Journal of Cellular Biochemistry 18:25–35 (1982)
Cellular Recognition 489–499

Deposition of Basement Membrane Proteins in Attachment and Neurite Formation of Cultured Murine C-1300 Neuroblastoma Cells

K. Alitalo, M. Kurkinen, I. Virtanen, K. Mellström, and A. Vaheri

Departments of Virology (K.A., M.K., K.M., A.V.) and Pathology (I.V.), University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki 29, Finland

The deposition of the basement membrane glycoproteins, laminin, fibronectin, and type IV procollagen was studied by indirect immunofluorescence microscopy during the attachment and differentiation of murine C-1300 neuroblastoma cells. A typical cytoplasmic perinuclear staining for the basement membrane antigens was seen both in undifferentiated and differentiated cells. Freshly seeded suspended cells lacked surface fluorescence but in two hours after plating, distinct punctate laminin deposits became discernible on the ventral surface of the cells. Notably, in sparsely seeded undifferentiated cultures, the cell-associated extracellular laminin deposits could only be detected under the primary attaching cells, whereas daughter cells in clonal cell colonies lacked such fluorescence. In cultures induced to neurite formation with dibutyryl cyclic AMP, laminin deposition was also detected in association with the growing cytoplasmic extensions. No distinct differences were found between the secreted proteins of cultures of differentiated and nondifferentiated neuroblastoma cells, but the patterns of fucosylation of high-molecular weight proteins in the two cultures were markedly different.

We conclude that cultured neuroblastoma cells both synthesize, secrete and deposit laminin. The distribution of laminin during neuroblastoma cell attachment and neurite extension suggests that this glycoprotein may be involved in cell-to-substratum interactions in C-1300 cell cultures.

Key words: substrate adhesion, basement membrane, laminin, collagen, extracellular matrix, neuronal cells

It was reported already in 1969 that cells derived from a spontaneous murine tumor of neuroectodermal origin were able to differentiate morphologically, when provided a solid substratum for growth [1]. Since then, neurite extension in neuroblastoma cultures has been induced by a variety of agents and treatments (see [2, 3]) that often elevate the level of intracellular cyclic AMP [2]. A firm cell-to-substrate adhesion is a necessary, though not a sufficient, condition for axonal elongation of

K. Mellström is now at the Department of Tumor Biology, The Wallenberg Laboratory, University of Uppsala, Box 562, S-75122 Uppsala, Sweden.

Received May 8, 1981; accepted July 21, 1981.

0730-2312/82/1801-0025$03.50

neuronal cells [4]. Treatments that seem to increase adhesion have been reported to lead to a morphologically differentiated state [5]. One of such recipes is to grow cells in conditioned medium derived from cultures of the C3 rat glial cell line [6] or from chick embryo heart cells [7]. From both conditioned media, the activity is bound to the culture substratum. In one study, nerve growth factor was found to induce changes both in the cell–substratum adhesion and in the polypeptides of the substrate-attached material of the PC-12 pheochromocytoma cell line [8]. Two studies have examined the surface glycoproteins in the N18 clone of C-1300 neuroblastoma cells, but found no fibronectin [9, 10]. However, glycoprotein of similar apparent size was present and could be labeled with radioactive fucose [9, 10], and fibronectin has also been reported in the adhesion sites of mouse neuroblastoma cells [11]. Growth and differentiation of neuroblastoma cells also occurs in serum-free preparations of defined media that contain fibronectin [12, 13].

The importance of deposition of the extracellular matrix for differentiation of Schwann cells in culture with neuronal cells has also been emphasized by Bunge and others [14]. A requirement for pericellular matrix for attachment may be a general prerequisite for the differentiation of neuronal [15], epithelial [16], and even hematopoietic [17, 18] cells under culture conditions.

We have studied the matrix proteins deposited by differentiating neuroblastoma cells C-1300, clone NB41A3. No consistent differences were found between polypeptides secreted by undifferentiated and differentiated cells. Deposits of laminin, but not of fibronectin or type IV collagen antigenicity, were found under attaching and differentiating cells.

MATERIALS AND METHODS

Cell Culture and Radioactive Labeling

The mouse neuroblastoma cells (C-1300, clone NB41A3, American Type Culture Collection, CCL 147) were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics. To induce morphologic differentiation, the neuroblastoma cells were plated sparsely and cultured in the presence of 1 mM N^6, O^2-dibutyryl adenosine 3′,5′-monophosphate (dbcAMP; Sigma, St. Louis, Missouri). In some experiments, 30 μg/ml sodium ascorbate (Fluka AG, Buchs, Switzerland) and 50 μg/ml of β-aminopropionitrile fumarate (Sigma) were added to the medium, as indicated.

