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Plasma membrane redox activity correlates with N-*myc* expression in neuroblastoma cells

Miguel Angel Medina, Antonio del Castillo-Olivares and Lothar Schweigerer

Sektion Onkologie/Immunologie, Universitäts Kinderklinik, Ruprecht-Karls-Universität, INF 150, W-6900 Heidelberg, Germany

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In different neuroblastoma cell lines and transfected clones, an increasing plasma membrane redox activity correlates with amplification and enhanced expression of the N-*myc* oncogene. Furthermore, plasma membrane redox activity is partially inhibited by retinoic acid in neuroblastoma cells with multiple copies of the N-*myc* oncogene but not in neuroblastoma cells with only one copy of this gene.

Neuroblastoma; Plasma membrane redox system; N-*myc* oncogene; Basic fibroblast growth factor; Retinoic acid

1. INTRODUCTION

Plasma membrane redox system (PMRS) activities have been found in every cell examined [1]. They can now be related to several vital functions, including the control of cell growth [2,3]. As a matter of fact, PMRS activities seem to be modified in transformed cells, as compared with those shown by normal untransformed cells [4,5].

Neuroblastoma is a common childhood solid tumor [6]. Amplification of the N-*myc* oncogene resulting in high N-*myc* expression is thought to be causally involved in the progression of neuroblastoma to advanced stages of malignancy [7].

Different neuroblastoma cell lines carrying different numbers of copy for the N-*myc* oncogene have been established. This fact makes neuroblastoma an optimal model system for the study of the changes in PMRS related to cell proliferation.

In the present study, we have measured PMRS activities of neuroblastoma cells containing different numbers of N-*myc* oncogene, ranging from one copy in cells, corresponding to very initial stages in the evolution of malignancy, to more than 100 copies in highly malignant cells. The effects of retinoic acid and basic fibroblast growth factor (b-FGF) are also shown.

2. MATERIALS AND METHODS

2.1. *Materials*

Culture dishes were from Falcon (Oxnard, CA, USA). RPMI-1640 medium, fetal calf serum, and antibiotics were from Gibco (Eggenstein, Germany). All other reagents were from Sigma (Deisenhofen, Germany).

Correspondence address: M.A. Medina, c/o Dr. L. Schweigerer, Im Neuenheimer Feld 326, 2.OG, W-6900 Heidelberg, Germany.

2.2. *Neuroblastoma cell lines*

Three well-established cell lines were used: SH-EP (a homogeneous SK-N-SH-derived subline), IMR-32, and Kelly, which carry 1, 25 and more than 100 copies of N-*myc*, respectively. In some experiments, two transfected clones derived from SH-EP cells were used: the 007 clone, transfected with a control plasmid; and the WAC2 clone, transfected with a plasmid carrying five exogenous copies of N-*myc*. Cell culture conditions and the establishment of the transfected clones have been described elsewhere [8].

2.3. *Discontinuous assay of PMRS activity*

Confluent cells were trypsinized, washed twice and suspended in 0.1 M Tris-HCl (pH 7.4). Quartz cuvettes with 0.1 mM ascorbate in a final volume of 2 ml of 0.1 M Tris-HCl buffer were incubated, in the absence or presence of 5×10^4 cells, at 37°C under 5% CO_2 atmosphere. Absorbance at 265 nm was read at 5 min intervals for 45 min. Under the conditions used in the experimental procedure, the first 10 min allowed for equilibration, and afterwards the changes in absorbance remained linear throughout the rest of the experimental time; for this reason, the rates were calculated using the measurements in the range 10–45 min. The extinction coefficient was 14 $mM^{-1} \cdot cm^{-1}$. The decrease in the autooxidation rate of ascorbate in the presence of cells is a measure of semi-hydroascorbate reduction by the cells [9].

2.4. *Short-term continuous assay of PMRS activity*

Ascorbate (0.1 mM) autooxidation in the absence or presence of 10^5 cells was continuously monitored for 5 min at 37°C as previously described [10].

