Molecular Mechanisms of Different Sensitivity of Tumor Cells to Dexamethasone

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The response of two cell lines, CSML-0 (does not express metastasin) and CSML-100 (high expression of metastasin) to cytolytic action of glucocorticoid was studied. Dexamethasone (1 μM) induced apoptosis of CSML-0 cells, while CSLM-100 cells were resistant to its cytolytic action. Apoptotic death of CSLM-100 cells was induced by incubation with dexamethasone in the presence of Ca-ATPase inhibitors, vanadate or thapsigargin. Metastasin, a proteins of the S-100 family, activated Ca-ATPase and ATP-dependent Ca^{2+} transport in plasmolemmal fraction of CSML-100 cells. Experiments showed that metastasin-induced activation of Ca-ATPase is a possible mechanisms of CSML-100 cell resistance to cytolytic action of dexamethasone.

Key words: *apoptosis; dexamethasone; calcium; Ca-ATPase; metastasin*

Tumor cells are characterised by uncontrolled proliferation, dedifferentiation, invasion and metastatic spreading, and impaired mechanisms of apoptotic death. Specific characteristics of transformed cells, *i. e.* a set of specific tumor markers, are used for molecular diagnostics and choice of antineoplastic therapy. One of these markers is metastasin (Mts-1), an intracellular protein with molecular weight (M_r) 11 kD belonging to the S-100 family [11]. Expression of this marker closely correlates with metastatic potential of tumor cells [8]. Two cell lines of murine adenocarcinoma with different metastatic capacities and Mts-1 content were obtained: CSML-100 and CSML-0 characterised by high metastatic frequency and superexpression of *mts*-1 gene, or by low metastatic capacities and trace amounts of Mts-1 protein, respectively [14].

The studies of potential targets for metastasin action that this protein can play a role in the regulation of Ca^{2+} metabolism in malignant cells [8]. p37, a target proteins (M_r=37 kD) isolated from CSML-100 cells, specifically changes Ca^{2+}-binding properties and tertiary metastasin structure [3]. Sequencing of 8 N-terminal amino acids of p37 and comparison of these data with data base on proteins with known structure revealed maximum homology between p37 and the 5th isoform of membrane Ca-ATPase. Since M_r of four examined Ca-ATPase isoforms is 100-150 kD, p37 protein can present a fragment of Ca-ATPase molecule, though it exhibited no ATPase activity. Recently obtained data on mapping monoclonal antibody epitopes to Ca-ATPase of plasma membrane show that sequenced p37 fragment is located within 330-353 amino acid residues, which corresponds to acid phospholipid and regulatory protein binding site of Ca-ATPase [5].

At present, there are convincing evidence that intracellular Ca^{2+} level is effected by various regulatory systems controlling apoptosis, which presents the main pathway of elimination of transformed cells. Increase in Ca^{2+} plasma membrane permeability is an early cells response during apoptosis induced by antineoplastic chemotherapy and γ-irradiation [7]. Moreover, it was shown that Ca^{2+} ionophores (ionomycin, A23187) and Ca-ATPase blockers induce apoptosis in various cell lines [12].

The aim of the present study was to compare calcium metabolism and sensitivity to cytolytic effects of dexamethasone of two CSML cell lines with different levels of Mts-1 expression.

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MATERIALS AND METHODS

Inverted vesicles of plasma membranes (PM) were isolated by differential centrifugation. Mts-1 was isolated from CSML-100 cells as described elsewhere [4]. To determine ATP-dependent Ca^{2+} transport in PM fraction vesicles were loaded with fluorescent Ca^{2+} ion indicator Fluo-3 to a final intravesicular concentration of 5-7 μM. The concentration of calcium ions in vesicles ($[Ca^{2+}]_I$) was calculated by the formula [15]:

$$[Ca^{2+}]_I = K_d \times (F_{max} - F_{530})/(F_{530} - F_{min}),$$

where F_{530} is fluorescence at 530 nm; F_{max} is fluorescence after probe saturation with calcium measured in the presence of 30 μM digitonin and 1 mM $CaCl_2$; F_{min} is fluorescence intensity in a calcium-free medium (in the presence of 5 mM EGTA and 5 μM A23187); K_d is Fluo-Ca dissociation constant (0.42 μM). Before measuring of Ca^{2+} incorporation, the samples were preincubated at 37°C for 30 min to ensure the balance in the medium-vesicle system. To calculate the amount of Ca^{2+} in vesicles their volume (W; 92 μl/mg protein) was determined in preliminary experiments. Ca-ATPase activity was determined spectrophotometrically by the formation of inorganic phosphate [9] in 400 μl medium of the following composition: 0.1 M KCl, 5 mM $MgCl_2$, 50 mM MOPS (pH 7.0), 5 mM ATP, 4 μM A23187, 0.4 μg oligomycin, 0.1 mM EGTA, 0.1 mg protein, and 1-200 μM $CaCl_2$ (concentration of calcium ions measured with Fura-2 probe [7] varied from 0.01 to 81 μM). Apoptotic changes were estimated by measuring cell volume [2], detection of DNA fragmentation by electophoresis [1] and counting viable cells. Cell death was evaluated microscopically by positive staining with 0.04% trypan blue and fluorometrically by ethidium bromide incorporation at 505 and 590 nm excitation and emission wavelength, respectively. Statistical processing of the results was performed using Student's *t* test.

RESULTS

Dexamethasone (1 μM) induced apoptosis of CSML-0 cells containing no Mts-1, while CSML-100 cells with high metastasin expression were resistant to the hormone. The following apoptotic changes were observed: significant ($p<0.05$) decrease in cell volume by 21-27%, internucleosomal DNA fragmentation (Fig. 1), and decrease in the number of viable cells by 65-72% after 3-, 4-, and 12-h incubation with dexamethasone, respectively. Significant decrease of the number of dead CSML-0 cells was observed in calcium-free medium in the presence of 1 μM dexamethasone and 1 μM phorbol 12-myristate 13-acetate (PMA; Fig. 2, *a*). In the control (1 μM dexamethasone, 12-h incubation) this parameter was 12-16% as estimated by positive trypan blue staining.

Basal cytoplasmic concentration of free Ca^{2+} ions was 104±9 nM. Ca response of CSML-0 cells consisted of two phases. Starting from the 5th min of incubation $[Ca^{2+}]_I$ gradually increased to 130-144 nM due to Ca^{2+} entry from the medium (phase I). After 2-h incubation, $[Ca^{2+}]_I$ sharply increased to 195-225 nM (184-198% of the initial level) due to Ca^{2+} mobilisation from intracellular stores, *i. e.* from endoplasmic reticulum and mitochondria (phase II).

Dexamethasone in a concentration of 1 μM induced no apoptosis in CSML-100 cells (Fig. 2, *b*). Addition of Ca-ATPase inhibitors, sodium vanadate (1 mM) and thapsigargin (100 nM) to dexamethasone-containing medium induced an apoptotic response in CSML-100 cells (48-56% viable cells). Ca response of CSML-100 cells was reduced: phase I was practically absent and $[Ca^{2+}]_I$ increase was transient and insignificant, while after 120-150-min incubation the rise of $[Ca^{2+}]_I$ did not exceed 60% of the basal level (157-169 nM).

Activity of calcium transport systems was evaluated using Fluo-3, a Ca-specific fluorescent probe possessing a number of advantages over other methods. Calcium accumulation in plasmolemmal vesicles is often determined by radioligand method with

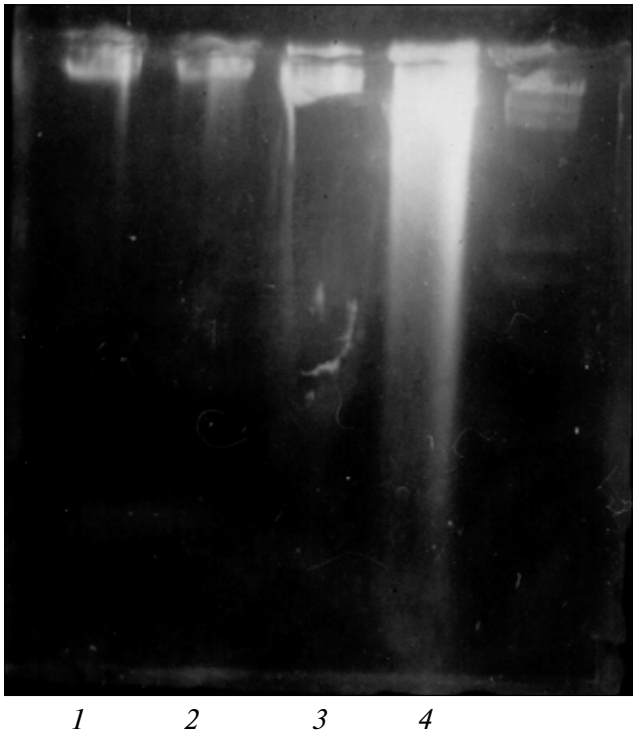

Fig. 1. Electrophoregram of DNA from CSML-100 and CSML-0 cells after 4 h incubation without (bands *1* and *3*) and with 1 μM dexamethasone (bands *2* and *4*). Ethidium bromide staining (1 μg/ml).

