A sequence of changes in cytoskeletal components during neuroblastoma differentiation

Mari-Madeleine Portier, Bernard Croizat and François Gros

Laboratoire de Biochimie Cellulaire, Collège de France, 11, Place Marcelin Berthelot, 75231 Paris Cedex, France

Received 27 May 1982; revision received 30 July 1982

Cytoskeleton *Neuroblastoma* *Differentiation* *Isotubulin* *Actin* *Vimentin*

1. INTRODUCTION

One of the major goals of developmental neurobiology is to understand the mechanisms controlling the acquisition of the morphological and biochemical properties of a competent neuronal cell. The properties include modulation of the cytoskeletal and membrane structures. The growth of axons depends on the assembly of microtubules and their elongation on the actin microfilaments [1]. Moreover, the shape and motility of cells [2] depend on the redistribution of cytoskeletal and contractile structures.

Brain maturation involves the integration of mechanisms that are much more complex than those involved in the modulation of the cell shape and motility. For instance, immature neurons migrate, then settle in their ultimate site and finally establish an extraordinary network of interconnections. These successive steps cannot be explained without the concept of 'cell recognition' which consists, simply speaking, in the integration and transmission of external signals through the membrane and cytoskeletal structures. Thus, several studies have shown that brain maturation is accompanied by modulations in the rate of synthesis of isotubulins, actins neurofilaments and cytoskeleton associated proteins [3–10].

Neuroblastoma clones are routinely considered as a convenient model for studying the genetic expression during terminal differentiation of neuron [11–14]. Despite some abnormalities, due to their neoplastic character, they have proved very useful in the study of formation of neurites, the appearance of enzymes that synthesize neurotransmitters and the acquisition of membrane excitability [15–17]. However, if studies on brain maturation have shown large modulations in the various cytoskeletal components, comparable studies concerning the neuroblastoma are generally limited to the isotubulins and report moderate changes in their microheterogeneity concomitant with cell differentiation. Considering neuroblastoma as a model of neuronal differentiation, we thought it would be interesting to further examine the changes in the cytoskeletal proteins of several neuroblastoma clones treated with 1-methyl-cyclohexane carboxylic acid (CCA), a new inducer of neuroblastoma differentiation [18], or under other inducing conditions. Comparisons of the effects observed in different clones cultured in various conditions allowed us to suggest a sequence of events occurring during the morphological differentiation of neuroblastoma.

Abbreviations: CCA, 1-methyl-cyclohexane carboxylic acid; DMSO, dimethyl sulfoxide; CM CLA SU, suspension cells in a complete medium containing CCA; WS SU, suspension cells in a medium without serum; CM CCA ML, monolayer cells in a complete medium containing CCA

2. MATERIALS AND METHODS

2.1. *Cell culture*

We used the N1E115, N18 and N1A103 clones from mouse neuroblastoma C1300. The conditions

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

of cultures have been described in [19]. The cells were grown in suspension in the medium or attached to a culture dish. In this second situation, clones N1E115 and N18 extend neurites when the serum is withdrawn from the medium or when CCA or dimethyl sulfoxide (DMSO) is added to the serum containing medium. Suspension cells were grown in the serum containing medium or they were blocked in the stationary phase when kept in a medium without serum or in a medium containing CCA.

2.2. *Drug treatment*

2.2.1. CCA-treated cultures

The CCA concentration was 0.1%. The cells were maintained for 3 days in these conditions prior to labeling, then kept 1 more day with labeling, in the presence of CCA. Cultures were changed with fresh medium every 24 h.

2.2.2. DMSO-treated cultures

Cultures were kept for 7 days in 2% DMSO-containing medium prior to labeling in the same medium. The medium was changed every 24 h.

2.3. *Protein labeling and cell extracts preparation*

The cells were labeled for 24 h with [^{35}S]methionine (25 μCi/ml medium) prior to harvesting. The 15 cm diam. dishes were inoculated so that they contained $\sim 5 \times 10^6$ cells at the time of harvesting. Cells were collected in the following buffer: 2-(*N*-morpholino)ethanol sulfonic acid (MES) 0.1 M (pH 6.4); EGTA 1 mM; EDTA 0.1 mM; β-mercaptoethanol 1 mM; $MgCl_2$ 0.5 mM; GTP 1 mM, then lysed by addition of 0.1–0.2% Nonidet P40; the lysate was recovered while the pellet was suspended in a small volume of the same buffer. Acid-precipitable radioactivity was measured in each case by hot trichloroacetic acid precipitation. Both supernatant and pellet were immediately treated for isoelectric focusing according to [20] and stored at −30°C.

2.4. *Polyacrylamide gel analysis of cell extracts*

The experimental protocol was described in [21] and the technical conditions were used as in [22] to obtain a good separation of proteins allowing the measurement of the radioactivity contained in the spots.

