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Antizyme protects against abnormal accumulation and toxicity of polyamines in ornithine decarboxylase-overproducing cells

(polyamine transport/antizyme gene transfection)

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ABSTRACT **Exposure of ornithine decarboxylase (ODC; L-ornithine carboxy-lyase, EC 4.1.1.17)-overproducing mouse FM3A cells to micromolar levels of spermine or spermidine caused abnormal accumulation and toxicity of polyamines. This was apparently due to the inefficiency of negative feedback control of polyamine transport by polyamines in ODC-overproducing cells. Since antizyme is the only protein thus far recognized that can interact with ODC, depletion of free antizyme was regarded as the reason for the abnormal accumulation of polyamines. Accordingly, ODC-overproducing cells were transfected with pMAMneoZ1 possessing rat antizyme cDNA under the control of a glucocorticoid-inducible promoter. In the transfected cells, the addition of dexamethasone caused an increase in the amount of antizyme with an apparent molecular mass of 27 kDa, a decrease in the amount of ODC, a decrease in the polyamine transport activity, and the recovery of growth inhibition or cell death. The results indicate that antizyme can regulate not only the amount of ODC but also the activity of polyamine transport.**

Polyamines are essential for cell growth (1, 2). The polyamine content in cells is maintained by both polyamine biosynthesis and its transport. Ornithine decarboxylase (ODC; L-ornithine carboxy-lyase, EC 4.1.1.17) catalyzes the conversion of ornithine to putrescine, the first step of polyamine biosynthesis and a major regulatory site. The amount of ODC is regulated not only by various growth stimuli but also by polyamines themselves at several levels, namely transcription, translation, and enzyme degradation (3). It is also known that eukaryotic cells generally contain an inducible and saturable transport system that incorporates all three polyamines and that the K_m values for three polyamines are in the micromolar range (4). Decrease in polyamine content in cells caused by the inhibitors of polyamine biosynthesis leads to a marked increase in the velocity of polyamine uptake, apparently without affecting the affinities for the substrates (5, 6). Then, as polyamine content in cells increases, the uptake activity decreases. This feedback mechanism is not yet well understood.

Antizyme is known to be induced by polyamines and to inhibit ODC activity by forming an ODC–antizyme complex (7). It also became clear that antizyme is involved in the rapid degradation of ODC by 26S proteasome (8–13). Recently, it has been reported that ODC-overproducing cells easily accumulate polyamines under certain circumstances, and cell death occurs (14, 15). Furthermore, it has been suggested that antizyme may function as a negative feedback regulator of polyamine transport, since the polyamine-stimulated and unstable protein is involved in the regulation (16).

We isolated ODC-overproducing mouse FM3A cells termed EXOD-1 (17). Since exposure of these ODC-overproducing cells to micromolar levels of spermine or spermidine caused abnormal accumulation and toxicity of polyamines and since overproduction of ODC may deplete antizyme levels, we tried to determine if antizyme is a negative regulator of polyamine transport that inhibits the overaccumulation of polyamines, using EXOD-1 cells transfected with antizyme cDNA. In fact, it was clearly demonstrated that antizyme negatively regulates polyamine uptake.

MATERIALS AND METHODS

Cell Culture and Transfection. ODC-overproducing mouse FM3A cells (EXOD-1) were previously isolated as described (17). The cells (2×10^4 cells per ml) were cultured in ES medium (Nissui Pharmaceutical, Tokyo) supplemented with 50 units of streptomycin and 100 units of penicillin G per ml and 2% (vol/vol) heat-inactivated fetal calf serum (FCS) at 37°C in an atmosphere containing 5% CO_2. The Mg^{2+} concentration in the medium was adjusted to 0.9 mM, since Mg^{2+} has been found to influence polyamine transport (18). pMAMneoZ1, possessing the majority (93%) of the antizyme coding region downstream from the promoter of the dexamethasone-inducible mouse mammary tumor virus long terminal repeat, was prepared as described (12). Transfection of EXOD-1 cells with pMAMneoZ1 was performed by the calcium phosphate coprecipitation method according to the manufacturer's instructions (Stratagene) with DNA at 20 μg/ml per 2.5×10^6 cells on a 10-cm plate, and cells were cultured overnight in ES medium containing 0.5% FCS and the above antibiotics. Cells were cultured further in ES medium containing 2% FCS and the antibiotics for 24 h. Cell lines were cloned from foci isolated after additional growth for 10 days in 0.5% soft agar containing ES medium, the antibiotics, 5% FCS, 5 mM α-difluoromethylornithine (DFMO), and 1 mg of G418 (geneticin) per ml, and ≈80 clones containing pMAMneoZ1 were isolated. ODC-overproducing EXOD-1 cells and a pMAMneoZ1 transfectant (AZ-2) were cultured in ES medium containing 2% FCS and 5 mM DFMO for 24 h in the presence and absence of 1 μM dexamethasone, and the effects of spermine and spermidine on cell growth were then examined in the presence of 1 mM aminoguanidine, an inhibitor of amine oxidase in serum (19). DFMO was kindly provided by Marion Merrell Dow (Cincinnati).