The cells were labeled with 1 μCi/ml of L-[^{3}H]fucose or 10 μCi/ml of [2-^{3}H]glycine and L-[5-^{3}H]proline. Labeling with L-[^{35}S]methionine (600 Ci/mmol) was carried out using methionine-free medium, in which the cell cultures were maintained for at least 1 hr prior to labeling.

Immunological Methods

Preparations of antibodies to mouse laminin [19], fibronectin [20], and procollagens and collagens [21] have been described. Laminin and fibronectin radioimmunoassays were as described in [19] and [20], respectively. The specificities of the antisera and lack of their cross-reactions between the various antigens, as well as their interspecies cross-reactions, have been established in several previous reports [19, 22, 23].

Immunofluorescence Microscopy

To stain extracellular antigens using indirect immunofluorescence microscopy, the cells were labeled unfixed at 0°C with the antibodies or antisera. The cell layers were then fixed with 3% paraformaldehyde in 0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.4 (Pi/NaCl), at room temperature for 20 min and incubated with fluorescein-conjugated anti-rabbit IgG antibodies (Wellcome, Beckenham, United Kingdom) for 30 min. For staining of both extra- and intracellular antigens, the cells were fixed with paraformaldehyde and treated with acetone (5 min at −20°C). For staining controls, both normal rabbit serum and affinity-purified anti-ovalbumin antibodies were used with consistently negative results. For fluorescence microscopy we used a Zeiss Universal microscope equipped with an epi-illuminator III RS and an HBO 200-W lamp for specific fluorescence excitation together with a filter for fluorescein isothiocyanate (490 nm). The distribution of antigens was related to culture topography by sequential use of immunofluorescence and phase-contrast optics without changing the objective level of the focus. Mouse tissues were processed for immunofluorescence microscopy by the method of Sainte-Marie [24] as described previously [25].

Immunoelectron Microscopy

Immunoferritin stainings for laminin and transmission electron microscopy were carried out as described previously [26].

Isolation and Analysis of Newly Synthesized Proteins

Labeled culture medium was harvested by centrifugation and secreted high molecular weight proteins were precipitated with $(NH_4)_2SO_4$ (281 mg/ml) overnight at 4°C in the presence of gelatin as carrier (50 μg/ml, type I, Sigma) and 1 mM EDTA, 0.9 mM N-ethylmaleimide, and 0.2 mM $PhCH_2SO_2F$ as proteinase inhibitors as well as 1 mM $\alpha_1\alpha'$-dipyridyl [23]. Precipitates collected by centrifugation were washed twice with 70% ethanol and analyzed like cell layers in sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis according to Laemmli [27] using fluorography [28] for the visualization of protein bands.

RESULTS

Laminin Deposition

In indirect immunofluorescence microscopy, intracellular laminin was detected in all undifferentiated and differentiated neuroblastoma cells (Fig. 1a). No surface-associated laminin was seen on freshly plated suspended cells, but already within 2 hr after after seeding they had produced punctate laminin deposits on their under-surface, often distributed in a ring-like pattern close to the periphery of the attaching cell (e and f). Presence of dbcAMP in the culture medium had no effect on the development of these initial laminin deposits. When sparsely seeded cells were grown to small colonies, only one patch of laminin deposits was detected in each colony (g and h). On the other hand, when the cells were plated at a high density and then surface stained, distinct laminin deposits were detected only under some of the cells and with no obvious topographical relationship to the cells (i to k). Laminin deposits were also seen under the proximal cones of the growing neurites in