3. RESULTS

The proteins mentioned in this study are visualized on a two-dimensional electrophoretogram (fig.1). Data on methionine incorporation into these proteins are plotted in fig.2.

3.1. *Modulations in isotubulins and actin*

There is a reduction in the incorporation of methionine into α- and β-isotubulins and into actin in every condition where the cells have stopped dividing.

These modulations were equally promoted by CCA, DMSO and serum deprivation. These reductions can occur in the absence of cell adhesion as shown by cells cultured in suspension, either in the presence of CCA (CM CCA SU) or in the absence of serum (WS SU). These reductions are also seen in the absence of cell differentiation when the cells adhere to the substratum, as shown in N1A103 cells (CM CCA ML) which stop dividing in the presence of CCA but are genetically unable to extend neurites.

The decrease in the methionine incorporation into isotubulins is generally more pronounced in the β-isoform than in the two α-isoforms. α_1 decreases significantly more than α_2.

Fig.1. Electrophoretogram showing the proteins discussed. The proteins are shown on a two-dimensional gel loaded with a pellet from N1E115 neuroblastoma extract. Cells were labeled for 24 h with L-[^{35}S]methionine and lysed with Nonidet P40. The crude extract thus obtained was separated into pellet and supernatant, which were treated for isoelectric focusing according to [20]. Except for isotubulins, actin and neurofilament, equally distributed in both pellet and supernatant, the proteins studied here are insoluble and thus exclusively recovered in the pellet. About 10^6 cpm corresponding to ^{35}S-labeled proteins are loaded on each gel. The cpm corresponding to each spot are counted and the percentages of each protein are calculated *vs* total cpm loaded on the first-dimensional gel. The data are expressed relative to the values found in a control culture (cells grown in a complete medium and maintained in monolayer conditions). Graphic representation of the data is shown in fig. 2. α_1, α_1-isotubulin; α_2, α_2-isotubulin; β, β-isotubulin; Vi, vimentin; act., actin; 130K, vinculin; nF, 70 000 M_r neurofilament.