Assay for Polyamine Transport. After washing FM3A cells with NaCl buffer (135 mM NaCl/1 mM $MgCl_2$/2 mM $CaCl_2$/10 mM glucose/20 mM Hepes, pH adjusted to 7.2 with Tris), the polyamine transport activity was measured as described (6) with 2×10^6 cells and 5 μM [^{14}C]spermine or [^{14}C]spermidine. The amount of radioactivity in the cells was

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Abbreviation: ODC, ornithine decarboxylase.
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measured in 10 ml of Aquasol-2 (DuPont/NEN) after sonication with 1 ml of 1% SDS. Initial rates of the transport were measured by incubation of the reaction mixture for 10 min and the K_m values were calculated from Lineweaver–Burk plots. Protein was determined by the method of Lowry *et al.* (20).

Measurement of Polyamines, Mg^{2+}, and ATP. FM3A cells (6×10^6) were harvested and extracted with 0.3 ml of 0.2 M $HClO_4$. The supernatant thus obtained was used for the following assays. Polyamines (putrescine, spermidine, and spermine) were measured as described (21). Mg^{2+} was analyzed in the presence of strontium chloride (1 mg/ml) by means of atomic absorption spectrometry. ATP was measured by the luciferase enzyme system (22) after neutralization with 1 M KOH containing 50 mM K_2HPO_4. The rate of light emission was linearly related to the square of the ATP concentration.

Immunoblot (Western Blot) Analysis of ODC and Antizyme. Cell lysates containing 4 μg and 40 μg of protein were used for Western blot analysis of ODC and antizyme, respectively. Rabbit polyclonal antibodies for mouse FM3A ODC and recombinant Z1 antizyme were prepared as described (11, 23). Western blot analysis of ODC and antizyme was performed as described (24) with goat anti-rabbit antibody conjugated with horseradish peroxidase (Bio-Rad) and the ProtoBlot Western blot AP system (Promega), respectively.

Purification of Antizyme. Cells were homogenized with a buffer containing 10 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, and protease inhibitors [20 μM FUT-175, 6-amino-2-naphthyl-4-guanidobenzoate dihydrochloride (25), and 0.5 mM phenylmethylsulfonyl fluoride], and the homogenate was centrifuged at 150,000 × *g* for 30 min. The supernatant (2 mg of protein) was then applied to a 0.1-ml HO-101 Affi-Gel 10 column, an affinity column ligating anti-ODC antibody (26), and the column was washed extensively with the above buffer containing 1 M NaCl. Antizyme was coeluted with ODC by using 0.5 ml of the above buffer containing 3 M $MgCl_2$. Antizyme in the supernatant (40 μg of protein) and the ODC-bound antizyme (0.1-ml aliquot) were analyzed by Western blot as described above.

Identification of Expression of Antizyme cDNA by PCR. Complementary DNA was prepared according to the method of Gubler and Hoffman (27) with 5 μg of poly(A)-rich RNA isolated by chromatography on oligo(dT)-cellulose. First PCR was performed using two primers complementary to the sequence downstream of the promoter of pMAMneo vector (primer P1) and the sequence upstream of the simian virus 40 polyadenylylation region of pMAMneo vector (P2); the second PCR was carried out with two primers complementary to the 5′ side in the open reading frame of Z1 antizyme (P3) and the downstream region of the termination codon of Z1 antizyme (P4) (28). The nucleotide sequences of P1 to P4 were: P1, 5′-ACAGGGACCCTCGGATAAGTGACCC-3′; P2, 5′-TAGAGCTTTAAATCTCTGTAGGTAG-3′; P3, 5′-ACCATGCCGCTTCTTAGTCAGCACA-3′; P4, 5′-TAGAATTAAAGCCACCACTTGAGTG-3′.