3. RESULTS AND DISCUSSION

Table I shows the results obtained in the discontinuous assay. All the data significantly differ from each other and it can very clearly be observed that an increase in N-*myc* amplification (that is, an increase in malignancy) perfectly correlates with an increase in PMRS activity. This result was confirmed in short-term continuous assays (see Table II). However, since SH-EP, IMR-32, and Kelly cells have different origins, it would be possible to argue that the different PMRS activity levels exhibited by these cells could be explained

Table I

Relative rates of ascorbate oxidation in the discontinuous assay in the presence of different neuroblastoma cell lines

Cell line	Copies of N-*myc*	Ascorbate oxidation (%)
None		100 ± 2
SH-EP	1	84 ± 1[a]
IMR-32	25	66 ± 11[a,b]
Kelly	>100	28 ± 1[a,b,c]

Assays were carried out in the absence or presence of 5×10^4 cells as described in Materials and Methods. Data are given as percentages, taking ascorbate autooxidation rate in the absence of cells as 100%. Figures are means ± S.D. for three different experiments.
[a] Significant vs. control experiments without cells ($P < 0.01$).
[b] Significant vs. experiments with SH-EP cells ($P < 0.01$).
[c] Significant vs. experiments with IMR-32 cells ($P < 0.01$).

mainly by the differences in their origins more than by the degree of N-*myc* amplification. To rule out this possibility, PMRS activity was also measured in two transfected clones derived from the homogeneous SH-EP subline [8]. Table II shows that once again the PMRS activity level correlates with N-*myc* amplification. Since 007 and WAC2 clones have the same origin and they only differ in the expression of N-*myc* (that is, in their malignancy levels), it seems reasonable to conclude that PMRS activity in neuroblastoma cells increases with amplification and enhanced expression of N-*myc*. The physiological and/or pathological relevances of this correlation remains to be elucidated.

Retinoids appear to induce variable effects on the proliferation of normal cells but they inhibit the growth of cells treated with a tumoral promoter, or spontaneously transformed cells, and they prevent tumor promotion [11,12]. On the other hand, bFGF behaves as a paracrine and autocrine growth factor to different tumor cells and it is one of the most potent of angiogenic factors known [13–15]. The possible differential

Table II

Ascorbate oxidation rates in the continuous assays in the presence of different neuroblastoma cell lines

Cell line	Copies of N-*myc*	Ascorbate oxidation (%)
None		100 ± 9
SH-EP	1	34 ± 3[a]
IMR-32	25	22 ± 5[a,b]
007	1	41 ± 9[a]
WAC2	1+5	20 ± 3[a,c]

Assays were carried out in the absence or presence of 10^5 cells as described in Materials and Methods. Data are given as percentages, taking ascorbate autooxidation rate in the absence of cells as 100%. Figures are means ± S.D. for three different experiments.
[a] Significant vs. control experiments ($P < 0.01$).
[b] Significant vs. experiments with SH-EP cells ($P < 0.01$).
[c] Significant vs. experiments with 007 cells ($P < 0.01$).

Table III

Inhibition of PMRS activity in two neuroblastoma cell lines by retinoic acid and bFGF

	SH-EP cells	IMR-32 cells
Retinoic acid (10 μM)	−2 ± 5	50 ± 3[a]
bFGF (5 ng/ml)	28 ± 11	23 ± 20

Continuous assays in the absence of presence of the different compounds were carried out with or without 10^5 SH-EP or IMR-32 cells. Data are given as percentages of inhibition of the activities obtained in control experiments with cells and no compound added. The addition of the compounds did not change ascorbate autooxidation rate significantly. Figures are means ± S.D. for three different experiments.
[a] Significant vs. experiments with SH-EP cells ($P < 0.01$).

effects of retinoic acid and bFGF on PMRS activities were tested in two neuroblastoma cell lines (SH-EP, and IMR-32) by measuring the inhibition of the redox activity in the presence of these compounds at frequently used concentrations [12,13,16]. As Table III shows, 10 μM retinoic acid induced significant inhibitions of PMRS activity in IMR-32 cells (those containing 25 copies of N-*myc*) and had no effect on PMRS activity in SH-EP cells (those containing only one copy of N-*myc*). These results are in agreement with the proposed anti-tumor activity of this compound [11], and they support the idea that the observed correlation between tumor progression and PMRS activity should be physiologically and/or pathologically relevant. In contrast, bFGF produced similar levels of inhibition in both cell lines, irrespective of their malignancy levels.

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