Is the induction of neuroblastoma differentiation by CCA mediated by its effects on the electrochemical gradient?

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Various mitochondrial inhibitors are tested in neuroblastoma cells. Their effects on the mit-proteins and some cytoskeletal proteins are compared to those of CCA, a differentiation inducer. This comparison favours the hypothesis that the primary effect of CCA induction is an alteration of the electrochemical gradient.

Cytoskeleton *Neuroblastoma* *Differentiation* *Mitochondria* *Electrochemical gradient* *Deoxyglucose*

1. INTRODUCTION

Neuroblastoma clones are routinely considered as a convenient model for studying genetic expression during terminal differentiation of neurons [1–4]. In previous studies we tested, in murine neuroblastoma cells, the inducing effects on neurogenesis of various pharmacologically active substances known for their antianoxic effect on rat brain. One of them, the compound CCA, is a potent inducer, causing ultimate modifications of the membrane-bound and cytoskeletal proteins [5–7]. Prior to any visible change in cell growth and morphology of the developing neuroblastoma, CCA promotes a significant increase in cellular accumulation of the complex between phosphorylated deoxy[^{14}C]glucose and hexose phosphate isomerase [8]. In [9], this accumulation is said to reflect glucose utilization.

Consequently, our attention was drawn to a possible general relationship between mitochondrial function and neurogenesis, and that led us to examine in particular the fate of mitochondrial (mit)-proteins during the course of neuroblastoma differentiation. We selected 3 major mit-proteins (4, 5 and C_1), which could act as major sites for a developmentally controlled modification since methionine incorporation into these markers increases during the course of CCA-induced neurogenesis [10,11]. This result supports the view that the inducing effect of CCA can be correlated to mitochondrial metabolism. In an attempt to elucidate the molecular mechanisms underlying this correlation, we examined the effects of various mitochondrial inhibitors on neuroblastoma cells. Here, we compare the effects of these inhibitors to those of CCA on the mit-proteins and some cytoskeletal and insoluble proteins which exhibit changes during CCA treatment.

2. MATERIALS AND METHODS

2.1. *Cell culture*

We used the N1E115 clone from mouse neuroblastoma C1300. The conditions of cultures have been described [12]. The cells were grown attached to a culture dish. In this situation, clone N1E115 extends neurites when CCA is added to the serum-containing medium.

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Abbreviations: CCA, 1-methylcyclohexane carboxylic acid; DNP, dinitrophenol; mit-protein, mitochondrial protein

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2.2. *Drug treatment*

2.2.1. CCA-treated cultures

The cells were maintained for 4 days in the presence of 0.1% CCA, then kept for the last 6 h with labeling in the presence of CCA. Cultures were changed with fresh medium every 24 h.

2.2.2. Antimitochondrial drug treatment

The cells were maintained for 4 days with various antimitochondrial agents (5 μM nonactin, 1 mM dinitrophenol (DNP), 0.6 μM oligomycin, or 1 mM NaN_3) added to the serum-containing medium.

2.3. *Protein labeling and cell extract preparation*

Control cells growing in a serum-containing medium, CCA-differentiated cells and cells treated with an antimitochondrial drug were labeled for 6 h with [^{35}S]methionine (50 μCi/ml medium) prior to harvesting. The 10 cm diameter dishes were inoculated so that they contained 2.5×10^6 cells at the time of harvesting. Cell extracts were prepared and analysed on two-dimensional electrophoresis as in [5].

2.4. *Preparation of mit-proteins*

Purification of mit-proteins was achieved as in [13]. Cells prelabeled with [^{35}S]methionine were homogenized in 0.25 M sucrose/0.3 mM EDTA (pH 7.4) and spun at $600 \times g$ for 10 min. The supernatant was centrifuged at $100000 \times g$ for 10 min, and the resulting pellet was washed twice. The last pellet was resuspended in 50% (w/w) sucrose/0.3 mM EDTA and layered under a step gradient of 44.5 and 41% sucrose. This was centrifuged for 2 h at 45000 rpm in a Beckman type 50 rotor. The material banding between 41 and 44.5% sucrose was diluted into 0.25 M sucrose/0.3 M EDTA, centrifuged at $10000 \times g$ for 10 min and the resulting pellet was dissolved for protein analysis in 8 M urea/20% Nonidet P-40/2% ampholines (LKB, 3.5–10/1% mercaptoethanol.

