these cell-substratum separations is supported by the elegant micromanipulation work of Harris [10].

A similar range of intensity occurs in interference reflection images of neurons. When neurons are cultured on untreated glass, small areas of close contact, appearing black or dark gray, are usually seen beneath the growth cone (figs 5–8). The images of axons are white, suggesting
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of a nerve fiber may also be adherent to the substratum. Microspikes extended from a growth cone appear white or have no image at all, again suggesting great separation from the substratum. Most cell bodies of neurons cultured on untreated glass were situated on non-neuronal cells [14, 19], however, interference reflection images of the few neurite-bearing cell bodies that were directly on the glass revealed black areas of presumed adhesion beneath these somata (figs 9 and 10).

Neurons cultured on polylysine-coated glass have extensive areas of cell-substratum adhesion. Neuronal somata are flattened at the margins and have large areas of close contact with the substratum (figs 11–12). The dark reflection image often extends to the edge of the phase image, and is interrupted by white circular areas of greater separation. The interference reflection image of nerve fibers is variable. Dark bands representing 100 Å separation may extend many microns beneath an axon (figs 13, 14, 18, 20). However, these adhesions may be narrower than the axon itself, or long zones of close contact may be interrupted by small white areas of greater separation. Microspikes extended from the side of a nerve fiber may also be adherent to the substratum.

The interference reflection images of growth cones cultured on polylysine are complex, ranging from white to black in many areas. Beneath the bases of growth cones are large black zones of close contact, though the most interesting areas are at the peripheral sites of extension and retraction of microspikes and lamellipodium-like veils (figs 15–20). Microspikes often appear all black, possibly indicating close contact all along their length, or they appear banded, suggesting limited adhesive contact beneath microspikes (fig. 16). Figs 19 and 20 illustrate a growth cone extending its margin in veils. The edges of these veils are white, while dark, elongated adhesive contacts are seen beneath the center of the veil or at the lateral margins (see also figs 23, 24, 25). The convex margins of the veil are analogous to similar portions of fibroblast lamellipodia, which are thought to be the sites of membrane extension or expansion [1, 2, 12, 13]. The white color suggests that these convex margins are widely separated from the substratum, in accord with Ingram’s observations [12] that the extending edge of a lamellipodium does not contact the substratum.

It should be noted that the elongated contacts beneath microspikes and veils often continue inward beneath the central or proximal regions of the growth cone (figs 18, 20, 23, 24). As described in the following section on whole mount electron microscopy, these linear adhesive contacts may be structurally related to intracellular bundles of microfilaments.

Because these membrane veils and microspikes are thin, reflections from the interface between the dorsal cell surface and the culture liquid may contribute to the interference images and confuse interpretation.
of the adhesive contacts of these membrane expansions. However, the numerical aperture of the illumination system was high in these studies, eliminating the higher order reflections described in [1, 13]. Time-lapse cinematography of the interference reflection images of growth cones will help clarify growth cone-substratum adhesion, by indicating whether these presumed adhesive regions appear and disappear as occurs with fibroblasts [13].

Concave portions of a leading lamella are thought to be undergoing retraction [1]. Note in fig. 27 a large concave region of a growth cone which is mostly widely separated from the substratum, but has regions of presumed adhesion at the lateral edges. The growth cone in fig. 18 has many microspike-like extensions separated by concave catenary-like portions of the cell margin. Several of the concave regions are white, indicating separation from the substratum, while microspikes bordering these concave areas appear to be adherent to the substratum. However, other concave regions have a black border, suggesting close contact with the substratum. Again, time-lapse cinematography will be useful in clarifying the progression of growth-cone substratum contact during motility.

The bulbous growth cones of neurons cultured on untreated glass will flatten and spread to resemble those of neurons cultured on polylysine-coated glass when the calcium content of the culture medium is elevated to 20 mM [14]. With interference reflection optics I have seen that this morphological transformation begins within minutes of increasing calcium levels and is accompanied by an increased extent of adhesion of microspikes, the growth cone, and the axon to the glass (figs 21–25). It is noteworthy that parts of the axon many microns proximal to the growth cone also become closer to the glass.

**Electron microscopy**

An important finding from the interference reflection studies of fibroblasts is that areas of closest contact are often coincident with intracellular stress fibers visible in phase and Nomarski optics [13]. This correlation is especially strong for the termination points of stress fibers near the cell margin and focal points of adhesion [2, 13]. Electron microscopy indicates that these focal contacts are located at the insertion sites of microfilament bundles at the lower cell surface [11].

Examination of thin sections from cultured neurons led to the conclusion that actin bundles or stress fibers of microfilaments are not formed in nerve cell bodies, axons, or growth cones [19]. However, note was made of localized areas of linear orientation in the microfilament network of growth cones [19, 26]. When viewed in whole mounts of neurons cultured on a polylysine-treated surface these linear areas of 50–70 Å microfilaments begin to resemble filament bundles (figs 28–33). Compared to the stress fibers or actin cables of fibroblasts these bundles are narrow and contain few filaments, but the organization of long straight bundles of roughly parallel

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Figs 30-34. Electron micrographs of a single whole growth cone.

