THE CYTOTOXIC AND CYTOLYTIC ACTIVITY OF EQUINATOXIN II FROM THE SEA ANEMONE Actinia equina

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ABSTRACT

The cytotoxic and cytolytic effects of equinatoxin II (EqT II) from the sea anemone Actinia equina L. were studied on exponentially growing and synchronized V-79-379 A cell line in culture. The cell viability test and the determination of the cytolytic effect by cell counting confirmed both cytotoxic and cytolytic activity of EqT II. Additionally, cytocidal and cytostatic effects depending on the toxin concentration were observed. The presence of fetal calf serum in the cell culture medium reduced both cytocidal and cytostatic effects by two magnitudes and prevented cytolysis. Combining EqT II and serum resulted in an insoluble complex which was cytostatic even when isolated and resuspended in the culture medium, while the supernatant retained both cytocidal and cytostatic activity. No significant difference in sensitivity between synchronized and exponentially growing cells could be detected after EqT II treatment.

INTRODUCTION

Three lethal toxins, equinatoxin (EqT) I, II and III were isolated from the sea anemone Actinia equina L. (Maček and Lebez, 1988). EqT II, i.e. equinatoxin according to Ferlan and Lebez (1974), is a basic protein with a molecular weight of 19,000 (Maček and Lebez, 1988). It is hemolytic in neutral and alkaline pH (Maček and Lebez, 1981) and causes aggregation and lysis of platelets (Teng et al., 1988). In addition to lethal effects (an i.v. LD₅₀ of 35 μg/kg in mice), potent cardiotropic action (Sket et al., 1974; Ho et al., 1988) and induction of pulmonary edema (Lafranconi et al., 1984) were reported for the toxin. EqT II is biochemically and biologically closely related to other cytolytic toxins isolated from several sea anemones whose cytolytic activity can be inhibited with membrane phosphatides, in particular sphingomyelin (Bernheimer and Rudy, 1986; Kem, 1988). It has been also demonstrated for EqT II that serum (Giraldi et al., 1976), serum lipoproteins (Turk et al., 1989), suspension of sphingomyelin and some other phosphatides (Turk and Maček, 1986) affect the cytotoxic and hemolytic activity.

In contrast to some other species of toxins, in particular bacterial, there are only a few reports on the effects of Coelenterate venoms or toxins on in vitro
cultured cells (Giraldi et al., 1976; Neeman et al., 1980; Olson et al., 1985; Avila et al., 1988; 1989). Both the work of Giraldi et al. (1976) and our preliminary studies (Batista et al., 1986; 1987) have clearly demonstrated the lytic potency of EqT II upon various cell lines. However, the aim of the present study was to elaborate in detail the toxicity of a pure sea anemone cytolytic toxin on exponentially growing and synchronized diploid lung fibroblasts of the Chinese hamster.

MATERIALS AND METHODS

Toxin

EqT II was isolated from the sea anemone *Actinia equina* L. according to the method of Maček and Lebez (1988) and its activity was assayed as described previously (Maček and Lebez, 1981).

Cells

The cells V-79-379 A (diploid lung fibroblasts of Chinese hamster) were grown in Eagle MEM (minimal essential medium, Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS, Gibco, Paisley, Scotland), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a CO₂ incubator.

Dye exclusion test for cell viability (Phillips, 1973)

The cells were centrifuged at 90 g for 10 min and resuspended in a buffered isotonic saline. EqT II at final concentrations of 4.3x10⁻¹¹ - 4.3x10⁻⁹ mole/l and EqT II (1.1x10⁻⁶ mole/l) preincubated with 10% FCS (10 min at 37°C) were added to the cell suspension (3.5x10⁵/ml) at 37°C. Time dependent cytotoxicity was determined by a dye exclusion test using trypan blue solution (0.4% w/v) diluted 1:6 (v/v) in isotonic saline. Stained and nonstained (viable) cells were counted at different time intervals. At the beginning of the experiment, the viability of the control cells was between 86-92 % as determined by the dye exclusion test.