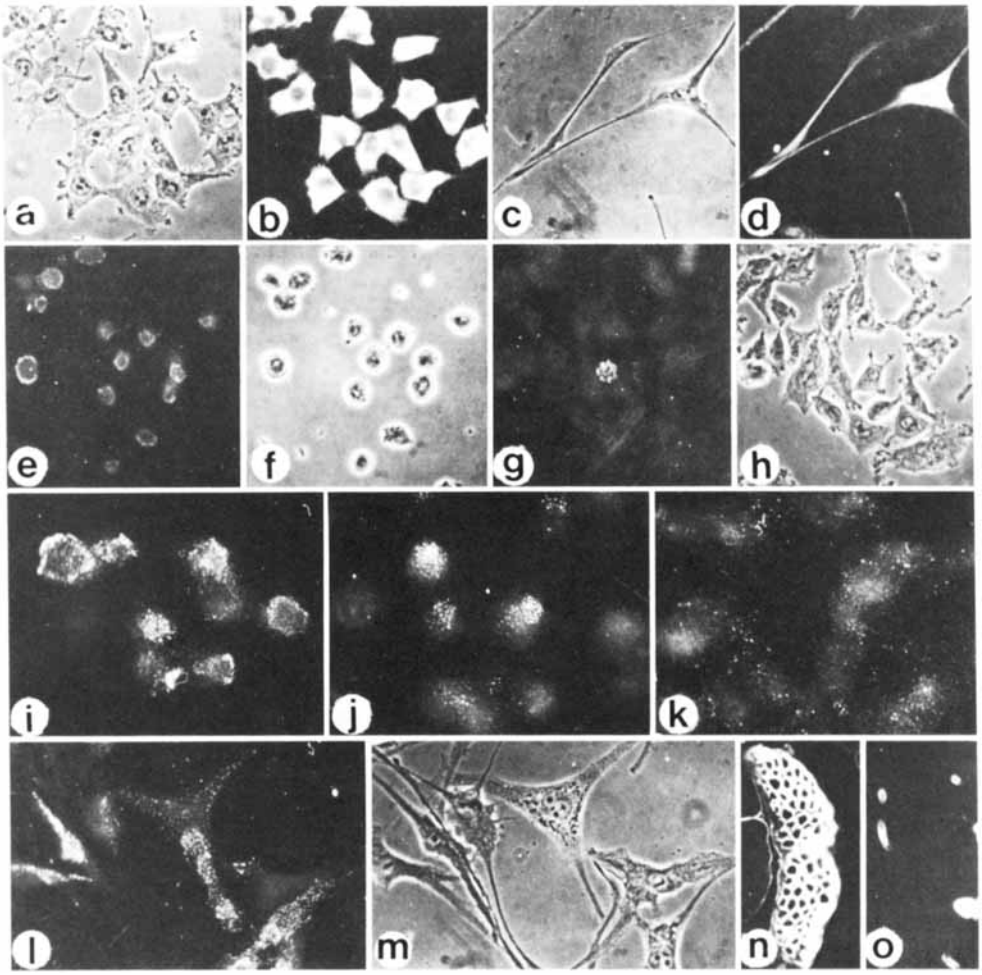

Fig. 1. Indirect immunofluorescence for laminin (a, b, e–o) and type IV collagen (c, d) in neuroblastoma cultures. Figures a, c, f, h, and m show phase-contrast micrographs of the fields b, d, e, g, and l, respectively. Cells shown in a–d were grown for 3 days in the absence (a, b) or presence (c, d) of dbcAMP and then stained for intracellular immunofluorescence. The positive staining for type IV collagen shown in d was obtained only after hyaluronidase treatment of the fixed cell layer. Figures e, i, j, and k show freshly attached cells seeded at different densities (10,000, 25,000, 100,000 cells/ml, respectively) and stained for extracellular laminin. Figures g, h, l, and m, surface staining for laminin in sparsely plated cultures grown for 3 days in the absence (g, h) or presence (l, m) of dbcAMP. Photomicrographs n and o show laminin immunofluorescence in tissue sections of mouse peripheral nerve and brain, respectively.

cell cultures induced to differentiated with dbcAMP (l and m). However, the deposits retained their relationship to cell extensions only for the first days of the differentiation process, and no correspondence of laminin deposits to cellular morphology could be found later in the cultures (not shown). In line with the immunofluorescence results, distinct clusters of ferritin particles were seen at the contacting cell processes of attaching C-1300 cells at the ultrastructural level using antilaminin antibodies in indirect ferritin-immunoelectron microscopy (Fig. 2).