RESULTS

Increase in Polyamine Toxicity in ODC-Overproducing EXOD-1 Cells. Since it has been reported that low concentrations of spermine or spermidine caused cell death in ODC-overproducing cells isolated from rat hepatoma cells (14) and from L1210 cells (15) under normal and hyposmotic conditions, respectively, we also examined whether or not our ODC-overproducing EXOD-1 cells isolated from mouse FM3A cells (17) become sensitive to polyamine toxicity. We recently reported that 1–2 mM spermine and 5–10 mM spermidine were toxic for parent FM3A cells because of their overaccumulation when cells were cultured in the presence of 0.9 mM Mg^{2+} (18). As shown in Fig. 1, 10 μM spermine or 30 μM spermidine inhibited the cell growth of EXOD-1 cells by ≈90% when they were cultured under the same conditions as for parent FM3A cells. Accordingly, sensitivity to polyamine toxicity of EXOD-1 cells increased 70- to 100-fold over the parent FM3A cells. Moreover, when the concentrations of spermine and spermidine in the medium were increased to 30 μM and 90 μM, respectively, a transient decrease in cell number was seen to occur on day 1.

Table 1 shows polyamine contents in EXOD-1 cells. The addition of 30 μM spermine or 90 μM spermidine to the medium resulted in their overaccumulation in cells. However, polyamine content in cells gradually decreased as their culture was continued. Although there was an initial time lag, the decrease in polyamine content eventually became nearly parallel with the recovery of cell growth.

The ATP content was seen to decrease upon the addition of spermine or spermidine (data not shown), as has been observed when cell growth is inhibited by polyamines or polyamine derivatives such as N^1,N^{12}-bis(ethyl)spermine (18, 29, 30). The ATP content then also recovered as cell culture was extended.

The polyamine transport activity of EXOD-1 cells treated with spermine and spermidine was then measured (Table 1). In the parent FM3A cells, spermidine transport activity decreased greatly from 10.7 to 1.37 pmol/min per mg of protein by the treatment of cells with 45 μM spermine for 48 h (29). Compared with the parent FM3A cells, both spermine and spermidine uptake activities of EXOD-1 cells were very high. With 12 h of culture, the inhibition of polyamine uptake activity by 30 μM spermine or 90 μM spermidine was weak (about 50–60% inhibition), but the uptake activity gradually decreased as the cell culture was extended. The K_m values of spermine for the transport with the parent FM3A cells and EXOD-1 cells were nearly equal; both were about 1.7 μM. When EXOD-1 cells were cultured with 10 μM spermine for 12 h, the uptake activities of spermine and spermidine were 32.4 and 47.1 pmol/min per mg of protein, respectively. The results indicate that polyamine toxicity increases in conjunction with polyamine uptake activity and also that negative control of the uptake activity by polyamines is weak in EXOD-1 cells.

Recovery by Antizymes from Overaccumulation and Toxicity of Spermine in EXOD-1 Cells. Since sensitivity to poly-

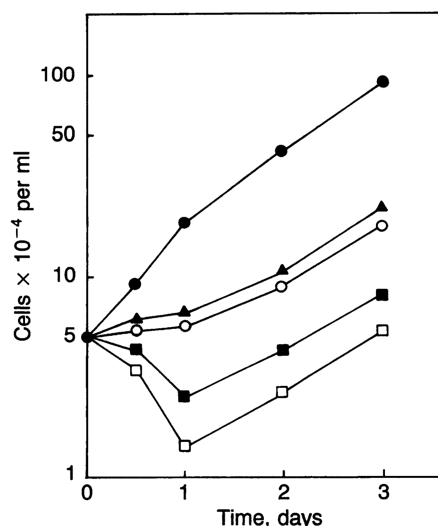

FIG. 1. Effect of spermine and spermidine on cell growth of ODC-overproducing EXOD-1 cells. Cells were cultured under standard conditions. ●, Control; ○, 10 μM spermine; □, 30 μM spermine; ▲, 30 μM spermidine; ■, 90 μM spermidine.