Determination of the cytolytic effect

The cytolytic effect of the EqT II (final concentrations of 4.3x10⁻¹¹ - 4.3x10⁻⁹ mole/l) and EqT II (1.1x10⁻⁶ mole/l) preincubated with 10% FCS for 10 min at 37°C was measured by cell counting. Cells were prepared by the same procedure as for the dye exclusion test. In a given area on the hemocytometer the cells were counted at different time intervals. The ratio between the remaining toxin treated and untreated cells was calculated.

Colony forming ability test - cytocidal effect

The cells were seeded in plastic Petri dishes (300-400 cells/dish - 50 mm in triplicates). After 3 hours the cells were washed with the culture medium without serum. The cells were then incubated with the media without serum, containing different concentrations of EqT II (4.3x10⁻¹² - 4.3x10⁻¹⁰ mole/l) or EqT II (4.7x10⁻¹¹ - 2.3x10⁻⁷ mole/l) preincubated with 10% FCS (10 min at 37°C). After one hour FCS was added at the final concentration of 10% if the
cells had been treated with EqT II without serum. Cells were maintained in a CO₂ incubator for 6 more days. The colonies were stained with a 10% Giemsa (Kemika, Zagreb, Yugoslavia) solution and counted. The cytotoxic effect was expressed as a surviving fraction (SF) and was calculated according to Olah et al. (1978) as follows:

\[ \text{SF} = \frac{\text{plating efficiency of treated culture}}{\text{plating efficiency of untreated culture}} \]

Inhibition of cell proliferation - cytostatic effect

The V-79-379 A cells \((7 \times 10^4)\) were plated in plastic Petri dishes \((50 \text{ mm})\) in triplicates and incubated overnight at 37°C in a CO₂ incubator. After 24 hours, different final concentrations of EqT II \((4.3 \times 10^{-11} - 4.3 \times 10^{-9} \text{ mole/l})\) or EqT II \((2.7 \times 10^{-10} - 9.2 \times 10^{-9} \text{ mole/l})\) preincubated with 10% FCS \((10 \text{ min at } 37°C)\) were added. After one hour, FCS was added at the final concentration of 10% if the cells were treated with EqT II without serum. Cells were incubated for 24 hours. The net cell increase was determined by cell counting following trypsinization. Growth factors (Chapman et al., 1971) were calculated as follows:

\[ \text{GF} = \frac{N(x) - N_0}{N_c - N_0} \times 100\% \]

\(N(x)\) stands for cell count/dish after 24-hour growth in the presence of the EqT II concentration \(x\), \(N_c\) for cell count/dish after 24-hour growth in the absence of EqT II and \(N_0\) for cell count/dish at time of addition of EqT II.

Results are given as growth factors at different EqT II concentrations.

Interaction between EqT II and fetal calf serum (FCS)

EqT II, at a final concentration of \(5 \times 10^{-8}\) or \(3.3 \times 10^{-7} \text{ mole/l}\), was first preincubated with culture medium containing 10% FCS for 10 min at 37°C and the mixture was then centrifuged at 15,000 g for 30 min. Supernatant and sediment, resuspended in Eagle MEM with 10% FCS, were tested separately for colony forming ability. After 6 days the colonies were washed twice in buffered isotonic saline and counted. The cytotoxic effect was expressed as the surviving fraction (Eq. 1). The total cell protein content was determined using a method of Oyama and Eagle (1956) and the percentage of protein/colony was calculated.