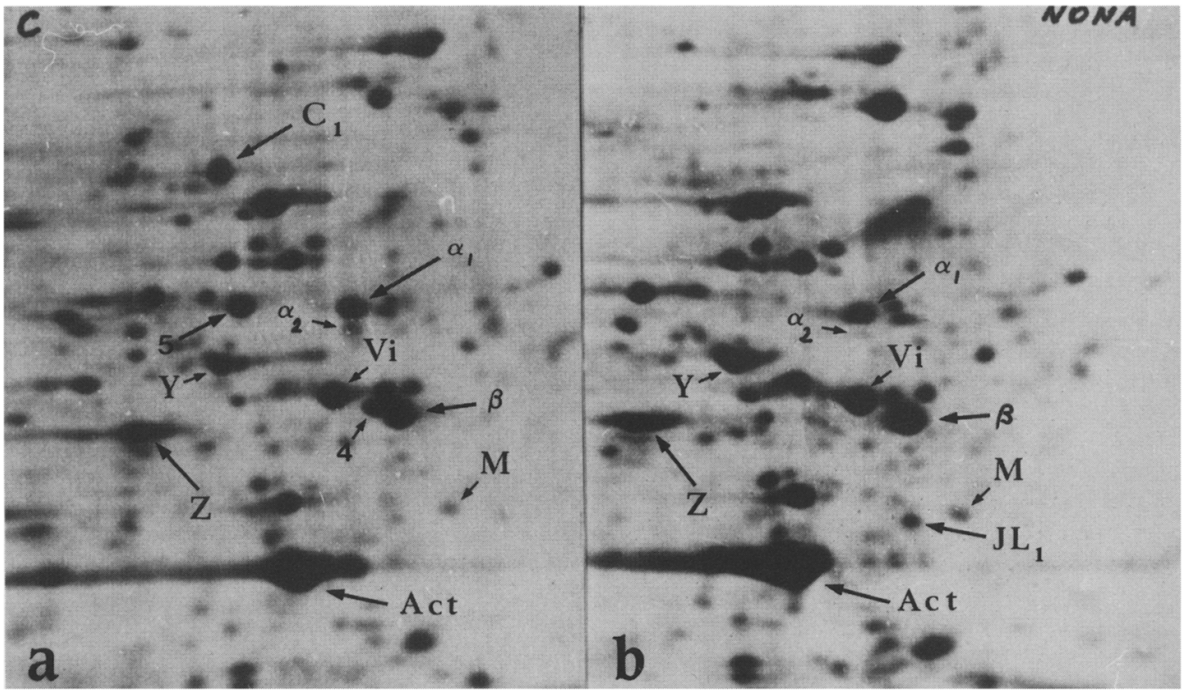

Fig.1. Areas from two-dimensional gels loaded with N1E-115 neuroblastoma extracts. Cells were labeled for 6 h prior to harvesting; 7×10^5 cpm corresponding to ^{35}S-labeled proteins were loaded on each gel. (a) Control culture growing in a complete medium. (b) Nonactin-treated cultures. Protein JL_1 is detectable in the latter culture conditions. act, actin; α_1, α_1-isotubulin; α_2, α_2-isotubulin; β, β-isotubulin; Vi, vimentin; 4, mit-protein 4; 5, mit-protein 5; C_1, mit-protein C_1; M, protein M; Y, protein Y; Z, protein Z.

3. RESULTS

Various antimitochondrial agents have been tested: dinitrophenol (the classical uncoupler), oligomycin (a mitochondrial ATPase inhibitor), nonactin (a K^+ ionophore), and NaN_3 (a cytochrome inhibitor). NaN_3 directly blocks the electronic flow while the other 3 specific inhibitors alter coupling between the oxidations and ATP synthesis.

The proteins studied are shown in fig.1. The first group is composed of the mit-proteins 4, 5 and C_1 (described in [10,11]) and of the insoluble proteins M and Z which copurify with the mitochondria. The synthesis of these proteins is enhanced when neuroblastoma differentiation is induced by CCA. A second neuronal group of proteins consists of: (i) the cytoskeletal components isotubulins, actin and vimentin; (ii) the insoluble protein Y characterized in our laboratory as specific of neuronal cells from the peripheral nervous system [14]; (iii) the Nonidet P40, insoluble protein JL_1. The latter is detectable after the addition of either CCA or antimitochondrial drugs to the cultures. Changes in the rates of synthesis of these cytoskeletal and associated proteins occur during neuroblastoma development. The sequence of these events has been described [7]. In addition to their effects on protein synthesis, antimitochondrial agents like CCA block cell division.

The effects of CCA and the antimitochondrial agents are shown in table 1:

(i) Group I: Methionine incorporation is enhanced in all of these proteins after CCA treatment. Oligomycin has a similar effect. In contrast, the synthesis of mit-proteins 4, 5 and C_1 is completely inhibited by nonactin and DNP, and strongly reduced by NaN_3. Differential effects have been observed on the insoluble proteins M and Z copurifying with mit-proteins. The synthesis of Z is not altered by antimitochondrial agents, but synthesis of M is enhanced in the presence of nonactin or DNP.

