# Suppression of nuclear ADP-ribosyltransferase activity in Ehrlich ascites tumor cells by 5-azacytidine

## Modification of DNA as a cause of suppression

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Received 18 January 1990

Exposure of Ehrlich ascites tumor cells to 5-azacytidine for 5 h resulted in a partial loss of ability of DNA to stimulate ADP-ribosyltransferase activity, as assessed in a reconstituted in vitro enzyme system consisting of purified calf thymus enzyme, calf thymus whole histone and DNA isolated from the cells. The degree of suppression in vitro varied depending on the amount of histone and DNA added and it reached a maximum with a value of 83% and 62% of control for DNAs from cells exposed to 10 μM and 30 μM 5-azacytidine, respectively, at a histone/DNA mass ratio of 0.4. In the absence of histone (conditions of auto-ADP-ribosylation of the enzyme), no suppression was detectable.

ADP-ribosylation reaction, in vitro; 5-azacytidine-modified DNA; Histone requirement; (Ehrlich ascites tumor cell)

## 1. INTRODUCTION

5-ACR, an antileukemic agent, is known as an effective trigger of cellular differentiation. It can be incorporated into DNA and thus DNA substituted for cytosine by 5-azacytosine strongly inhibits the activity of DNA-cytosine methyltransferase by forming a tightly bound DNA:transferase complex. The hypomethylation of genomic DNAs that occurs leads to gene activation as a possible cause of differentiation (reviews [1,2]).

We previously found a time- and dose-dependent suppression of ADPRT (EC 2.4.2.30) activity in the nuclei of 5-ACR-treated EAT cells [3] and regenerating rat liver after partial hepatectomy [4]. ADPRT is, like DNA methyltransferase, a chromatin-bound enzyme which catalyzes the polymerization of an ADP-ribose moiety of NAD to produce poly ADP-ribosylated chromatin proteins, including ADPRT itself (auto-ADP-ribosylation of enzyme) [5]. The enzyme absolutely requires DNA with strand breaks for its activity as a cofactor and a role in the central biological processes such as differentiation, DNA replication and DNA repair in particular has been suggested for this reaction (reviews [6,7]). Although the mechanisms through which ADPRT is activated by DNA is not exactly explained, the binding of enzyme to the cleaved DNA chain termini seems responsible for the enzyme function [8,9].

In the present study we reconstituted an in vitro enzyme system consisting of the purified ADPRT from calf thymus, calf thymus histones and DNA preparations from 5-ACR-treated and non-treated EAT cells and could show that a modification of DNA by 5-ACR is responsible for the nuclear suppression of ADPRT activity we previously observed.

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*Abbreviations:* ADPRT, ADP-ribosyltransferase; 5-ACR, 5-azacytidine; EAT, Ehrlich ascites tumor

## 2. MATERIALS AND METHODS

### 2.1. *Preparation of ADPRT*

ADPRT was purified 450-fold from calf thymus by the method described by Buki et al. [10] with slight modifications. The purified enzyme had an apparent molecular weight of 116000 as determined by SDS polyacrylamide gel electrophoresis with β-galactosidase as a marker protein. The enzyme preparation was free of DNA and its activity was entirely dependent on the exogenously added DNA.

### 2.2. *Incubation of EAT cells with 5-ACR for the preparation of DNA*

EAT cells were propagated in vivo in the peritoneal cavity of female NMRI mice as previously described [11]. The cells were incubated at 37°C for 5 h in a humidified atmosphere of 5% $CO_2$ + air in 50 ml RPMI 1640 medium (dutch modification, Gibco BRL), supplemented with 5% fetal calf serum (Gibco BRL), 100 IU/ml penicillin G, 100 μg streptomycin (Flow Lab.), 2 mM L-glutamine and 5-ACR (Sigma Chemie) at concentrations of 0, 10 or 30 μM at a cell density of 7–9 × $10^6$/ml.

### 2.3. *Isolation of cell nuclei and DNA*

Cell nuclei were prepared by the method of Konstantinovic and Sevaljevic [12]. Nuclei (1 × $10^8$) were lysed in 10 ml TE buffer (50 mM Tris-HCl, pH 7.5), centrifuged and the pellets were

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/90/$3.50 

resuspended in 10 ml TE buffer. Proteinase K (Sigma Chemie) and SDS were added to a final concentration of 0.25 mg/ml and 1% (w/v), respectively, and the mixtures were incubated at 37°C for 12 h. Further procedures of DNA isolation were carried out according to the standard protocol described by Maniatis et al. [13] and the DNA preparations thus obtained were stored in TE buffer.