Fig. 1

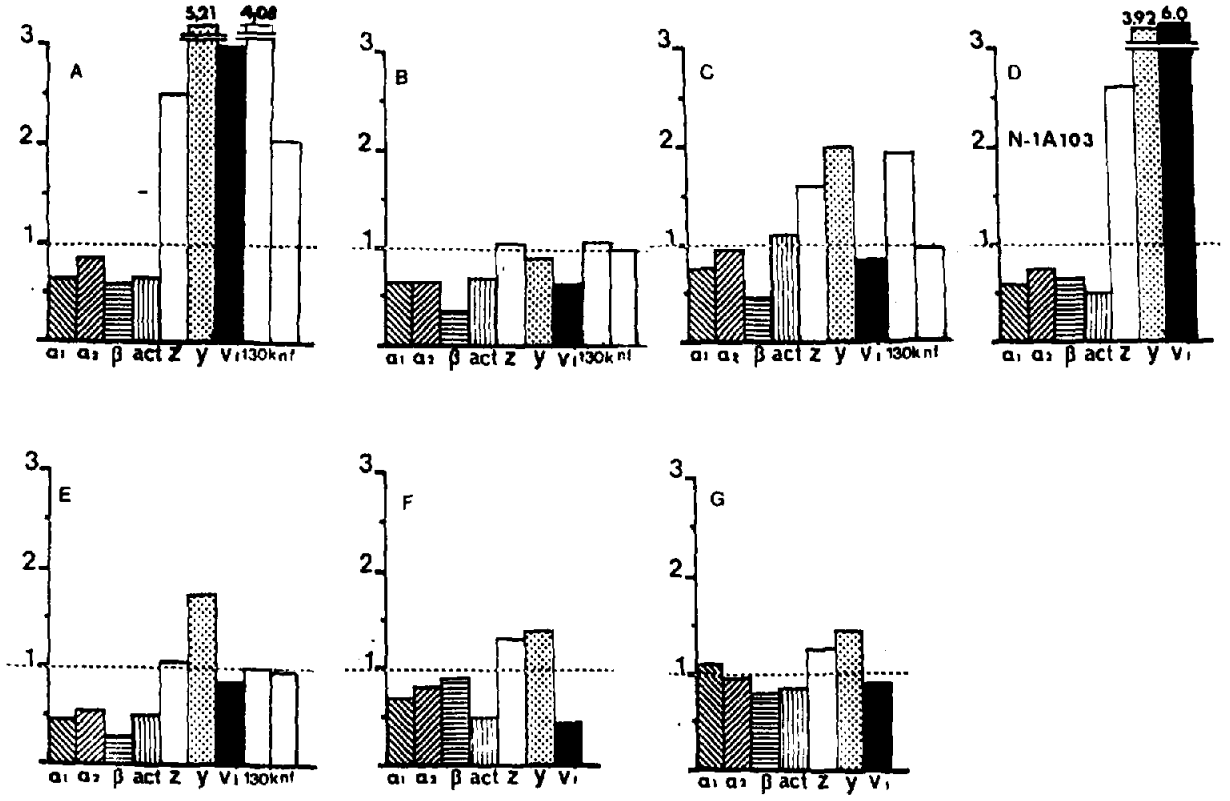

Fig.2. Graphic representation of the methionine incorporation into some proteins in various culture conditions. The effects of various culture conditions are shown on the methionine incorporation into isotubulins, actin and some other proteins. The data are expressed relative to the values found in control cultures which can then be considered = 1 (---). Control cultures are cells grown in complete medium and maintained in monolayer conditions. The experiments were made with clone N1E115 except when mentioned N1A103: (A) monolayer culture in a complete medium containing CCA; (B) monolayer culture in a complete medium containing DMSO; (C) monolayer culture in a medium without serum; (D) monolayer culture of N1A103 cells in a complete medium containing CCA (cells are blocked in a stationary phase without expressing any morphological differentiation); (E) suspension cells in a complete medium containing CCA; (F) suspension cells in a medium without serum; (G) suspension cells in a complete medium; α_1, α_1-isotubulin; α_2, α_2-isotubulin; β, β-isotubulin; act, actinin; Vi, vimentin; 130 k, vinculin; nf, 70 000 M_r neurofilament.

3.2. *Modulations in other cytoskeletal components*

CCA promotes a specific stimulation of methionine incorporation into vimentin, the insoluble proteins Y and Z, and presumably into vinculin and the 70 000 M_r neurofilament. These effects require the capacity of the cells to adhere to the surface of the dish but not necessarily to differentiate (as shown in the N1A103 monolayer cultures (CM CCA ML)).

We have identified the intermediate filament vimentin by peptide analysis according to [24]. The insoluble proteins Y and Z, exclusively recovered in the pellet from cell extracts, are not yet identified. Their M_r-values are 57 000 and 50 000, respectively. Antibodies to Y and Z are in preparation. We have found Y in neuroblastoma and in primary cultures of neurons from neonatal rat cervical ganglion but not in non-nervous cells. The most significant stimulation of methionine incorporation into Y occurs in the presence of CCA, in particular when the cells adhere to the substratum (5.2 × stimulation). Identification of the two other

proteins showing an increased methionine incorporation is in progress. Nevertheless, their migration properties on a two-dimensional gel, as well as their comigration with the corresponding purified protein, make it very probable that they are vinculin (or 130 000 M_r) and the 70 000 M_r triplet of neurofilaments. Vinculin is an anchorage protein linking the actin microfilaments to the inner surface of the membrane.

3.3. *Neurite extension*

Using clone N1A103, a mutant which is unable to extend neurites, we have shown that the changes in the incorporation of methionine into isotubulins, actin and the other proteins can occur in the absence of morphological differentiation, as shown in N1A103 monolayer cultures (CM CCA ML) (fig.2).

The results presented here for clones N1E115 and N1A103 and confirmed using clone N18 (not shown) can be summarized as follows:

(i) Modulations in isotubulins and actin depend on cell division;
(ii) Modulations in other cytoskeletal components depend on cell adhesion;
(iii) Neurite extension is the final step occurring (or not) after the previous molecular events.

Thus we can postulate the following sequence of events:

			N1A103* :	
Cell division →	Post mitosis modulation of tubulins and actins →	Adhesion modulation of Z, Y, vimentin and presumably 130 k, 70 k NF	:	→ Morphogenesis

4. DISCUSSION

We have shown that CCA allows an immature round neuroblast to develop into a larger cell that spreads on the substratum and extends long processes [18]. The surface of interaction between cell membrane and the substratum was significantly more increased with CCA than with any other inducing conditions. Accompanying these morphological changes, modulations in some cytoskeletal and membrane components were observed [20].

Here, using CCA, other inducing conditions and various clones, we describe the successive steps in the modulations of the cytoskeletal components and 2 insoluble proteins (Y,Z) which are presumably associated with the membrane. These modulations are closely related to the changes in cell morphology which occurs during the course of neuroblastoma differentiation.

(1) We have shown that the decrease of methionine incorporation into actin and isotubulins, more precisely the relative decreases in the different α and β isoforms, strictly depends on an arrest of cell division. These changes correspond to those which are generally presented as 'variations in the tubulin microheterogeneity during neuroblastoma differentiation'. However, our results show that they can occur in the absence of cell adhesion and/or differentiation. The question whether neurite formation involves the de novo synthesis of isotubulins or the assembly of pre-existing subunits has long remained open. Recent studies favour the idea that the assembly of neuroblastoma microtubules that are necessary for neurite outgrowth would proceed from a pre-existing pool of tubulins. These experiments, although not designed to answer this question, do not support the hypothesis of de novo synthesis.