Table 1. Polyamine contents and its uptake activity in ODC-overproducing EXOD-1 cells

Culture conditions			Polyamine content, nmol/mg of protein			Polyamine uptake, pmol/min per mg of protein	
Time, h	Spermidine, μM	Spermine, μM	Putrescine	Spermidine	Spermine	Spermidine	Spermine
24	—	—	1.25	9.24	10.8	74.8	52.1
48	—	—	1.08	8.24	10.8	69.7	50.4
12	90	—	2.95	31.2	10.6	37.8	22.8
24	90	—	<0.1	24.0	10.2	26.2	15.4
48	90	—	<0.1	18.0	7.2	22.5	12.4
12	—	30	0.30	7.3	37.8	34.1	21.1
24	—	30	<0.1	5.4	30.4	25.7	13.4
48	—	30	<0.1	2.1	21.3	19.1	11.7

Each value is the average of three determinations. The standard error was within ±10% for each value.

amine toxicity increased 70- to 100-fold by ODC-overproduction in FM3A cells, and because antizyme is the only protein thus far identified that is induced by polyamines and can interact with ODC, we examined the possibility that antizyme is a negative regulator of polyamine transport. For this purpose, antizyme cDNA-transfected cells were isolated by using a truncated rat antizyme cDNA, Z1 (23). Approximately 80 transfectants were obtained from 2.5×10^6 cells, and they synthesized antizyme with an apparent molecular mass of 27 kDa, the same size as that of Z1 antizyme synthesized in rat hepatoma cells (9).

Dexamethasone strongly stimulated the synthesis of the 27-kDa antizyme in antizyme cDNA-transfected cells (AZ-2 cells) (Fig. 2*B*). It was also demonstrated that antizyme mRNA could be synthesized from an integrated plasmid DNA, pMAMneoZ1 (Fig. 2*A*). A PCR product of 1100 nt was obtained from cDNA and two primers with complementary sequences to the pMAMneo vector (lane 1). Then a PCR product of 700 nt was obtained from the PCR product in lane 1 by using two primers with complementary sequences for antizyme cDNA (lane 2). The sizes of the two products were as expected, indicating that antizyme mRNA synthesized from pMAMneoZ1 exists in AZ-2 cells.

Then we examined whether the 27-kDa antizyme behaves in a similar manner to the natural antizyme with an apparent molecular mass of 24 kDa, which has been reported previously (11). When total and ODC-bound antizymes in the cytosol (150,000 × *g* supernatant) were analyzed by Western blot analysis, the ratio of 27 kDa to 24 kDa in the two fractions (Fig. 2*C*) was almost the same as that in the cell lysate (Fig. 2*B*). The results suggest that the 27-kDa antizyme has almost the same affinity for ODC as the natural 24-kDa antizyme.

Next, we examined whether or not antizyme can restore cell growth and viability. Sensitivity to spermine was slightly different in the EXOD-1 and AZ-2 cells in the absence of dexamethasone. Partial cell death of EXOD-1 was observed with 30 μM spermine (Fig. 1), and that of AZ-2 cells was observed with 10 μM spermine (Fig. 3). Expression of antizyme by the addition of dexamethasone clearly prevented the death of AZ-2 cells caused by spermine (Fig. 3). To get clear results, it was necessary to preincubate the cells with dexamethasone for 24 h. On the other hand, the addition of dexamethasone did not influence the amount of antizyme in EXOD-1 cells (Fig. 2*B*) nor the growth inhibition or death of cells caused by spermine (data not shown). AZ-2 cells pretreated with dexamethasone for 24 h to induce antizyme synthesis increased the amount of 27-kDa antizyme (arrow in Fig. 4), and the amount of ODC decreased by 30% (first and second lanes in Fig. 4). AZ-2 cells treated with 10 μM spermine for 12 h stimulated the synthesis of 24-kDa antizyme (asterisk in Fig. 4), and the amount of ODC decreased by 80% (third to sixth lanes in Fig. 4). Similar results were obtained when cells were treated with spermine for 24 h, although the amount of ODC was slightly greater.

