ABERRANT ENERGY METABOLISM IN A VARIANT EPIDERMAL GROWTH FACTOR RECEPTOR-NEGATIVE FIBROBLASTIC CELL LINE

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Received 18 March 1981

1. Introduction

A variant 3T3 cell line has been isolated [1] lacking mitogenic response to epidermal growth factor [2,3]. This non-responder variant (NR-6) lacked epidermal growth factor (EGF) receptors, but retained the ability to respond to other mitogens, and displayed growth control in culture [1]. NR-6 cells have been used as specificity controls in identification of the EGF receptor [4–6] on 3T3 cells and for studies on insertion of exogenous hormone receptors into receptor-negative mutant cells [7]. We were interested in further characterizing this variant cell line, and examined whether the lack of EGF receptors is accompanied by other losses as well. Such studies may provide new insights into receptor genetics and the regulatory mechanisms involved in receptor biosynthesis. Here we report our preliminary findings on the energy metabolism characteristics of NR-6 cells. We find that compared with the parent 3T3 cells, the variant NR-6 cells are extremely deficient in cytochrome *c* oxidase, a key enzyme in the oxidative phosphorylation pathway, but this deficiency is compensated for by an exceptionally high rate of aerobic glycolysis. This aberrant energy metabolism does not appear to be associated with any transformed growth characteristics.

2. Materials and methods

2.1. *Materials*

Calf serum was from Gibco. [^{3}H]Thymidine (20 Ci/mol) and α-[^{3}H]aminoisobutyrate (2.5 Ci/mmol) were from New England Nuclear and ICN, respectively. Lactate dehydrogenase, β-NAD, ATP, fructose 6-phosphate, β-NADH, aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, and cytochrome *c* were obtained from Sigma Chemical Co.

2.2. *Cell culture*

Monolayer cultures of Swiss mouse 3T3 cells and mouse NR-6 cells were grown and maintained at 37°C in a 10% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 3% fetal calf serum and 7% newborn calf serum. Cells were subcultured by trypsinization. Routine tests showed the cells to be free of mycoplasma contamination.

2.3. *Assay of cytochrome* c *oxidase*

The enzyme in cell homogenate was assayed by measuring spectrophotometrically the decrease in absorbance of reduced cytochrome *c* at 550 nm at 25°C [8]. In a total vol. of 600 μl, the reaction mixture contained 36 mM reduced cytochrome *c*, 80 mM sodium phosphate buffer, pH 7.0, and 5–10 μl of cell homogenate. After the addition of cell homogenate, the fall in absorbance was recorded at 10-s intervals. The reaction rate was linear up to 3 min. 1 unit of enzyme activity represented oxidation of 1 nmol of reduced cytochrome *c*/min under these standard conditions. Extinction coefficient for reduced cytochrome *c* at 550 nm is $27.7 \times 10^6\ M^{-1} \cdot cm^{-1}$.

2.4. *Measurement of lactic acid production*

The reaction conditions of [9] were used. In a total vol. of 100 μl the reaction mixture contained the following: 2 mM $MgSO_4$, 2 mM ATP, 5 mM potassium phosphate (dibasic), 1 mM NAD, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 100 mM glucose and 10–90 μl of 24 000 $\times$ *g* supernatant (see table 1). After incubation at 37°C for 1 h, 0.9 ml of water was added to the tubes and the lactic acid produced was measured as in [10].

2.5. *Assay of phosphofructokinase in cell extracts*

Cells were suspended (7×10^6 cells/ml) in a hypotonic buffer (10 mM Tris-HCl, pH 7.4), allowed to stand at 0°C for 10 min, homogenized in a Dounce homogenizer (pestle B), and then centrifuged at 24 000 × *g* for 30 min. The supernate was adjusted to 0.1 mM EDTA/0.1 mM $MgSO_4$/0.1 mM dithiothreitol and assayed for phosphofructokinase activity be measuring the disappearance of β-NADH spectrophotometrically at 340 nm at 25°C [11], using the reaction conditions of [12]. The reaction mixtures (minus fructose 6-phosphate) were preincubated at 25°C for 10 min. The fall in absorbance was measured 1 min after the addition of fructose 6-phosphate. The reaction rates were generally linear upto at least 7 min after substrate addition.