### 2.4. *Assay for ADPRT reaction*

ADPRT was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 0.1 mM [adenine-U-$^{14}$C]NAD (8000 cpm/nmol) (Amersham Buchler), 1 mM dithiothreitol, 10 mM $MgCl_2$ (where indicated), 2.7 $\mu$g ADPRT protein and DNA from EAT cells with or without calf thymus whole histone (Sigma Chemie, Type IIa) in the amount indicated in a final volume of 0.1 ml. After the incubation at 25°C for 5 min, the reaction was terminated by the addition of 0.8 ml ice-cold 15% (w/v) trichloroacetic acid and 20 $\mu$l 5% bovine serum albumin as a carrier. The acid-insoluble material was collected on Whatman GF/C glass microfiber filter, washed with 5% trichloroacetic acid and 95% ethanol and the radiolabel was counted in a scintillation spectrometer.

### 2.5. *DNA and protein assay*

DNA was determined fluorometrically by the method of Labarca and Paigen [14] using Hoechst 33258 (Sigma Chemie). Protein content was estimated by the method of Lowry et al. [15] after precipitation of protein with trichloroacetic acid in the presence of deoxycholate.

## 3. RESULTS AND DISCUSSION

### 3.1. *Characterization of the purified ADPRT and DNA preparations isolated from EAT cells*

The complete in vitro assay mixture for the ADP-ribosylation reaction contained purified ADPRT, activated calf thymus DNA, histone and $Mg^{2+}$ as active constituents (table 1). In the absence of added DNA, the ADPRT exhibited no activity. $Mg^{2+}$ was rather inhibitory in the presence of all other constituents. Omission of histone alone had no effect, probably because of the known preferential auto-ADP-ribosylation of the enzyme. However, histone stimulated the reaction 2-fold in the absence of $Mg^{2+}$ [16]. The addition of 5-ACR at concentrations up to 1 mM to this in vitro enzyme system had no effect on the reaction rate, confirming the previous observation on the isolated EAT cell nuclei [3].

The nuclear ADPRT activity of EAT cells exposed for 5 h to 10 $\mu$M and 30 $\mu$M 5-ACR under the conditions described in section 2 was found to be 82% and 73% of control, respectively. DNAs isolated from these nuclear preparations are now referred to as 0-DNA, 10ACR-DNA and 30ACR-DNA for those from the cells cultured without 5-ACR, with 10 $\mu$M and 30 $\mu$M 5-ACR, respectively. The absorbance ratio 260/280 nm of these DNAs was found to be 1.98, 1.93 and 1.84, respectively, and thus they were highly pure. They were electrophoretically heterogenous but showed the same gel pattern and had apparent molecular masses higher than $15 \times 10^6$ Da, as estimated by agarose gel electrophoresis using *Hin*dIII-digested lambda DNA as a reference marker.

Table 1

ADP-ribosyltransferase reaction in vitro in the presence of activated calf thymus DNA

| Addition | Poly(ADP-ribose) synthesis | |
|---|---|---|
| | (nmol/min per mg enzyme protein) | (% of control) |
| ADPRT, DNA, histones, $Mg^{2+}$ | 109 ± 9 | 100 |
| ADPRT, histones, $Mg^{2+}$ | nd | 0 |
| ADPRT, DNA, $Mg^{2+}$ | 117 ± 11 | 107 |
| ADPRT, DNA, histones | 146 ± 8 | 134 |
| ADPRT, DNA | 72 ± 5 | 66 |

The complete reaction mixture contained 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 $\mu$g activated calf thymus DNA, 10 $\mu$g calf thymus whole histone, 1 mM dithiothreitol, 0.1 mM [adenine-U-$^{14}$C]NAD and 2.7 $\mu$g purified ADPRT protein in a total volume of 0.1 ml. nd, not detectable. Data are the means ± SD for 3 enzyme reactions