**Fig. 30.** This low power picture illustrates that the flattened growth cone margin contains a network of microfilaments. Bundles of filaments with a distribution similar to linear adhesive contacts seen with interference reflection (arrowheads). ×3700.

**Fig. 31.** Higher power view of growth cone area at left in fig. 30. Areas of microfilament bundles and unorganized filament lattice work can be distinguished. Arrowheads indicate strings of vesicles oriented along microfilament bundles. ×10500.

**Figs 32 and 33.** Stereo pair of region outlined in fig. 30. Also contained in fig. 31. Illustrates microfilament bundles passing through an expanded extension from growth cone margin. ×22000.
microfilaments is certainly different from the surrounding filament lattice, which consists of branched or crossed microfilaments. The straight microfilaments are linked at many points by short branches to each other, the surrounding filament network, and the lower cell surface. Also, like the close contacts seen with interference reflection (figs 18, 20, 23–25), these bundles continue from the central growth cone region out to the cdc of the cell margin and pass down microspikes as a core of linearly oriented microfilaments. Interference reflection observation and subsequent electron microscopy of the same neurons will be required to confirm this relationship between microfilament organization and the adhesive contacts of growth cones.

Note in figs 31 and 32 numerous vesicles aligned along a microfilament bundle which passes outward from the central growth cone region. The origin and movement of these vesicles cannot be determined. However, their presence in association with the filament bundles suggests that adhesive contacts influence the position of other structures within growth cones in addition to microfilaments.

DISCUSSION

How do these studies help understand the role of cell-substratum adhesion in nerve fiber growth?

According to current models, adhesive interactions anchor microspikes and other extensions from the growth cone margin. Thus stabilized against withdrawal or retraction, these protrusions comprise expansion of the growth cone surface, and coupled with subsequent additions to internal neurite structures, these events constitute neurite elongation [6, 16, 25]. Using interference reflection I have visualized the adhesive events associated with extension of the growth cone margin and, together with electron microscopy of whole neurons, have arrived at a clearer picture of growth cone function.

In line with previous reports I observe adhesive contacts beneath the central region of growth cones cultured on untreated glass, while the growth cone margin, microspikes and nerve fibers tend to be far from the substratum and weakly adherent, if at all [5, 17, 20]. If microspikes and other areas of the growth cone margin do make close contacts with the substratum, they must be very brief or infrequent, as they are not seen. It is unclear whether such fleeting adhesive contacts would stabilize the growth cone, as proposed in the models. Thus, the close contact beneath the central part of the growth cone may be the significant adhesive interaction, and indicates that neurite elongation on untreated glass is limited by the advance of this central adhesive region. It is unclear why nerve fibers, proximal to the growth cone, do not form adhesive contacts to untreated glass, because growth cones and glial cells show a high adhesive affinity for nerve fibers [24].

When neurons are cultured on polylysine-coated glass, the relationship between extension and adhesion is more apparent. Large areas of close contact are seen beneath growth cones, including many peripheral adhesions which may stabilize the high-ly spread margin. Even more revealing are adhesive contacts beneath microspikes and the veils which expand from the sides of microspikes and between adjacent microspikes. Although portions of these extensions (including the extending edge itself) are far from the substratum, the adhesive sites may provide anchorage, allowing subsequent extension to further advance the growth cone margin. The very long micro-
spikes observed on polylysine-coated glass may form because the firm adhesions, which occur beneath the microspikes, allow extension of an individual microspike tip to be prolonged. The frequent spreading of membrane veils on polylysine can be explained by the occurrence of membrane expansion all along the linear adhesive contacts, beneath the center of a veil, or at its lateral borders. These linear adhesions are rarely seen on untreated glass, and the production of veils is similarly uncommon (except when Ca\(^{2+}\) levels are high). In addition, the location of adhesions at the edges of areas undergoing withdrawal suggests that these contacts can limit retraction of the cell margin.

If we speculate that the elongated adhesive contacts beneath microspikes and veils are structurally related to the bundles of microfilaments within growth cones, as established for fibroblasts [2, 11, 13], then a role for adhesive events in nerve fiber elongation can be proposed. Adhesive sites may coincide with intracellular microfilament bundles, because cell-substratum adhesion triggers assembly or redistribution of microfilaments into bundles by a transmembrane event [2, 4], or because preexisting bundles are stabilized by association with adhesive contacts. Actual cause and effect in this relationship is unknown. Nevertheless, by influencing microfilament organization, cell-substratum adhesion could influence microfilament function in the following manner. The activity of microfilaments in neurite extension may include extension and retraction of the cell margin, movements of the cell surface, and transport and positioning of intracellular materials, such as precursors of neurite structures [5, 21, 26]. The crucial effect of adhesion may be that the rates or directions of these activities are different within the linearly or-organized microfilament bundles, compared to within the surrounding branched or criss-crossing filament lattice.

Thus, growth cone-substratum adhesion participates in neurite elongation in two major ways. Adhesions stabilize extensions of the growth cone margin, promoting expansion of the cell surface, and may also influence microfilament organization and hence, function in growth cone motility and the assembly of neurite structures. This model can be applied to propose regulatory roles for cell adhesion in such phenomena as the stimulation of neurite formation by conditioned medium [8], the induction of branch points and steering of neurites with microneedles [25], and the guidance of nerve fiber elongation [3, 15].

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