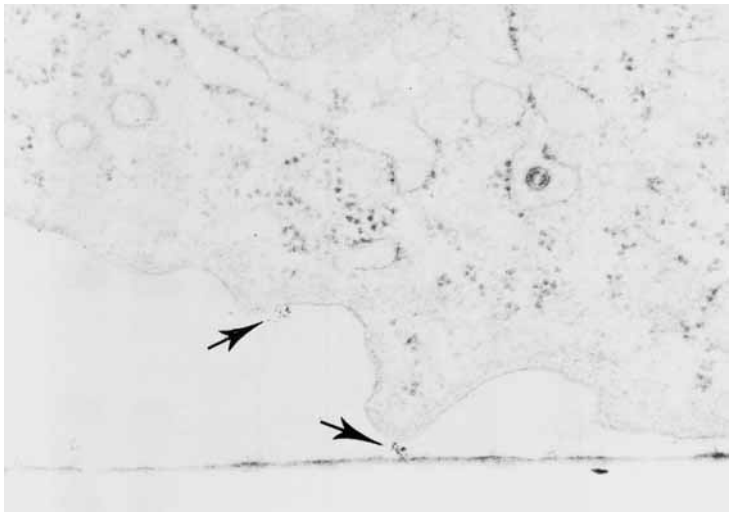

Fig. 2. Immunoferritin electron micrograph of a typical localization of laminin in nondifferentiated neuroblastoma cell cultures (arrows) (magnification × 10,000).

Distribution of Fibronectin and Collagen Type IV

In indirect immunofluorescence, fibronectin was found diffusely distributed throughout the cultures, but neither in attaching nor in differentiating cultures could fibronectin deposits be found to have similar relationships to the cells (not shown). Intracellular collagen type IV was stained only after hyaluronidase (2 mg/ml, P_i/NaCl; Serva, Heidelberg, Federal Republic of Germany), pretreatment (30 min at room temperature) of the coverslips (Fig. 1c,d). Essentially no extracellular type IV collagen was found by immunofluorescence.

Secretion and Fucosylation of Neuroblastoma Proteins

As reported earlier [29], the overall pattern of protein secretion did not appreciably vary over a wide range of labeling times and was not affected by the presence of 1 mM dbcAMP in the cultures (Fig. 3). No apparent differences were found in the fibronectin or laminin molecules in $NaDodSO_4$ gel electrophoresis (Fig. 4) or in their radioimmunoassay binding properties (data not shown). However, distinct differences were found in the patterns of fucosylated polypeptides between differentiated and undifferentiated cell layers. There was a marked reduction of fucose incorporation into a high molecular weight polypeptide that barely entered 5% polyacrylamide gels. Essentially no fucosylation of fibronectin or laminin polypeptides was found in either case (Fig. 5).

Laminin in Nervous Tissue In Vivo

In peripheral nerves the only fluorescence for laminin localized to the peri- and endoneurium but spared again the nervous tissue (Fig. 1n). No laminin could be detected by immunofluorescence in adult mouse brain, except for in the basement membranes of the brain vessels (Fig. 1o).