Table 2

Activation of purified ADP-ribosyltransferase by the limited DNase I digestion of 0-DNA, 10ACR-DNA and 30ACR-DNA

| DNA | Poly(ADP-ribose) synthesis (nmol/min per mg enzyme protein) | | Degree of activation (-fold) |
|---|---|---|---|
| | − DNase I | + DNase I | |
| 0-DNA | 8.2 ± 2.0 | 108.9 ± 11.3 | 13.3 |
| 10ACR-DNA | 8.7 ± 3.9 | 99.4 ± 8.2 | 11.4 |
| 30ACR-DNA | 10.5 ± 1.5 | 118.7 ± 2.6 | 11.3 |

DNA preparations (0.5 $\mu$g) were first treated with or without 0.5 ng DNase I for 20 min at 37°C. The digestion was terminated by cooling in an ice-water bath and the poly(ADP-ribose) synthesis was assayed under the standard conditions in the presence of 2.7 $\mu$g enzyme protein but without histone. The data are the means ± SD for 3 incubations

### 3.2. *ADP-ribosylation reaction in vitro in the presence of EAT DNAs*

First we studied the effect of DNase I on the in vitro enzyme activity in the presence of 3 EAT DNA preparations, as the extent of strand breakage of the isolated DNAs strongly affects the ADPRT activity. Table 2 shows that the synthesis of poly(ADP-ribose) was almost equally (to an extent of 11–13-fold) stimulated by DNase I. Thus the possibility of the different extent of strand breakage could be excluded as a cause of ADPRT suppression which we observed in the nuclei of 5-ACR-treated EAT cells [3]. This result is also consistent with the same agarose gel pattern of all 3 DNAs as mentioned above.

As clearly seen in table 1, the 2-fold stimulation of enzyme reaction by histone was partly reversed by $Mg^{2+}$. Our separate experiments suggested that $Mg^{2+}$ inhibits the ADPRT-DNA-histone interaction (data not shown). In the experiment shown in fig.1, therefore, the effect of increasing amounts of histone on the rate of ADP-ribosylation was studied in the presence of a

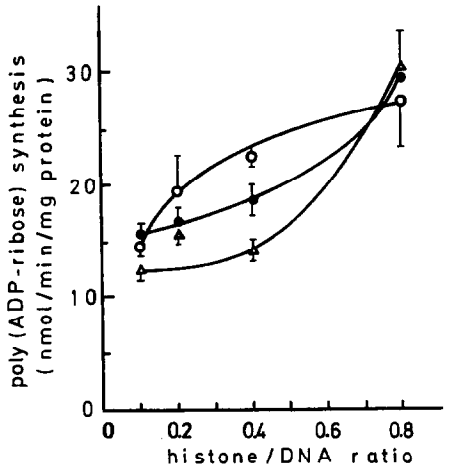


Fig.1. Stimulation of the in vitro ADP-ribosylation reaction by 0-DNA, 10ACR-DNA or 30ACR-DNA in the presence of histone. The enzyme reaction mixture contained 1.35 $\mu$g ADPRT protein, 2.5 $\mu$g DNA from EAT cells and increasing amounts of histone as indicated in a final volume of 0.1 ml and the reaction was run as described in section 2. The data are the means ± SD for 3 incubations. (○—○) 0-DNA; (●—●) 10ACR-DNA; (△—△) 30ACR-DNA.

constant amount of EAT-DNAs but without addition of $Mg^{2+}$. In the presence of 0-DNA (2.5 $\mu$g/tube), histone stimulated the rate of reaction in a dose-dependent fashion and saturated at a histone/DNA mass ratio of approximately 0.8. By contrast, in the presence of 10ACR-DNA and 30ACR-DNA (2.5 $\mu$g/tube), the histone effect was rather sigmoidal and not saturated at least up to the ratio of 0.8 tested so far. Thus, 10ACR-DNA and 30ACR-DNA were able to support the ADP-ribosylation to an extent of only 83% and 62%, respectively, of control 0-DNA at a histone/DNA ratio of 0.4. These degrees of suppression coincided roughly with those measured directly on the isolated nuclei, from which the DNAs in question were isolated, although the histone/DNA mass ratio of the nuclei from various sources is reported to be approximately 1.0 [17]. In the absence of histone (conditions of auto-ADP-ribosylation of ADPRT), however, we found no significant difference in the reaction stimulation between the control and 5-ACR-DNAs at DNA concentrations ranging from 0.01 to 8 $\mu$g/tube (data not shown). All these results strongly suggest that the ADPRT-DNA-histone interaction, which is essential for the enzyme function in chromatin, was markedly disturbed presumably as a result of 5-ACR incorporation into DNA [3].

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