(2) CCA promotes a much greater stimulation of methionine incorporation into Y, Z and 130 000 M_r than do any other inducing conditions; CCA is the only agent which stimulated incorporation into vimentin and 70 000 M_r. These modulations are strictly dependent on an interaction between cell membrane and substrate. This dependence leads us to postulate that CCA causes come important modifications in the neural surface. This is supported by our morphological observations showing better adhesion to the substratum of cells differentiated by CCA than of cells differentiated by DMSO or serum deprivation [18]. Concomitant with these membrane modulations which probably include variations in the synthesis of other membrane bound proteins, neurite extension can proceed more efficiently: the neurites are more stable than those formed after induction by other sub-

* Clone N1A103 cannot extend neurites: The sequence of events is thus blocked before morphogenesis

stances. It must be noted that the general effect of CCA on surface structures seems specific for neural differentiation since we did not observe any similar effect in non-neural cells such as glial cells and myoblasts deriving from the mouse teratocarcinoma PCC3. The sequence of events presented here, which occur during neuroblastoma differentiation and in close relationship with membrane/substratum interactions, seems particularly interesting considering that development and activity of a neural cell implies an extraordinary capacity of integrating signals through membrane, synaptic and cytoskeletal structures.

ACKNOWLEDGEMENTS

This work has been supported by a grant from SANOFI. We thank Denise Baron for typing the manuscript.

REFERENCES

[1] Yamada, K.M., Spooner, B.S. and Wessels, N.K. (1970) Proc. Natl. Acad. Sci. USA 66, 1206–1212.
[2] Porter, K.R. (1976) Cold Spring Harbor Conf. Cell Prolif. 3, 1–28.
[3] Gozes, I. and Littauer, U.Z. (1978) Nature 276, 411–413.
[4] Gozes, I. and Littauer, U.Z. (1979) FEBS Lett. 99, 86–90.
[5] Dahl, J.L. and Weibel, V.J. (1979) Biochem. Biophys. Res. Commun. 3, 822–828.
[6] Denoulet, P., Eddé, B., Jeantet, C. and Gros, F. (1982) Biochimie 64, 165–172.
[7] Shelanski, M.L. and Liem, R.K.H. (1979) J. Neurochem. 33, 5–13.
[8] Dahl, D. and Bignami, A. (1982) 13th Annu. Meet. Am. Soc. Neurochem. abst.
[9] Peters, A. and Vaughn, J.E. (1967) J. Cell Biol. 32, 113–119.
[10] Liem, R.K.H., Pachter, J.S. and Hutchinson, S.B. (1982) 13th Annu. Meet. Am. Soc. Neurochem. abst.
[11] Tocco, A. and Sato, G. (1969) Proc. Natl. Acad. Sci. USA 64, 311–315.
[12] Klebe, R.J. and Ruddle, F.M. (1969) J. Cell Biol. 43, 69a.
[13] Nelson, P., Ruffner, W. and Nirenberg, M. (1969) Proc. Natl. Acad. Sci. USA 64, 1004–1010.
[14] Schubert, D., Humphreys, S., De Vitry, F. and Jacob, F. (1971) Dev. Biol. 25, 514–546.
[15] Schubert, D., Humphreys, S., Baroni, C. and Cohn, M. (1969) Proc. Natl. Acad. Sci. USA 64, 316–323.
[16] Blume, A., Gilbert, F., Wilson, S., Faber, J., Rosenberg, R. and Nirenberg, M. (1970) Proc. Natl. Acad. Sci. USA 67, 786–792.
[17] Minna, J., Glazer, D. and Nirenberg, M. (1972) Nature New Biol. 235, 225–231.
[18] Coirzat, B., Berthelot, F., Ferrandes, B., Eymard, P., Sahuquillo, C. and Gros, F. (1979) Compt. Rend. Acad. Sc. Paris 289 D, 1283–1286.
[19] Croizat, B., Berthelot, F., Felsani, A. and Gros, F. (1977) Eur. J. Biochem. 74, 405–412.
[20] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[21] Portier, M.M., Eddé, B., Berthelot, F., Croizat, B. and Gros, F. (1980) Biochem. Biophys. Res. Commun. 96, 1610–1618.
[22] Portier, M.M., Jeantet, C. and Gros, F. (1980) Eur. J. Biochem. 112, 601–609.
[23] Eddé, B., Portier, M.M., Sahuquillo, C., Jeantet, C. and Gros, F. (1982) Biochimie 61, 141–151.
[24] Cleveland, D.W., Fischer, S.G. Kirschner, M.W. and Laëmmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.