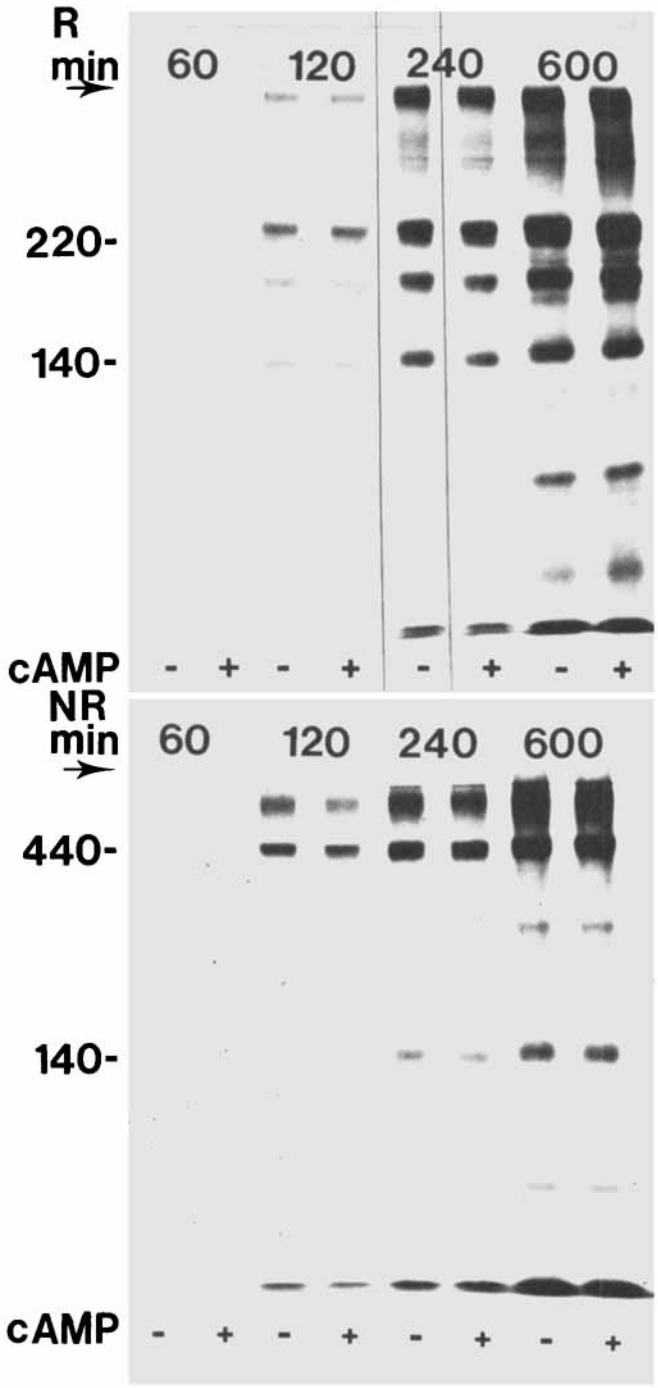

Fig. 3. NaDodSO$_4$-polyacrylamide gel analysis in reducing (R) and nonreducing (NR) conditions of polypeptides in neuroblastoma cell culture media labeled with [^{35}S]methionine in the absence (−) or presence (+) of dbcAMP for the indicated periods of time (min). Numbers on the left refer to the positions of monomeric (220-kd) and dimeric (440-kd) fibronectin as well as to a monomeric polypeptide of molecular weight 140,000.

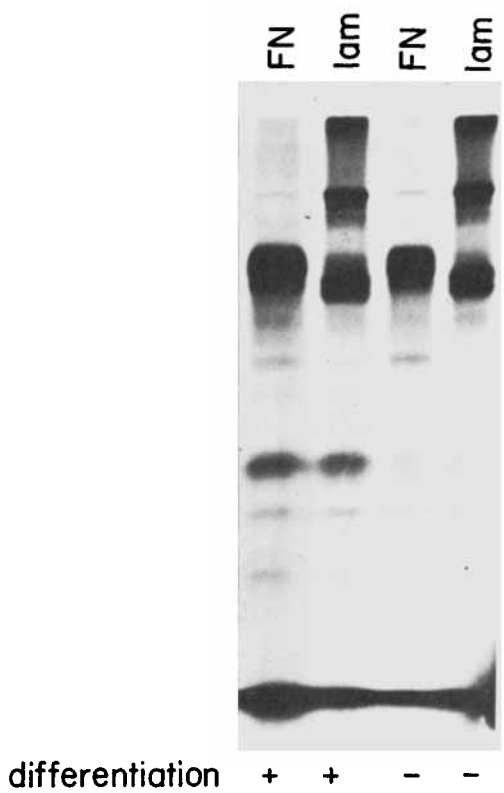

Fig. 4. Immunoprecipitates of ^{35}S-labeled fibronectin (FN) and laminin (lam) from differentiated (+) and nondifferentiated (−) neuroblastoma cells analyzed in 5% polyacrylamide gel electrophoresis in reducing conditions.

DISCUSSION

The major secreted extracellular matrix glycoproteins of C-1300 neuroblastoma cultures have been shown to be fibronectin, laminin, and procollagen type IV [29]. In the present study, deposition of laminin antigenicity was localized to the primary attachment sites of neuroblastoma cells and also to areas under the extending neurites formed in response to dibutyryl cyclic AMP.

As reported in earlier studies on neural cell lines, differentiation induced changes in the fucosylation in cellular high molecular weight polypeptides [9, 10, 30, 31]. However, we found only negligible incorporation of fucose into the extracellular matrix proteins of neuroblastoma cells; the major changes in fucosylation occurred in molecules barely entering the 5% polyacrylamide gels used in the electrophoretic analysis.

In vivo, laminin and type IV collagen are found only in basement membranes [19, 32]. Immunoelectron microscopy has located laminin to the lamina lucida, the zone abutting the epithelial cells [33].