2.6. *Protein determination*

Protein was determined according to [13] using bovine serum albumin as the standard.

3. Results and discussion

3.1. *Cytochrome c oxidase activity and aerobic glycolysis in NR-6 cells*

In these studies, cytochrome *c* oxidase activity and lactate production were measured in NR-6 cells and normal Swiss 3T3 cells grown to confluence (table 1). Cytochrome *c* oxidase activity, a measure of mitochondrial oxidative phosphorylation in the parent 3T3 cell line, closely agreed with the values reported in [14] in rat liver cells and BHK cells, hence the activity in 3T3 cells can be considered as normal. However, cytochrome *c* oxidase activity in variant NR-6 cells was only about 20% of that in 3T3. The low cytochrome *c* oxidase activity was found to be associated with an exceptionally high rate of aerobic glycolysis, as measured by lactic acid production. Lactate production in NR-6 cells was about 600% of that in 3T3 cells.

We were interested in examining whether the NR-6 cells also contained a high level of phosphofructokinase, a key enzyme in the regulation of glycolysis. Table 2 shows that the parent 3T3 cells contained a normal level of phosphofructokinase activity [12]; but the activity in NR-6 cells was 7–9-fold higher than that in the parent 3T3 cells. This difference in phosphofructokinase activity between NR-6 and 3T3 cells was observed over a wide range of assay substrate (fructose 6-phosphate) concentrations (table 2).

3.2. *DNA replicative quiescence in NR-6 cell monolayers*

Since enhanced rates of aerobic glycolysis have been associated with tumor cells [15] and growth-stimulated normal cells [12], we were interested in examining whether the NR-6 cells in monolayers were truly quiescent and density inhibited. Table 3 shows that the rate of [^{3}H]thymidine incorporation into DNA in NR-6 cell monolayers was low, and similar to that in the quiescent 3T3 cells. Moreover, nuclear autoradiographic experiments showed that a large majority (greater than 95%) of the cells in NR-6 cell monolayers

Table 1
Cytochrome *c* oxidase activity and aerobic glycolysis in 3T3 and NR-6 cells

Cell type	Cytochrome *c* oxidase activity (units/mg protein)[a]	Lactic acid formed (nmol/h per mg protein)[a]
Swiss 3T3	149 (100%)	192 (100%)
NR-6	32 (21%)	1200 (625%)

[a] The homogenate was centrifuged at 24 000 × *g* for 30 min, and the supernate was assayed for lactate production as in section 2

Confluent monolayers of 3T3 and NR-6 cells in 10-cm dishes were incubated at 37°C for 24 h with 10 ml of Dulbecco's modified Eagle's medium containing 1% fetal calf serum. At the end of incubation, the cells were washed 3 times with 0.15 M NaCl/10 mM Tris-HCl, pH 7.4, and scraped off the dish using a rubber policeman. Cells were suspended (7×10^6 cells/ml) in a hypotonic buffer (10 mM Tris-HCl, pH 7.4), allowed to stand at 0°C for 10 min, homogenized in a Dounce homogenizer (pestle B), and then tested for activity. Cytochrome *c* oxidase activity in cell homogenates was assayed as in section 2. Data in parentheses are % 3T3 activity

Table 2
Phosphofructokinase activity in NR-6 and 3T3 cells

Cell type	Frunctose 6-phosphate concn. in assay (mM)	Fructose 6-phosphate phosphorylated (nmol/min per mg extract protein)
3T3	0.05	4.5
	0.10	9.0
	0.15	14.0
	0.50	28.0
	1.0	30.0
	2.5	32.0
NR-6	0.05	33.0 (7.3)
	0.10	65.0 (7.2)
	0.15	104.0 (7.4)
	0.5	266.0 (9.5)
	1.0	283.0 (9.4)
	2.5	290.0 (9.1)

