Action of cobra venom cardiotoxin on chick embryonal fibroblasts transformed with a temperature-sensitive mutant of Rous sarcoma virus

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Received 26 August 1985; revised version received 9 October 1985

The cytolytic action of cardiotoxin analogue III from the venom of the Formosan cobra on chick embryonal fibroblasts transformed with a temperature-sensitive mutant of Rous sarcoma virus was investigated. The 50% effective dose of the toxin for the cells cultured at a non-permissive temperature (41°C) or for non-infected normal cells was about 8 μg/ml whereas the value was 2 μg/ml for the cells cultured at a permissive temperature (36°C). This indicates that the transformed cells became more susceptible to the cytolytic action of the toxin than the non-transformed cells.

Cobra venom cardiotoxin *Cytotoxicity* *Rous sarcoma virus* *Temperature-sensitive mutant* *Fibroblast* *Membrane lipid fluidity*

1. INTRODUCTION

Cobra venom cytotoxins (cardiotoxins) are highly basic membrane-active polypeptides consisting of 60–61 amino acid residues with 4 intramolecular disulfide linkages [1–3]. They exhibit in vitro a weak direct hemolytic effect and relatively preferential cytotoxicity toward tumor cells such as Yoshida sarcoma and other ascites hepatoma cells [4,5]. In previous studies, comparison of the cytolytic activity toward normal and transformed cells was carried out for different species or among different cell species [6]. Therefore, it was difficult to appreciate the difference in susceptibility between transformed and non-transformed cells. Here, we describe the cytolytic action of the major toxin, CTX-III (cardiotoxin of Narita and Lee [7]), on chick embryonal fibroblasts infected with a ts mutant of RSV with which a strict control experiment can be performed with the same cell species by only changing the temperature.

Abbreviations: ts, temperature-sensitive; CTX-III, cardiotoxin analogue III; RSV; Rous sarcoma virus; DPH, 1,6-diphenyl-1,3,5-hexatriene; FCS, fetal calf serum; PBS, Dulbecco's phosphate-buffered saline

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2. MATERIALS AND METHODS

2.1. *Materials*

CTX-III was purified from the venom of the Formosan cobra *(Naja naja atra)* as described [8]. It was found to be homogeneous by a high-performance liquid chromatography [9]. A ts mutant of RSV, strain LA-31, isolated by Wyke [10], belonging to the class T mutant (a defect in transformation) which can transform the cells at a permissive temperature (36°C), but not at a non-permissive temperature (41°C), was kindly supplied by Dr M. Yoshida (Cancer Institute, Tokyo).

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2.2. *Culture of fibroblasts*

Chick embryonal fibroblasts were prepared from 11-day embryos by trypsinization and cultured in medium 199 supplemented with 10% FCS and 10% tryptose phosphate broth as described [11]. Subconfluent cells were infected with the virus at 36°C for 15 h in the presence of polybrene (10 μg/ml), and cultured at 36°C for 2 weeks until all cells were morphologically transformed. Since long-term incubation at 36°C resulted in a decrease of cell viability and appearance of vacuoles in cytoplasm, transformed cells were incubated at 41°C for 2 days and transferred to 36°C for an additional 2 days before use. After mild treatment with trypsin (500 μg/ml, 20–30 s at room temperature), cells were suspended by careful pipetting in PBS containing 1 mM EDTA, and then washed twice with PBS containing 2% FCS and adjusted to approx. 2×10^6 cells/ml.

2.3. *Cytotoxicity assay*

A mixture of 50 μl each of a cell suspension and a serial dilution of CTX-III in PBS containing 2% FCS was incubated for 30 min at 37°C. The reaction was terminated by cooling the mixture in an ice bath, and then a 50 μl aliquot of 0.4% trypan blue in PBS was added for the dye exclusion test [8]. The number of intact and damaged cells were determined on a hemocytometer slide glass.