(ii) Group II: The insoluble proteins Y and JL_1 exhibit the same pattern of variation. Their synthesis is strongly enhanced by either CCA or the inhibitors which uncouple oxidations and ATP synthesis. In contrast, synthesis is reduced or suppressed by NaN_3. Vimentin synthesis is stimulated

Table 1

Comparative effects of CCA and of antimitochondrial agents on methionine incorporation into proteins from group I and II

	CCA (0.1%)	Oligomycin (0.6 μM)	Nonactin (5 μM)	Dinitrophenol (1 mM)	Sodium azide (1 mM)
Group I					
4	1.50	1.53	NS	NS	0.36
5	1.40	1.30	NS	NS	0.57
C_1	1.90	2.60	NS	NS	0.49
Z	2.00	1.20	1.00	1.05	0.90
M	2.20	2.50	2.00	1.60	0.84
Group II					
JL_1	S	S	S	S	NS
Y	4.00	3.20	2.60	2.60	0.75
Vi	3.75	1.75	0.70	0.43	0.85
Actin	0.58	1.25	0.77	0.70	0.88
α_1	0.57	1	0.94	0.63	0.63
α_2	1.20	1	0.69	0.67	0.67
β	0.88	1.20	0.72	0.60	0.53

The data are expressed relative to the values found in control cultures (cells growing in a complete medium) which can be considered as equal to unity. No quantitative data are shown for JL_1 since this protein is not detectable in control cultures. S, synthesized; NS, not synthesized

by CCA and oligomycin, and reduced by the other antimitochondrial drugs.

Actin and isotubulins are reduced by nonactin, DNP and NaN_3, but are not significantly modified by oligomycin. As shown [5], CCA also reduces the synthesis of these proteins except for α_2-isotubulin.

4. DISCUSSION

The electrochemical gradient generated by electron transport along the respiratory chain is used for ATP synthesis, transport of the substrates necessary for respiration, processing of mitochondrial proteins, and ion transport. The antimitochondrial drugs which block (oligomycin), discharge (nonactin, DNP) or suppress the electrochemical gradient (NaN_3) cause alterations to these functions.

Here, we have paid particular attention to the physiological and molecular effects of the drugs within the neuroblastoma cells. All of these agents block cell division. This is probably due to ATP depletion.

In contrast, changes in the synthetic rates of mit-proteins or cytoskeletal and associated proteins depend on the type of antimitochondrial agents. Therefore, these changes cannot be related just to ATP depletion (or arrest of cell division), and thus alteration of the electrochemical gradient does not affect uniquely mitochondrial functions. This extensive analysis allows us to conclude that the electrochemical gradient strictly controls the processing of mit-proteins 4, 5 and C_1 and may affect significantly most of the cytoskeletal components and associated proteins. Consequently, the regulations depend not only on the aerobic ATP production but also on the ionic repartitions across the mitochondrial membrane.

A comparison of the effects of the antimitochondrial agents and those produced by CCA reveals a marked similarity between the potent neural differentiation inducer and the mitochondrial ATPase inhibitor, oligomycin. This suggests that a common molecular mechanism operates at least at one level: CCA may affect the electrochemical gradient, like oligomycin. This hypothesis is supported by oxygraphic measurements (not shown). However, the similarity does not involve all of the proteins studied. Furthermore, oligomycin is not a neural inducer. We postulate that CCA causes an alteration in the electrochemical gradient and, thereby, promotes changes in the ionic environment of the mitochondrion. This change would, in turn, cause a rearrangement of the filamentous proteins leading to the differentiated state.

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REFERENCES

[1] Tocco, A. and Sato, G. (1969) Proc. Natl. Acad. Sci. USA 64, 311–315.
[2] Klebe, R.J. and Ruddle, F.M. (1969) J. Cell Biol. 43, 69a.
[3] Nelson, P., Ruffner, W. and Nirenberg, M. (1969) Proc. Natl. Acad. Sci. USA 64, 1004–1010.
[4] Schubert, D., Humphreys, S., De Vitry, F. and Jacob, F. (1971) Dev. Biol. 25, 514–546.
[5] Portier, M.M., Eddé, B., Berthelot, F., Croizat, B. and Gros, F. (1980) Biochem. Biophys. Res. Commun. 96, 1610–1618.
[6] Gros, F., Croizat, B., Portier, M.M., Berthelot, F. and Felsani, A. (1982) in: Molecular Genetic Neurosciences (Schmidt, F.O. et al. eds) pp.335–347, Raven, New York.
[7] Portier, M.M., Croizat, B. and Gros, F. (1982) FEBS Lett. 146, 283–288.
[8] Croizat, B., Berthelot, F., Portier, M.M., Ohayon, H. and Gros, F. (1981) Biochem. Biophys. Res. Commun. 103, 1044–1051.
[9] Sokoloff, L., Reivich, M., Kennedy, C., Des Rosiers, M.H., Patlak, C.S., Pettigrew, K.D., Sakurada, O. and Shinohara, M. (1977) J. Neurochem. 28, 897–916.
[10] Vayssière, J.L., Berthelot, F., Portier, M.M., Denoulet, Ph., Croizat, B. and Gros, F. (1984) Communication Intern. Symp. Contractile Proteins, Sassari (Italy), in press.
[11] Vayssière, J.L., Berthelot, F., Croizat, B. and Gros, F. (1984) Biochem. Biophys. Res. Commun., in press.
[12] Croizat, B., Berthelot, F., Felsani, A. and Gros, F. (1977) Eur. J. Biochem. 74, 405–412.
[13] Anderson, L. (1981) Proc. Natl. Acad. Sci. USA 78, 2407–2411.
[14] Portier, M.M., Croizat, B., De Néchaud, B., Gumpel, F. and Gros, F. (1983) CR Acad. Sci. Paris 297, 57–61.