Confluent monolayers of 3T3 and NR-6 cells in 10-cm dishes were kept at 37°C for 24 h in 10 ml of Dulbecco's modified Eagle's medium containing 1% fetal calf serum. At the end of the incubation the cells were washed 3 times with 0.15 M NaCl/10 mM Tris-HCl, pH 7.4 and scraped off the dish using a rubber policeman. Cell extracts were prepared and were analyzed for phosphofructokinase activity as in section 2. The values within parenthesis represent NR-6 activity/3T3 activity ratio at each assay substrate concentration

were quiescent with respect to DNA replication (table 3). Thus the enhanced rate of aerobic glycolysis could not be correlated with a loss of growth control.

Studies on other characteristics of quiescence showed that membrane amino acid transport in NR-6 cells, as measured by α-[^{3}H]aminoisobutyrate uptake [16] was low (6.7 nmol/40 min per mg protein), and similar to that observed in density-inhibited 3T3 cells (6.9 nmol/40 min per mg protein). This provided an extra criterion for quiescence in the NR-6 cell monolayers.

4. Conclusions

The present work shows that the EGF receptor-negative variant NR-6 cell line is also a variant with respect to energy metabolism. These cells are abnormally deficient in cytochrome *c* oxidase activity. This deficiency is likely to lead to a decrease in the rate of oxidative phosphorylation and ATP production, which may cause an allosteric activation of phosphofructokinase [15], leading to a compensatory increase in aerobic glycolytic rate. This abnormal energy metabolism does not appear to be associated with any transformed growth characteristics in these cells.

Table 3
DNA replicatory quiescence in 3T3 and NR-6 cell monolayers

Cell type	DNA synthetic rate (cpm of [^{3}H]thymidine incorporated)[a]	% total nuclei labeled[b]
3T3	1800	1–2
NR-6	2900	3–4

[a] Monolayers of 3T3 cells or NR-6 cells in 16-mm dishes were incubated at 37°C for 48 h with 1 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum (DME-2% FCS). Then [^{3}H]thymidine (1 μCi/ml, 0.65 μM) was added to each dish and incubations were continued for an additional hour at 37°C. At the end of incubation, trichloroacetic acid-insoluble radioactivity was determined as described [5]

[b] Monolayers of 3T3 or NR-6 cells in glass coverslips were incubated at 37°C for 48 h with DME-2% FCS. Then, [^{3}H]thymidine was added to conditioned medium at a final concentration of 0.65 μM and 1 μCi/ml, and incubations were continued for an additional 20 h. At the end of incubation, the monolayers were washed twice with 0.15 M NaCl/10 mM Tris-HCl, pH 7.4, and fixed in methanol at 4°C for 5 min. The coverslips were air-dried, mounted on glass slides, and then treated with NTB2 emulsion (Kodak). After 24 h incubation in the dark, the slides were developed and fixed. Labeled nuclei were visualized as dark spots containing at least 5 dark grains. The unlabeled nuclei were stained with hematoxylin (Fisher). For each determination of percent labeled nuclei, at least 400 cells were counted

The results reported here may prove to be useful in studies on chromosomal localization of the murine EGF receptor gene, and characterization of the genetic lesion in murine NR-6 cells. In humans, binding of EGF has been shown to be associated with the presence of chromosome 7 [17]. Although the murine EGF receptor has been characterized and identified as a cell surface macromolecular polypeptide [4–6], little is known about its genetics and biosynthesis. Therefore, it may be of interest to examine the linkage of the murine receptor gene with other genes, especially those involved in the regulation of energy metabolism pathways.

Acknowledgements

We thank Mark Pittenger for technical assistance. This work was supported by grants AM-25819 and AM-25724 to M. D. from the National Institutes of Health. M. D. is the recipient of a Research Career Development Award from N. I. H. (AM-00693).

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