2.4. *Fluorescence labeling*

Cells were harvested in the same manner as described above until the first centrifugation step. The subsequent washing buffer was changed to PBS containing 5 mM $MgCl_2$ and 1 mM $CaCl_2$. These divalent cations were effective for maintaining cell viability during fluorescence labeling. After washing with this buffer the cell suspension was adjusted to approx. 0.8×10^6 cells/ml. A DPH dispersion in PBS (2 μM) was prepared according to [12]. Labeling was carried out for 1 h at 25°C as in [12] in the presence of 5 mM $MgCl_2$ and 1 mM $CaCl_2$. After washing with PBS containing 2% FCS, the cells were resuspended in the same buffer for the fluorescence measurement. The viability of the labeled cells was over 90%.

2.5. *Fluorescence polarization measurements*

Fluorescence polarization was measured with an Elscint model MV-1a microviscosimeter (Elscint, Israel). The degree of fluorescence polarization, P, was directly recorded. The rotational relaxation time (ϱ) of the fluorophore was calculated with the following Perrin equation;

$$\frac{r_0}{r} = 1 + \frac{3\tau}{\varrho}$$

where r_0 and r are the limited and measured fluorescence anisotropy, respectively, and τ is the fluorescence lifetime of the excited state of DPH [13]. The r_0 value was 0.362 [13] and r was calculated from P [14].

3. RESULTS AND DISCUSSION

Chick embryonal fibroblasts infected with the ts mutant of RSV were reversibly transformed on shifting down of the temperature from a non-permissive (41°C) to a permissive temperature (36°C). Non-transformed cells had a flat and smooth cell surface as non-infected cells, and transformed cells showed a round form with numerous microvilli which was evident after 2 days incubation at 36°C (fig.1). These morphological features were the same as those described [15]. The dose-response curves for the cytolytic activity of CTX-III toward these cells are shown in fig.2. With the infected cells cultured at 41°C, the effective dose for 50% lysis of the cells (ED_{50}) was about 8 μg/ml, which was almost identical to that for normal fibroblasts without viral infection. With the cells cultured at 36°C, the ED_{50} value decreased significantly to 2 μg/ml. At this concentration, the non-transformed cells retained over 90% viability. These changes in susceptibility toward the toxin were reversible, depending on the cultivation temperature. These facts may suggest that the increased susceptibility to CTX-III is one of the transformational phenotypes of the cells.

The membrane lipid fluidity of 3 kinds of cells was measured by the fluorescence depolarization method using DPH, and the relative fluidity was expressed in terms of the rotational relaxation time (ϱ) of the fluorophore (table 1). It was found that non-transformed cells gave similar ϱ values to those for normal fibroblasts, whereas transformed cells resulted in rather lower values at each temperature measured. This suggests that the membrane lipid fluidity increases upon transformation.

