Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 459-464

DELIVERY OF ANTI-GAP-43 ANTIBODIES INTO NEUROBLASTOMA CELLS REDUCES GROWTH CONE SIZE

Thomas B. Shea*

Laboratories for Molecular Neuroscience, McLean Hospital, Belmont,MA 02178
Department of Psychiatry, Harvard Medical School, Boston,MA 02115

Received July 8, 1994

We have previously demonstrated that antibodies to the growth-associated protein, GAP-43, introduced intracellularly using a lipid carrier inhibited neurite outgrowth in NB2a/d1 neuroblastoma cells, and that culturing of these cells on adhesive substrates such as laminin or poly-L-lysine overcame this restriction. These findings suggest that GAP-43 may faciliate neuritogenesis by increasing membrane adhesiveness. To address this issue, in the present study we examined the effect of intracellular delivery of this antibody on growth cone size. A statistically significant percentage of those neurites that did elaborate following intracellular delivery of GAP-43 exhibited either no observable growth cones or smaller growth cones versus cells receiving pre-immune IgG. These results support the hypothesis that the requirement for GAP-43 in neuritogenesis may be related to growth cone formation and membrane adhesiveness.

The neuronal growth-associated protein GAP-43, also referred to as B50, F1, pp46, GAP48, p57 and neuromodulin (7), has been implicated in neuronal development and synaptic plasticity. GAP-43 is expressed at high levels during axonal outgrowth and synaptogenesis (3,14), at which time the protein is concentrated along the entire axonal length and in growth cones (10,12,13,17,19). Levels decline in most neurons following the establishment of synaptic contacts (14), though in instances where axonal regeneration can occur, the protein is re-expressed at levels comparable to those seen during development (4,8).

The spatial and temporal correlation of GAP-43 expression with the development of neurites has led to the hypothesis that this protein may play an essential role in neurite outgrowth. However, controversial results have been obtained in studies in which GAP-43 levels and/or function have been altered experimentally. Transfection of PC12 cells with a GAP-43 transgene increased sensitivity to NGF and accelerated neurite outgrowth (26). Neurite outgrowth was also enhanced following transfection of a gene encoding high levels of GAP-43 into neuroblastoma cells (16), while alterations of membrane adhesion and outgrowth were observed when the GAP-

*To whom correspondence should be addressed at McLean Hospital.

0006-291X/94 $5.00

43 gene, or variants mutated at the protein kinase C phosphorylation site, was expressed in a variety of non-neuronal cells (25,27). Conversely, intracellular delivery of anti-GAP-43 IgG inhibited neurite outgrowth without altering already established processes in NB2a/d1 neuroblastoma (21), and antisense oligonucleotides complementary to portions of GAP-43 mRNA altered neuritic growth in PC-12 cells and in primary sensory ganglion neurons (1,15). However, Baetge and Hammang (2) reported that a variant PC12 cell line that does not express GAP-43 extended neurites when treated with NGF, and Ferreira et al. (11) showed that suppression of kinesin expression using antisense oligonucleotides still allowed neurites to elongate even though GAP-43 was no longer transported down the axon .

Suppression of GAP-43 translation by antisense oligonucleotide treatment of sensory ganglion neurons did not prevent neuritogenesis, but resulted in the elaboration of neurites that lacked growth cones (1). These neurites were hypothesized to have been generated by a mode of outgrowth driven primarily by cytoskeletal dynamics.

Our previous studies have indicated a role for GAP-43 in neurite outgrowth (21), but further indicate that this requirement may be supplanted by culturing of cells, and inducing neurite outgrowth, under conditions where substrate adhesiveness is increased (23). We therefore examined in the present study the effect of intracellular delivery of anti-GAP IgG on growth cone morphology.

MATERIALS AND METHODS

NB2a/d1 cells were cultured in Dulbecco's Minimal Eagle's Medium (DMEM) containing 10% horse serum as described previously (21) and induced to elaborate neurites by treatment for 4hr with 10μM dibutyryl cyclic AMP (dbcAMP) in DMEM in 10% serum or 4hr of serum deprivation of cells plated 24hr previously in Lab-Tek chamber slides.