By the pretreatment of AZ-2 cells with dexamethasone for 24 h, polyamine uptake activity decreased by 50–60% (Table

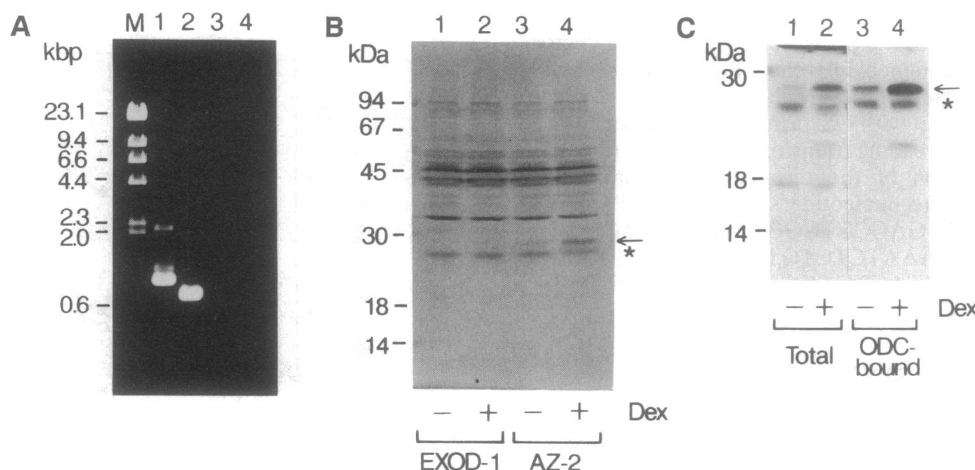

FIG. 2. Dexamethasone-dependent expression of the 27-kDa antizyme. (*A*) PCR product. Lanes: 1, PCR product from cDNA and two primers (P1 and P2) having complementary sequence to the pMAMneo vector; 2, PCR product from the lane 1 product and two primers (P3 and P4) having complementary sequence to antizyme cDNA; 3, PCR product from the lane 1 product and one primer (P3); 4, PCR product from the lane 1 product and the other primer (P4). (*B*) Western blot analysis of antizymes in cell lysate. EXOD-1 (lanes 1 and 2) and AZ-2 (lanes 3 and 4) cells were cultured in the absence (lanes −) and presence (lanes +) of 1 μM dexamethasone for 24 h. Arrow and asterisk indicate the 27-kDa and 24-kDa antizymes, respectively. (*C*) Western blot analysis of antizymes in cytosol (150,000 × *g* supernatant). The cytosol was prepared from the cell lysate in *B*. ODC-bound antizymes were prepared as described in *Materials and Methods*. Total (lanes 1 and 2) and ODC-bound (lanes 3 and 4) antizymes in the cytosol of AZ-2 cells cultured without (lanes −) or with (lanes +) 1 μM dexamethasone, respectively.

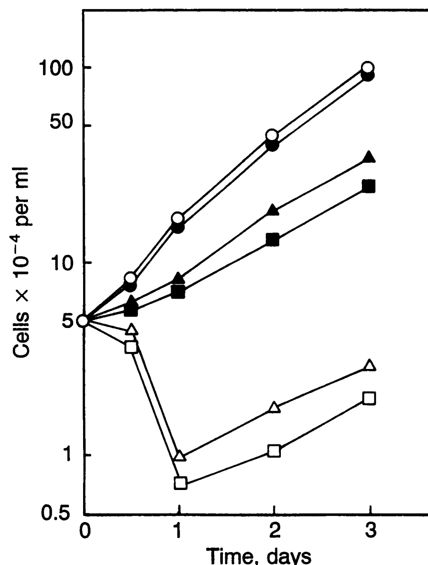

FIG. 3. Effect of spermine on cell growth of AZ-2 cells. Cells were cultured under standard conditions. ○, Control; ●, 1 μM dexamethasone; △, 10 μM spermine; ▲, 10 μM spermine and 1 μM dexamethasone; □, 30 μM spermine; ■, 30 μM spermine and 1 μM dexamethasone. Where dexamethasone was used, cells were pretreated with 1 μM for 24 h to induce antizyme synthesis.