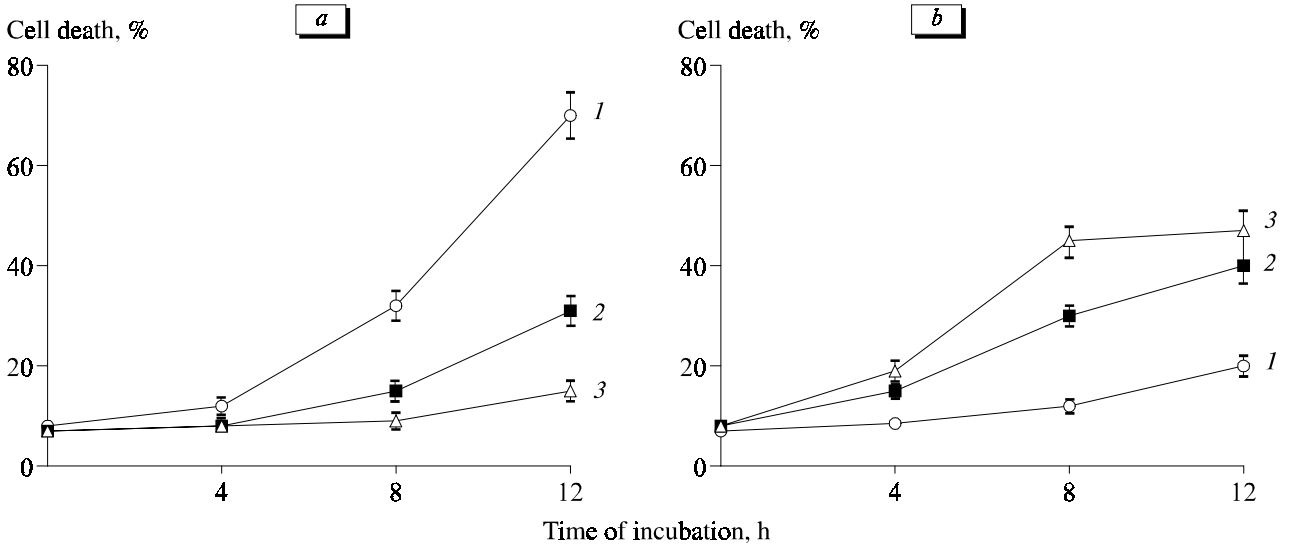

Fig. 2. Cytolytic effect of dexamethasone (1 μM) under various experimental conditions. *a*: CSML-0 cells (5×10^6/ml); *1*) control (dexamethasone), *2*) in the presence of 1μM PMA, *3*) in calcium-free medium (5 mM EGTA). *b*: CSML-100 cells (7×10^6 cells/ml); *1*) control (dexamethasone), *2*) in the presence of 100 nM thapsigargin, *3*) in the presence of 1 mM sodium vanadate.

^{45}Ca. Maximum rate of Ca^{2+} transport (V_{max}) determined by this method can differ by several times for the same specimen. V_{max} depends significantly on experimental conditions, while calculation of actual Ca^{2+} concentration in vesicles is hampered by the difficulties in measuring membrane-bound Ca^{2+}. Fluorescent probe Fluo-3 allows to specifically measure Ca^{2+} transport. In our experiments we obtained microsomal fraction of CSML-100 cells enriched by inverted plasmolemmal vesicles. Control experiments with Fura-2/AM and Fluo-3/AM fluorescent probes confirmed the presence of ATP-dependent transport systems providing Ca^{2+} accumulation in PM vesicles. In these control experiments the membranes were treated with blockers of sulfhydryl groups, calcium ionophore A23187, digitonin, La^{3+}, and calmodulin inhibitor trifluoperazine (data not shown).