In the present study, a striking finding was that laminin was deposited under

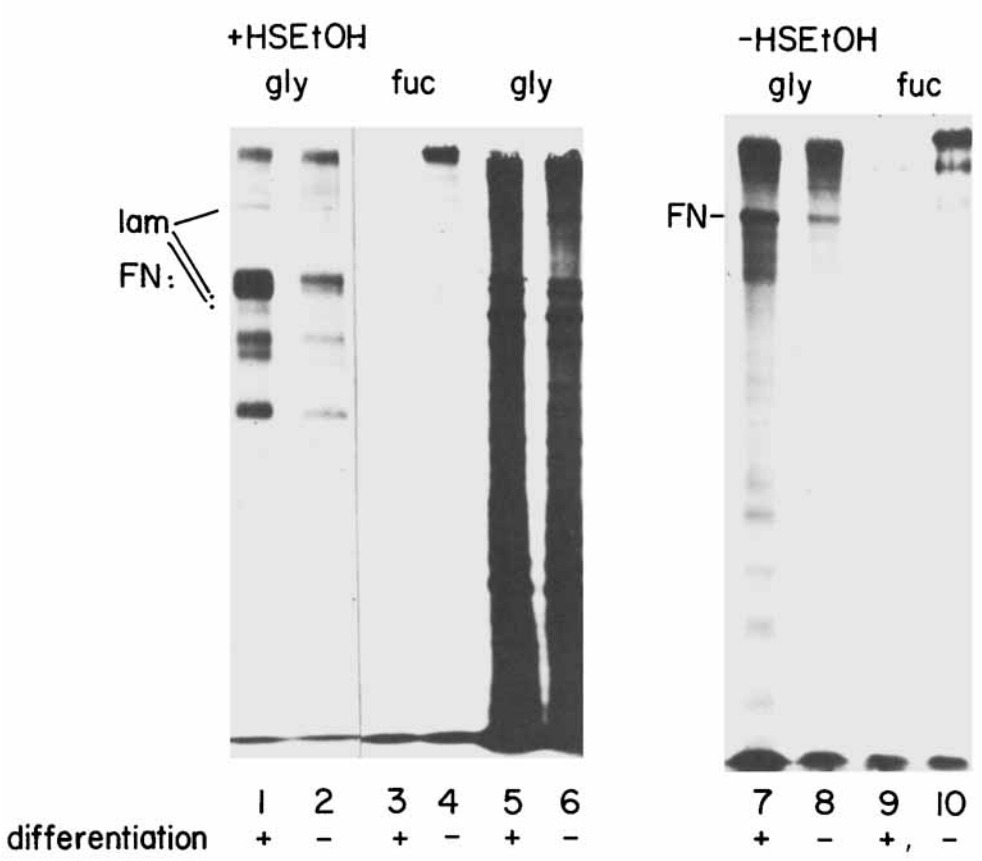

Fig. 5. $NaDodSO_4$-polyacrylamide gel analysis in reducing (+HSEtOH, lanes 1–6) and nonreducing conditions (– HSEtOH, lanes 7–10) of polypeptides in culture media and cell layers of differentiated (+) and nondifferentiated (–) neuroblastoma cell cultures labeled either with [^{3}H]glycine (gly) or [^{3}H]fucose (fuc). Lanes 1, 2, 7, and 8, medium; 3–6, 9, 10, cell layers.

the cells only during their primary attachment and during neurite elongation. In contrast, postmitotic daughter cells did not deposit such laminin plaques on the growth substratum. Furthermore, when the cells were seeded as a dense population, fewer laminin deposits were found deposited and only under some of the cells. These observations suggest that cell–cell contact modulated adhesion may proceed via a different mechanism than the primary cell–substratum adhesion, as is the case in chick neural retina cells in culture [34]. Alternatively, the secretory organelles of the neuroblastoma cell may become polarized upon attachment to the growth substrata inhibiting further secretion and deposition under the cell body. In these studies it was also found that parallel to the deposition of laminin, differentiation in neuroblastoma cell cultures was promoted by low cell density upon plating, as well as by the presence of the differentiation-inducing agent during the primary attachment stage (data not included).

Small amounts of fibronectin were detected in the medium of both undifferentiated and differentiated C-1300 cells. Culp et al [11] have reported on presence of

fibronectin in preparations of substrate-attached material on the N115 clone of C-1300 cells. Unlike in various other cell cultures [35], we found no deposition of fibronectin under the cells. Hence, we consider it unlikely that fibronectin could be involved in neuronal cell adhesion and neurite extension.