The IgG fractions derived from anti-GAP-43 antiserum raised in sheep and the respective pre-immune serum were diluted to 1:40 (values based on reconstitution to sheep serum IgG concentration) in serum-free DMEM and delivered into cells that had been transiently permeabilized as described (21) with minor modifications to allow treatment and monitoring of cells in Lab-Tek chamber slides rather than culture plates. Briefly, cells (50-75% confluent) in uncoated, laminin-coated and poly-L-lysine-coated Lab-Tek 8-well chamber slides were rinsed with serum-free DMEM and treated with 100μl of pre-warmed (37°C) 1.2M glycerol in phosphate-buffered saline (PBS; pH 7.4). The cells were then incubated at 0°C for 10 min (by placing the slides directly on ice), after which L-α-lysophosphatidylcholine (LPC) was added at a final concentration of 40μg/ml and the incubation was continued for an additional 8 min. Cultures then received 100μl of pre-warmed (37°C) serum-free DMEM containing either no further additions, anti-GAP-43 IgG, or pre-immune IgG. Following a 10 min incubation at 37°C, all cultures received an additional pre-warmed 100μl aliquot of media with and without serum and dbcAMP as described above to induce neurite outgrowth, and incubated for an additional 2hr.

Cultures were then fixed (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) and examined by phase-contrast microscopy for the presence or absence of neurites, or stained with Coomassie Brilliant Blue for examination of growth cone morphology. Between 100-200 cells were examined, and their neurites were scored as possessing growth cones provided that the distal terminus either elaborated filopodia or was wider in diameter than the neurite shaft. All reagents were obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

Under basal conditions, the majority of NB2a/d1 cells cultured on plastic either have no neurites or only short filopodia-like processes. Within 2hr of adding dbcAMP, cells extend neurites (see also ref. 21-23). As previously demonstrated, intracellular delivery of anti-GAP IgG suppressed neurite outgrowth (Fig. 1).

We compared the morphology of growth cones on those neurites that were elaborated by dbcAMP treatment on plastic following the intracellular delivery of anti-GAP versus those receiving pre-immune IgG. Axonal neurites elaborated by NB2a/d1 cells normally exhibit markedly heterogeneous growth cone morphology, ranging from numerous filopodia elaborating from expansions of membrane that are significantly wider than the diameter of the axonal shaft to no observable specialization at the distal terminus of the axonal shaft (e.g., Fig. 2). Nevertheless, a statistically significant percentage of neurites exhibited either no observable growth cones, or smaller growth cones with fewer filopodia following intracellular delivery of GAP-43 versus cells receiving pre-immune IgG [47.4±4.5% (n=72) versus 29.9 ±3.4% (n=59), respectively; $p<0.0005$, Student's t test].

DISCUSSION

Numerous developmental studies point to a strong correlation between axonal outgrowth or remodeling and GAP-43 expression (5). Various analyses have been carried out with cell cultures to address more directly the putative role of GAP-43 in neuritogenesis. Enhanced neurite

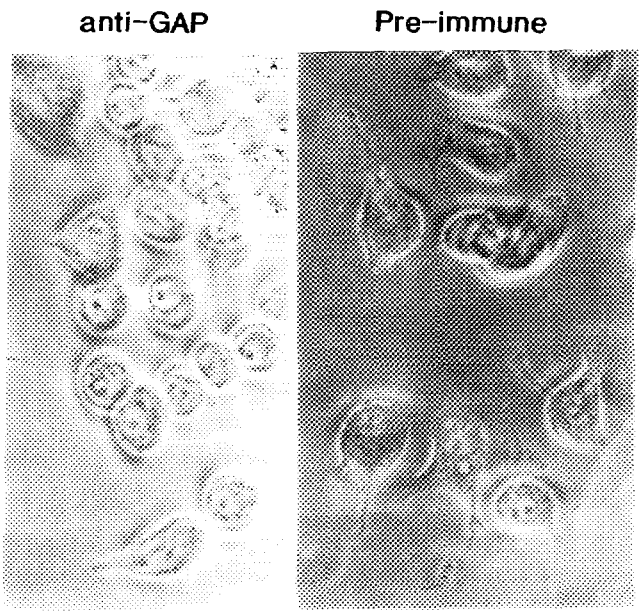

Fig. 1. Phase contrast micrographs of NB2a/d1 cells treated with dbcAMP for 2hr following the intracellular delivery of pre-immune or anti-GAP IgG as indicated. Neurite outgrowth induced by dbcAMP was reduced by intracellular delivery of anti-GAP but not by pre-immune IgG.