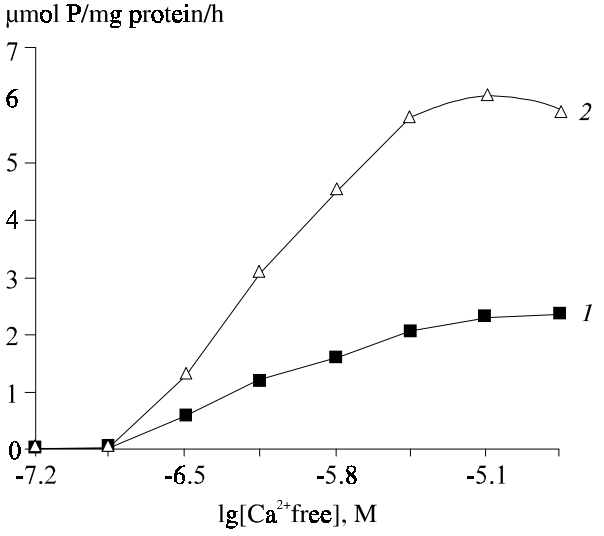

Fig. 3. Effect of metastasin on Ca-ATPase activity in plasma membranes of CSML-100 cells at 37°C in the absence or (*1*) or presence of 10 nM metastasin (*2*).

Metastasin (10 nM) markedly activated Ca-ATPase in CSML-100 PM (Fig. 3). In the presence of Mts-1 maximum Ca-ATPase activity at saturating calcium concentrations attained 6.2±0.2 μmol P_i/mg protein/h, while in the control (without metastasin) V_{max} was 2.43±0.09 μmol P_i/mg protein/h. Apparent K_m did not change (0.63±0.02 and 0.59±0.02 μM in the absence or presence of metastasin, respectively). Under these experimental conditions calmodulin inhibitor trifluoperazine (20 μM) decreased K_m and V_{max} of Ca-ATPase activity by on average 70%. This points to the presence of endogenous modulator of Ca-ATPase activity, calmodulin, in PM fraction and explains the absence of differences between the control and experimental K_m values.

Notably, potentiating effect of metastasin was not pronounced at low Ca^{2+} concentrations (30-100 nM) corresponding to basal Ca^{2+} level in rest cells. However, this effect peaked at higher Ca^{2+} concentrations (0.3-1.0 μM) observed in stimulated cells [6]. This fact points to a role of metastasin in the regulation of Ca^{2+} metabolism under physiological conditions.

Ca-ATPase creates a considerable Ca^{2+} gradient: Ca^{2+} concentration in the medium was 1 μM, while $[Ca^{2+}]_i$ after 10-min incubation attained 8.6±0.4 μM (Fig. 4). Metastasin in a concentration of 10 nM significantly increased $[Ca^{2+}]_i$ accumulation to 23.9±1.7 μM. Passive Ca^{2+} transport without ATP was 0.1 nmol/mg protein per 10 min, *i. e.* about 10% of Ca^{2+} accumulation in the presence of ATP. Study of correlation

between energy-dependent Ca^{2+} transport and metastasin content in the medium showed that 50% increase in Ca^{2+} transport can be induced by 8.5±0.4 nM protein. The concentrations of metastasin used in our experiments corresponded to intracellular level of S-100 proteins in target cells [12].

Thus, metastasin significantly activated Ca-ATPase in PM of CSML-100 cells and energy-dependent Ca^{2+} transport. Metastasin-mediated activation of Ca-ATPase and subsequent decrease of Ca^{2+} level is a possible molecular mechanisms of inhibition of apoptosis in tumor cells.

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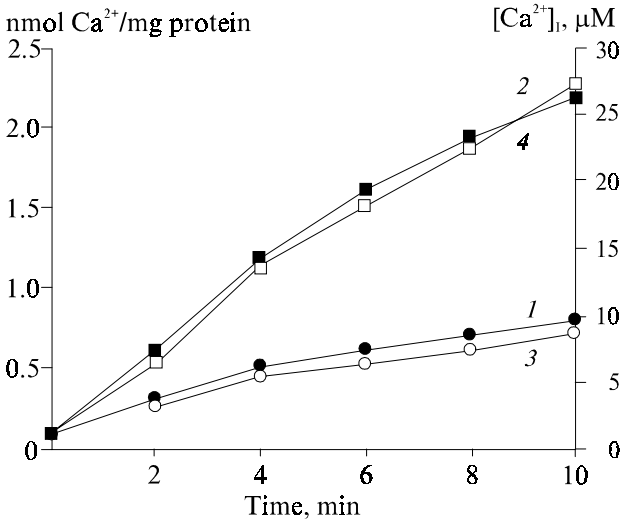

Fig. 4. Effect of metastasin (10 nM) on ATP-dependent Ca^{2+} accumulation in the fraction of plasma membranes of CSML-100 cells. *1, 3*) control, *2, 4*) experiment; *1, 2*) right, *3, 4*) left ordinate axes.

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