Synchronization procedure

The cells were synchronized in G₁, S and M phases of the cell cycle and the plateau phase of growth. The cells in the G₁ phase were obtained by serum deprivation (Mauck and Green, 1973), in the S phase following treatment with 1 mmole/l hydroxyurea (Sigma, St. Louis, USA). Mitotic cells were collected by mitotic detachment (Ashihara and Baserga, 1979). The cells attained a plateau phase of growth after 6 days in culture with contact inhibition. Degree
of synchrony was determined by a labeling index obtained with autoradiography and synchrony index (Lloyd, 1987). The cytocidal effect of EqT II preincubated with 10% FCS for 10 min at 37°C on synchronized cells was studied with the colony forming ability test.

RESULTS

The effect of EqT II on cell viability of V-79-379 A cell line following one hour exposure is shown in Fig. 1a. The ED<sub>50</sub> of EqT II was 8.8x10<sup>-10</sup> mole/l, i.e. 17 ng/ml. The cytolytic effect of a different final concentration of EqT II on V-79 cells after one hour exposure is shown in Fig. 1b. The number of cells in a suspension was decreased by raising the toxin concentration, and at an EqT II concentration of 4.3x10<sup>-9</sup> mole/l 50% of cells remained unlysed. When the V-79 cells were treated with EqT II preincubated with 10% FCS, cell lysis was not observed even when the EqT II concentration was 1.1x10<sup>-6</sup> mole/l.

**FIG.1 THE CYTOTOXIC AND CYTOLYTIC EFFECTS OF EqT II ON V-79-379 A CELLS IN SUSPENSION AFTER ONE HOUR TREATMENT.**

a) The effect of different final concentrations of EqT II (4.3x10<sup>-11</sup> - 4.3x10<sup>-9</sup> mole/l) on cell viability of V-79 cells after one hour treatment, measured by dye exclusion test (cell concentration: 3x10<sup>5</sup> cells/ml buffered isotonic saline).

b) The cytolytic effect of different final concentrations of EqT II (4.3x10<sup>-11</sup> - 4.3x10<sup>-9</sup> mole/l) on V-79 cells after one hour treatment, measured by cell counting (cell concentration: 3x10<sup>5</sup> cells/ml buffered isotonic saline).

Values represent the means of three experiments and standard deviations.
FIG 2. THE CYTOCIDAL EFFECT OF EqT II ON V-79 CELLS.

The cells were incubated with media without serum, containing different final EqT II concentrations of $4.3 \times 10^{-12}$ - $4.3 \times 10^{-10}$ mole/l (solid symbols) and EqT II of $4.7 \times 10^{-11}$ - $2.3 \times 10^{-7}$ mole/l, preincubated with 10% FCS (10 min at 37°C)(open symbols). After one hour, FCS was added at a final concentration of 10% if the cells had been treated with EqT II without serum. The cytotoxic effect was expressed as a surviving fraction (SF) (Eq. 1) in relation to the EqT II concentration (mole/l).

Values represent the means of four experiments each in triplicate and standard deviations.

Fig. 2 shows the cytocidal effects of EqT II and EqT II preincubated with 10% FCS on V-79 cells. The inhibitory effect of EqT II on the colony forming ability was observed in both experiments. The survival of cells was greatly affected. The presence of 10% FCS in the culture medium reduced the cytoidal activity of EqT II by at least 300 fold. ED$_{50}$ for EqT II was 2.4 μg/ml and for the FCS treated toxin 890 μg/ml. Increasing amounts of EqT II in the culture medium decreased cell growth factors as demonstrated in Fig.3. In addition to the cytocidal effect, EqT II caused cytostatic activity. Toxin at concentrations above $4.3 \times 10^{-11}$ mole/l exhibited both cytostatic and cytolytic effects. By increasing the toxin concentration, the cytotoxic effect prevailed, as indicated by negative growth factors (dotted line in Fig. 3). EqT II preincubated with 10% FCS did not lyse cells, although net cell proliferation was decreased (Fig. 3). In contrast, at low doses of EqT II slightly accelerated cell growth kinetics occurred as shown in Fig. 3. This was also seen in some other experiments (data not shown).
FIG. 3 THE CYTOSTATIC EFFECT OF EqT II ON V-79 CELLS.