Of interest was the finding that hyaluronidase pretreatment was needed to obtain positive fluorescence for intracellular type IV procollagen. Earlier, the same observation had been made in studies of type II collagen in tissues [22, 36] and on SV-40 virus-transformed cells in culture [37]. It may be that the great amounts of hyaluronic acid produced by transformed cells in general associate with collagenous antigens, rendering them unaccessible for antibodies.

An early work on neural embryology found production of basement membrane material by chick neural tube at the time of neural–mesenchymal interaction [38]. Also glycosaminoglycans, that are abundant in brain tissue, are produced in tissue culture of the neural tube [39] and in neuroblastoma cultures [40]. True basal lamina structures in the central nervous tissue occur only around blood vessels, the only site where lamina antigens can be found at the immunofluorescence level [41]. Matrix material in synapses of the central nervous system is poorly known [42]. In the peripheral nervous system acetylcholinergic neuromuscular synapse has an interposed basal lamina structure, where specific antigens are found [43]. Acetyl-cholinesterase having a collagen-like tail structure is located histochemically in the cleft [44]. Conceivably such a location would confer specificity for site of action of an extracellular enzyme.

In vivo laminin was detected in the endo- (and peri-) neural structures. Studies with normal nerve cells and Schwann cells grown both separately and in coculture led Bunge and co-workers [14] to conclude that neurons may contribute to the formation of basal laminae. Also, according to Bunge and co-workers [14], secretion of extracellular matrix material is required for Schwann cell differentiation seen as the formation of a myelin ensheathment of axons [45]. Previously we [26] along with others [47] have demonstrated that collagen, possibly the basement membrane type, is produced by cloned rat Schwann cells in culture. The present studies raise the possibility that some laminin of endoneurium may be derived from nerve cells.

Several studies have demonstrated the lack of deposition of pericellular matrix proteins by a variety of transformed cells (see [19, 48]). Treatment of cultures with dibutyryl cyclic AMP has been reported to cause a partial reversion to a morphologically normal phenotype and matrix deposition of mesenchymal cells [49].

The firm cell-to-substratum attachment is a prerequisite for neurite extension and has been found in cultures of neurons and neuronal cell lines [2, 4, 50]. Laminin has been shown to mediate the adhesion of epithelial cells to collagen type IV [51]. In the present study we detected laminin deposits under the growing neurites during their elongation. We suggest that laminin mediates the cell-to-substratum adhesion in the differentiation of neuroblastoma cells.

ACKNOWLEDGMENTS

We kindly thank Dr. Rupert Timpl for the generous gift of antilaminin antibodies and Ms. Anja Virtanen and Ms. Raili Taavela for expert technical assistance. This work was supported by the Finnish Cancer Foundation, the Medical Research

Council of the Academy of Finland, the Orion Corporation Research Foundation, and the National Cancer Institute, DHEW (Grant no. 24605).