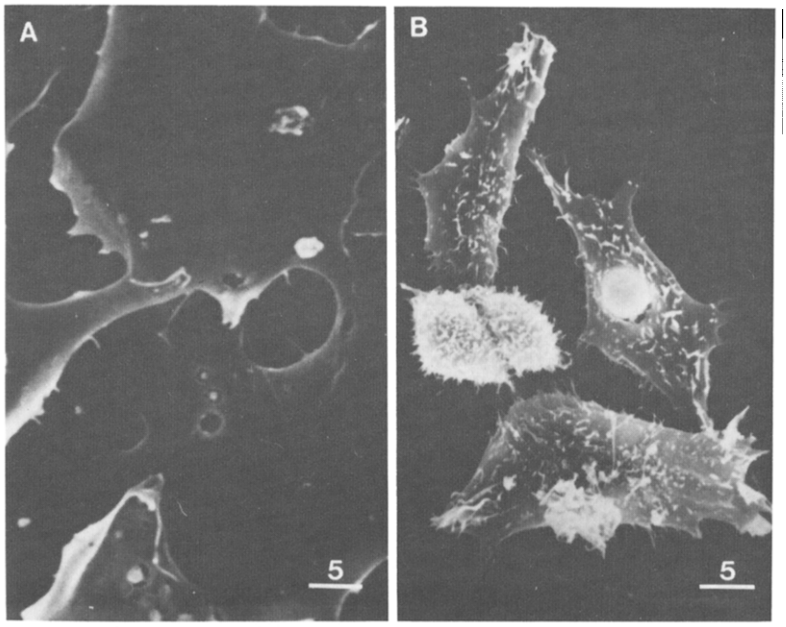

Fig.1. Scanning electron micrographs of chick embryonal fibroblasts infected with a ts mutant of Rous sarcoma virus, strain LA-31. Cells cultured at (A) 41°C for 2 days, (B) at 36°C for 2 days. Bar, 5 μm.

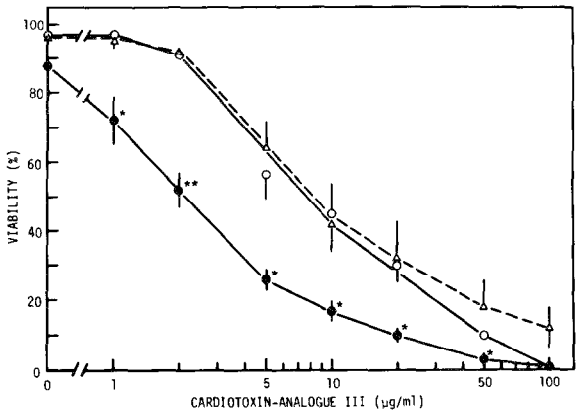

Fig.2. Dose-response curves of the cytolytic action of CTX-III on chick embryonal fibroblasts infected with the ts mutant of Rous sarcoma virus. The cell suspension (2×10^6 cells/ml) and toxin were incubated for 30 min at 37°C in PBS containing 2% FCS. Cytotoxicity was assayed by the dye exclusion test. Cells cultured at a (○) non-permissive temperature (41°C), (●) permissive temperature (36°C); (△) fibroblasts which were not infected with the virus. Data represent means ± SE for 3 independent experiments. The statistical differences between non-transformed (41°C) and transformed (36°C) cells were calculated according to Student's *t*-test; * $P<0.05$; ** $P<0.01$.

Table 1

Degree of fluorescence polarization *(P)* and rotational relaxation time (ϱ) of DPH embedded in the plasma membrane of chick embryonal fibroblasts infected with the ts mutant of Rous sarcoma virus

Temperature (°C)	Transformed cells		Non-transformed cells		Normal fibroblasts	
	P	ϱ (ns)	*P*	ϱ (ns)	*P*	ϱ (ns)
4	0.292	49	0.305	55	0.306	56
20	0.248	30	0.269	36	0.261	33
37	0.192	15	0.213	18	0.213	18

P was measured at 3 different temperatures for cells cultured at 36°C (transformed) and 41°C (non-transformed), and normal fibroblasts which were not infected with the virus. ϱ was calculated as described in the text assuming the fluorescence lifetime of DPH to be 11.0, 10.5 and 8.8 ns at 4, 20 and 37°C, respectively [13]. The data represent the mean values for 2 independent experiments

Recently, it was proposed that the interpretation of membrane lipid fluidity should be done with care when intact cells are investigated because hydrocarbon fluorophores such as DPH label the intracellular membrane region as well as the plasma

membrane. Grunberger et al. [16] described a method for distinguishing plasma membrane lipid fluidity from intracellular fluidity, and reported that the plasma membrane had almost the same lipid fluidity as intracellular membranes in the cases of 3T3 fibroblasts and macrophages. This method might be useful for further investigations to confirm our present results. It would be of interest to consider that the membrane lipid fluidity plays an important role in the cytolytic action of CTX-III and that cell transformation induces a change in lipid fluidity of the plasma membrane which may lead to modification of the susceptibility of the cells to the toxin.

ACKNOWLEDGEMENT

We thank Dr S. Saga (Nagoya University School of Medicine) for carrying out the scanning electron microscopy.

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