Various final EqT II concentrations of $4.3 \times 10^{-11} - 4.3 \times 10^{-9}$ mole/l (solid symbols) and EqT II of $2.7 \times 10^{-10} - 9.2 \times 10^{-8}$ mole/l, first preincubated with 10% FCS (10 min at 37°C) (open symbols) were added to the cells. After 24 hours the net cell increase was determined by cell counting. Growth factors (%) were calculated from Eq. 2. Values represent the means of three experiments each in triplicate and standard deviations.

Incubation of the toxin with FCS resulted in a precipitate which was isolated by centrifugation. The resulting supernatant and sediment, resuspended in culture medium, were tested for colony forming ability in comparison to EqT II. Fig. 4 a shows the cytocidal effects of EqT II, supernatant and sediment on V-79 cells. EqT II itself and the supernatant decreased cell survival, while the sediment, obtained after the treatment of FCS with $5 \times 10^{-8}$ or $3.3 \times 10^{-7}$ mole/l of EqT II, apparently did not affect colony forming ability, although changes of colony morphology were observed in all exposures (unpublished data). Colony forming ability (Fig. 4 a) implies a cytocidal effect of the supernatant, corresponding values of protein content/colony (Fig. 4 b) indicate cytostatic activity of the sediment and supernatant.

Labeling indices of 95% for cells in S phase and 1% for cells in plateau phase and synchrony indices of 0.86 for cells in $G_1$, 0.91 for cells in S phase and 1.0 for mitotic cells were obtained in synchronization experiments.

Cytocidal effects of EqT II preincubated with 10% FCS on synchronized cells, in comparison to exponentially growing cell culture, are shown in Fig. 5. The capability of cells to form colonies was diminished in both cases. No significant difference in sensitivity between synchronized and exponentially growing cells could be detected. ED$_{50}$s for mitotic cells was 0.7 $\mu$g/ml, for cells in S phase 0.6 $\mu$g/ml and plateau phase of growth 1 $\mu$g/ml. Cells in the $G_1$ phase of cell cycle and mitotic cells were exceptions. Even at the highest concentration of
FIG. 4a) THE CYTOCIDAL EFFECTS OF EqT II, THE SUPERNATANT AND THE SEDIMENT OF CENTRIFUGED FCS TREATED EqT II ON V-79 CELL LINE.

EqT II, the supernatant and the sediment, resuspended in culture medium with 10% FCS, were added to the cells (300-400 cells in 50 mm Petri dishes). Results are given as a surviving fraction (Eq. 1) in relation to the EqT II concentrations (mole/l). Values represent the means of three experiments each in triplicate and standard deviations.

FIG. 4b) THE PERCENTAGE OF PROTEIN/COLONY/CONTROL OF EqT II, THE SUPERNATANT AND THE SEDIMENT OF THE CENTRIFUGED FCS TREATED EqT II ON V-79 CELL LINE.

EqT II, the supernatant and the sediment, resuspended in culture medium with 10% FCS, were added to the cells (300-400 cells in 50 mm Petri dishes). Results are given as the percentage of protein/colony in relation to the EqT II concentrations (mole/l). Values represent the means of three experiments each in triplicate and standard deviations.

FIG. 5 CYTOCIDAL EFFECT OF EqT II ON EXPONENTIALLY GROWING AND SYNCHRONIZED V-79 CELLS.

Different final concentrations of EqT II (9.2x10^-9 - 9.2x10^-8 mole/l) were first preincubated with medium with 10% FCS (10 min at 37°C) and then added immediately to the exponentially growing cells (open circles), cells in G_1 (solid circles), S (open squares), M phase of cell cycle (solid squares) and plateau phase of growth (triangles). Results are given as a surviving fraction (Eq. 1) in relation to the EqT II concentrations (mole/l). Values represent the means of three experiments each in triplicate and standard deviations.
EqT II used, survival of cells in G1 did not fall below 56% and there was an inhibitory effect of EqT II on the surviving fraction (0.18) of mitotic cells.