REFERENCES

1. Schubert D, Humphreys S, Baroni C, Cohn M: Proc Natl Acad Sci USA 64:316, 1969.
2. Prasad KN: In Evans AE (ed): "Advances in Neuroblastoma Research." New York: Raven Press, 1980, p 135.
3. Prasad KN, Sinha PK: In Saunders GF (ed): "Cell Differentiation and Neoplasia." New York: Raven Press, 1978, p 111.
4. Letourneau PC: Exp Cell Res 124:127, 1979.
5. Schubert D, Whitlock C: Proc Natl Acad Sci USA 74:4055, 1977.
6. Monard D, Solomon F, Rentsch M, Gysen R: Proc Natl Acad Sci USA 70:1894, 1973.
7. Collins F: Proc Natl Acad Sci USA 75: 5210, 1978.
8. Schubert D: Brain Res 155:196, 1978.
9. Akeson R, Hsu W-C: Exp Cell Res 115:367, 1978.
10. Littauer UZ, Giovanni MY, Glick MC: J Biol Chem 255:5448, 1980.
11. Culp LA, Ansbacher R, Domen C: Biochemistry 19:5899, 1980.
12. Barnes D, Sato G: Cell 22:649, 1981.
13. Bottenstein JE, Sato GH: Exp Cell Res 129:361, 1980.
14. Bunge MB, Williams AK, Wood PM, Uitto J, Jeffrey JJ: J Cell Biol 84:184, 1980.
15. Hawrot E: Devel Biol 74:136, 1980.
16. Alitalo K, Kuismanen E, Myllylä R, Kiistala U, Asko-Seljavaara S, Vaheri A: (submitted for publication).
17. Dexter TM, Allen TD, Lajtha LG, Krizsa F, Testa NG, Moore MAS: Cold Spring Harbor Conf Cell Prolif 5:63, 1978.
18. Fliedner TM, Calvo W: Cold Spring Harbor Conf Cell Prolif 5:757, 1978.
19. Timpl R, Rohde H, Robey PG, Rennard SI, Foidart J-M, Martin GR: J Biol Chem 254:9933, 1979.
20. Vuento M, Vaheri A: Biochem J 183:331, 1979.
21. Timpl R: In "Methods in Enzymology." New York: Academic Press (in press).
22. Alitalo K: Biochem Biophys Res Commun 93:873, 1980.
23. Alitalo K, Vaheri A, Krieg T, Timpl R: Eur J Biochem 109:247, 1980.
24. Sainte-Marie G: Histochem Cytochem 10:250, 1962.
25. Kurkinen M, Alitalo K, Vaheri A, Stenman S, Saxén L: Develop Biol 69:589, 1979.
26. Hedman K, Kurkinen M, Alitalo K, Vaheri A, Johansson S, Höök M: J Cell Biol 81:83, 1979.
27. Laemmli UK: Nature (London) 227:680, 1970.
28. Bonner WM, Laskey RA: Eur J Biochem 46:83, 1974.
29. Alitalo K, Kurkinen M, Vaheri A, Virtanen I, Rohde H, Timpl R: Nature (London) 287:465, 1980.
30. Brown JC: Exp Cell Res 6:440, 1971.
31. McGuire JC, Greene LA: Cell 15:357, 1978.
32. Timpl R, Martin GR, Bruckner P, Wick G, Wiedemann H: Eur J Biochem 84:43, 1978.
33. Foidart JM, Bere EW, Yaar M, Rennard SI, Guillino M, Martin GR, Katz S: Lab Invest 42:336, 1980.
34. Middleton CA: Nature (London) 259:311, 1976.
35. Vaheri A, Alitalo K: In Rees D, Lloyd C (eds): "Cellular Controls in Differentiation." New York: Academic Press (in press).
36. von der Mark K, von der Mark H, Gay S: Develop Biol 53:153, 1976.
37. Krieg T, Aumailley M, Dessau W, Wiestner M, Müller P: Exp Cell Res 125:23, 1980.
38. Cohen AM, Hay ED: Develop Biol 26:578, 1971.
39. Manasek FJ, Cohen AM: Proc Natl Acad Sci USA 74:1057, 1977.
40. Augusti-Tocco G, Chiarugi VP: Cell Diff 5:161, 1976.
41. Shellswell GB, Restall DJ, Duance VC, Bailey AJ: FEBS Lett 106:305, 1979.
42. Kelly PT, Cotman CW: J Biol Chem 252:786, 1977.
43. Sanes JR, Hall ZW: J Cell Biol 83:357, 1979.
44. McMahan UJ, Sanes JR, Marshall LM: Nature (London) 271:172, 1978.

45. Moya F, Bunge MB, Bunge RP: Proc Natl Acad Sci USA 77:6902, 1980.
46. Kurkinen M, Alitalo K: FEBS Lett 102:64, 1979.
47. Church RL, Tanzer ML, Pfeifer SE: Proc Natl Acad Sci USA 70:1943, 1973.
48. Hayman E, Engvall E, Ruoslahti E: J Cell Biol 88:352, 1981.
49. Nielson SE, Puck TT: Proc Natl Acad Sci USA 77:985, 1980.
50. Prasad KN: In Fedoroff S, Hertz L (eds): "Cell, Tissue and Organ Cultures in Neurobiology." New York: Academic Press, 1977, p 447.
51. Terranova VP, Rohrbach DH, Martin GR: Cell 22:719, 1980.
52. Collins F, Garrett JE: Proc Natl Acad Sci USA 77:6226, 1980.
53. Krystosek A, Seeds NW: Science 213:1532, 1981.

NOTE ADDED IN PROOF

Since the submission of this manuscript the authors have become aware of two works that partially explain the mechanism of guiding and elongation of growth cones. One by Collins and Garrett [52] clearly documents the role of the neurotropic substratum associated material released from heart cells [7]. The other [53] implicates the release of plasminogen activator activity at the tips of elongating neurites as a way of releasing substrate-attachment in migration.