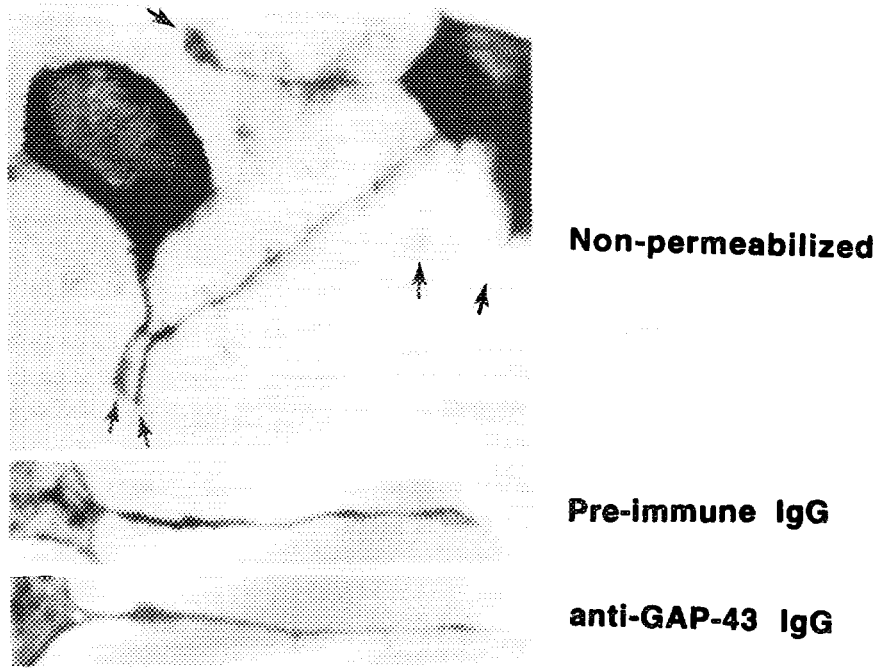

Fig. 2. Representative growth cones, visualized by Coomassie Brilliant Blue staining, of neurites elaborated in response to dbcAMP-treatment of unpermeabilized cells and cells that had been transiently permeabilized in the presence of pre-immune and anti-GAP-43 IgG as indicated. Note the heterogeneity of growth cone morphology in non-permeabilized cells.

outgrowth following transfection of PC12 or neuroblastoma cells with GAP-43 strongly suggests that this protein mediates certain aspects of neurite outgrowth (16,18,20,26). Intracellular delivery of anti-GAP-IgG into NB2a/d1 cells inhibited dbcAMP-induced neurite outgrowth (21). Further evidence that the protein plays a direct role in neurite outgrowth comes from studies showing that genetic or immunological manipulations that alter levels of the protein alter the growth characteristics of neurons and other cells (e.g., ref. 1). Nevertheless, apparent discrepancies in this relationship derive from studies showing that a variant line of PC12 cells extends processes in the absence of GAP-43 expression (2), and that blockage of GAP-43 transport down the axon fails to arrest neurite outgrowth (11).

While the intracellular delivery of anti-GAP-43 antibodies inhibited neuritogenesis in NB2a/d1 cells cultured on plastic, culturing these same cells on adhesive substrates such as laminin, poly-L-lysine or poly-ornithine minimized this inhibition (21,22), indicating that the role of GAP-43 in neurite outgrowth is likley to be related to membrane adhesiveness. In this regard, it is noteworthy that PC12 cells and primary neurons are routinely cultured on coated surfaces, rather than culture plastic, which, according to the above results in NB2a/d1 cells, would minimize any requirement for GAP-43 in neuritogenesis.

Transfection of non-neuronal systems provide the most compelling evidence that the *de novo* expression of that GAP-43 can modulate surface activity. GAP-43 enhanced the appearance of, and accumulated within, membrane irregularities and filopodial extensions following transient transfection of non-neuronal cells (25,27). This conclusion was further supported by the failure of filopodia to be induced when these cells were transfected with a GAP-43 sequence containing substitution mutations that prevented the protein's association with the submembrane skeleton (27). Overexpression of GAP-43 in PC12 cells induced membrane "blebs" and filopodia independent of NGF treatment (20).