**DISCUSSION**

Our study of the effects of the sea anemone cytolytic toxin, equinatoxin II, upon cultured lung fibroblasts corroborates previous observations of Giraldi et al. (1976) on equinatoxin in vitro cytotoxic and cytolytic activity in Ehrlich ascites carcinoma and Li210 leukemia cells. Equinatoxin II cytotoxicity on the V-79-379 A cells and on the tumour cells is very similar for comparable values of ED50s. Moreover, it seems that the potency of equinatoxin II to lyse tumour cells (Giraldi et al., 1976), sheep erythrocytes (Maček and Lebez, 1981) and erythrocytes of some other mammalian species (unpublished data), rabbit platelets (Teng et al., 1988) and V-79-379 A cells is of the same magnitude. This finding is consistent with the suggestion that sea anemone cytolytic toxins are channel forming polypeptides affecting mainly the phospholipid part of biological membranes (Michaels, 1979; Bernheimer and Rudy, 1986; Kem, 1988) and thus making them permeable for ions and non-electrolytes (Varanda and Finkelstein, 1980; Menestrina, in preparation). Recently, Zorec et al. (in press) showed that equinatoxin II cytotoxicity is mediated by increasing intracellular calcium activity by ion channel formation in cell membranes. If this is an exclusive mode of action of sea anemone cytolytic toxins in cells a significant cytotoxic selectivity for different cell types might not be expected. Once more our finding coincides with the above suggestion; the lack of significant differences in sensitivity in cells in relation to phases of the cell cycle supports the thesis that the toxin affects a part of the membrane or a process which is independent or only slightly connected to changes during the cell cycle. The small differences in sensitivity which were detected, however, are probably due to different physiological states of the cells following synchronization procedures, and detailed interpretation needs further experimentation.

Presence of serum, an obligatory additive in cell culture media, and a normal cell bathing solution in vivo, obviously decreased the cytotoxic effect and greatly reduced the cytolytic activity of equinatoxin II, a fact also reported by Giraldi et al. (1976) and Bernheimer and Avigad (1976) for the Stoichactis helianthus cytolysin. These findings and the formation of an insoluble complex in the mixture of serum and EqT II could be interpreted in terms of an interaction between the toxin and serum lipoproteins as shown by Turk et al. (1989). Our experiments demonstrate a great capacity of fetal calf serum to neutralize the cytotoxicity and lytic activity of EqT II, although it seems that the isolated complex retains at least a part of the cytostatic activity by a mechanism not known. Overall, this buffering ability of the serum, the cytostatic activity and even the slight acceleration of the cell proliferation observed in our experiments suggest a complexity of actions EqT II might exert upon living cells when applied sublethally.

Extracts or purified toxins from Coelenterates with very potent cytotoxic and cytolytic activity have been tested for antitumor activity in vitro and in vivo (Tabrah et al., 1974; Norton and Kashwagi, 1972; Quinn et al., 1974; Giraldi et al., 1976). The limited success in vivo as compared to in vitro antitumor activities opens the questions of cell selective cytotoxicity and toxin spatial
selectivity. In this respect, it is obvious that the cytotoxic selectivity of EqT II for different types of cells is poor and extensively decreased by serum, and very likely by other body solutions containing lipoproteins. Hence, only spatial selectivity achieved by site directed delivery, might be used for damaging target cells by a sea anemone cytolytic toxin, as has been true for intraperitonelly Ehrlich ascites carcinoma bearing mice treated with equinatoxin (Giraldi et al., 1976). Another more promising example of this strategy has been recently reported by Avila et al. (1988; 1989) who used immunotoxins, conjugates of *Stoichactis helianthus* cytolysin and antitumour specific immunoglobulins, for directing cytotoxicity.

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