These results suggest that GAP-43 may regulate neuritogenesis by modulating surface membrane adhesiveness. This possibility is consistent with the association of GAP-43 with the membrane skeleton following fractionation, and the preferential localization of GAP-43 within areas of the growth cone plasma membrane that exhibit tight substrate binding (17). Furthermore, although no overall difference in neurite outgrowth were observed, Baetge and Hammang (2) report that their GAP-43-deficient PC12(B) line is readily dislodged from the culture substratum by tapping, whereas the parental line is not and needs vigorous washing in Ca^{2+}- and Mg^{2+}-free buffer.

We observed in the present study a modest but statistically significant reduction in growth cone size in those neurites that were elaborated in response to dbcAMP following intracellular delivery of anti-GAP IgG versus those receiving pre-immune IgG. These results are in agreement with those of Aigner & Caroni (1), where growth cone morphology was altered following antisense-oligonucleotide mediated suppression of GAP-43 expression in cultured neurons. These findings support the suggestion (1) that GAP-43 may function in axonal neurite outgrowth by regulating growth cone interactions with the surrounding environment. Impaired growth cone–mediated neurite-substrate interactions may underlie the inhibition of neuritogenesis following intracellular delivery of anti-GAP-43 IgG (21).

ACKNOWLEDGMENTS

This research was supported by the National Science Foundation and by NIMH MH47349.

REFERENCES

1. Aigner L and Caroni P. (1993) J. Cell Biol. 123: 417-429.
2. Baetge EE and Hammang JP (1991) Neuron 6: 21-30.
3. Basi GS, Jacobson RD, Virag I, Schilling J and Skene JHP (1987) Cell 49: 785-791.
4. Benowitz LI, Lewis, E (1983) J Neurosci 3: 2153-2163.
5. Benowitz LI, Perrone-Bizzozero NI (1991) Prog Brain Res 89: 69-88.
6. Benowitz LI, Perrone-Bizzozero NI, Finklestein SP, Bird ED (1989) J Neurosci 9: 990-995.
7. Benowitz LI, Routtenberg A (1987) Trends in Neurosciences 10: 527-532.
8. Benowitz LI, Shashoua VE, Yoon MG (1981) J Neurosci 1: 300-307.
9. Burry RW and Perrone-Bizzozero NI (1993) J Neurosci Res 36:241-251.
10. Dani JW, Armstrong DM and Benowitz LI. (1991) Neuroscience 40: 277-288.
11. Ferreira A, Niclas J, Vale RD, Banker G and Kosik KS (1992) J Cell Biol 117: 595-606.
12. Fitzgerald M, Reynolds ML and Benowitz LI (1992) Neuroscience 41: 187-199.

13. Goslin K, Schreyer DJ, Skene JH and Banker G (1988) Nature 336: 672-674.
14. Jacobson RD, Virag I and Skene JHP (1986) J Neurosci 6: 1843-1855.
15. JapTjoen San ERA, Schmidt-Michels M, Oestreicher AB, Gispen WH and Schotman P. (1992) Biochem. Biophys. Res. Comm. 187: 839-846.
16. Kumagi C, Tohda M, Isobe M and Nomura Y (1992)J Neurochem 59: 41-47.
17. Meiri KF and Gordon-Weeks PR (1990) J Neurosci 10: 256-266.
18. Morton AJ and Buss TN. (1993) Eur. J. Neurosci. 4: 910-916.
19. Moya KL, Jhaveri S, Schneider GE, Benowitz, LI. (1989) J Comp Neurol 288: 51-58.
20. Nielander HB, French P, Oestreicher AB, Gispen WH, Scotman P (1993) Neurosci Lett 162: 46-50.
21. Shea TB, Perrone-Bizzozero NI, Beermann ML and Benowitz LI (1991) J. Neurosci. 11: 1685-1690.
22. Shea TB and Beermann (1993a) Neurosci Res Comm 11: 163-169.
23. Shea TB and Beermann (1993b) Mol Cell Biol 4: 171a.
24. Skene J.H.P. (1989) Ann. Rev. Neurosci. 12: 127-156.
25. Widmer F. and Caroni P (1993) J. Cell Biol. 120: 503-512.
26. Yankner BA, Benowitz LI, Villa-Komaroff L, Neve RL. (1990) Molec. Brain Res. 7: 39-44.
27. Zuber MX, Goodman DW, Karns LR and Fishman MC (1989) Science 244: 1193-1195.