2). Under these conditions, the K_m value of spermine (1.7 μM) for the transport did not change significantly. When AZ-2 cells were treated with spermine for 12 h together with dexamethasone, natural 24-kDa antizyme synthesis was induced (Fig. 4), and polyamine uptake activity was strongly suppressed (to about 30% of control) (Table 2). The accumulation of spermine in AZ-2 cells was less when cultured with than without dexamethasone, in concert with polyamine uptake activity (Table 2). From then, the uptake activity of AZ-2 cells in the presence of spermine gradually decreased with the spermine-dependent synthesis of natural 24-kDa antizyme. This was followed by the decrease in spermine accumulation (Table 2) and the restoration of the growth of cells cultured without dexamethasone from day 2 (Fig. 3).

The results taken together indicate that antizyme prevents cell death caused by polyamine overaccumulation through the inhibition of polyamine transport.

DISCUSSION

The mechanism(s) for hypersensitivity of EXOD-1 to polyamines was investigated. Growth inhibition was correlated with increase in polyamine transport activity. As antizyme is known to interact with ODC, we examined the possibility that ODC and polyamine transport protein may compete for the binding with antizyme. For this purpose, pMAMneoZ1, possessing the majority (93%) of the rat antizyme coding region, was transfected into EXOD-1 cells, and antizyme cDNA-transfected cells thus obtained (AZ-2 cells) were used for the experiments. When AZ-2 cells were cultured in the presence of 1 μM dexamethasone, the amount of antizyme increased (Fig. 2) and polyamine transport was inhibited (Table 2). In addition, the polyamine transport activity of AZ-2 cells gradually decreased with the addition of spermine, in conjunction with the spermine-dependent synthesis of the natural 24-kDa antizyme, and cell growth recovered. These results, taken together, indicate that antizyme inhibits polyamine transport. Therefore, the synthesis of antizyme protects cells from their growth inhibition or death caused by the overaccumulation of spermine.

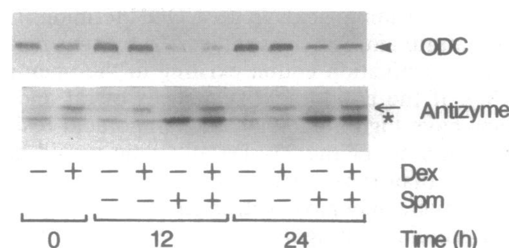

FIG. 4. Western blot analysis of ODC and antizymes. AZ-2 cells were cultured in the presence (+) and absence (−) of 10 μM spermine (Spm) and 1 μM dexamethasone (Dex) as shown. Dexamethasone "+" indicates that cells were pretreated with 1 μM dexamethasone for 24 h to induce antizyme synthesis. Arrowhead, arrow, and asterisk indicate ODC and the 27-kDa and 24-kDa antizymes, respectively.

The molar ratio of ODC to antizyme in AZ-2 cells was estimated by Western blot analysis of both proteins using reagents with different sensitivities and by the staining of ODC and antizymes with Coomassie brilliant blue R-250 after the elution of ODC–antizyme complex from anti-ODC affinity column chromatography. The ratio was estimated to be greater than 20:1. If antizyme competes for a binding site between ODC and the polyamine transport protein, it may have stronger affinity for the latter. Another possibility may be that the binding site of antizyme to ODC is different from that to the polyamine transport protein.

The activity of the 27-kDa antizyme in the degradation of ODC seems less effective than that of the 24-kDa antizyme (Fig. 4). The antizyme synthesized from pMAMneoZ1 (27-kDa antizyme) is identical to rat natural antizyme only in a

Table 2. Polyamine contents and its uptake activity in the antizyme cDNA transfectant AZ-2 cells

Dex pretreatment	Culture		Polyamine content, nmol/mg of protein			Polyamine uptake, pmol/min per mg of protein	
	Spermine	Time, h	Putrescine	Spermidine	Spermine	Spermidine	Spermine
−	−	0	0.50	18.7	8.8	84.4	66.2
+	−	0	<0.1	14.5	6.8	42.3	25.3
−	−	12	0.25	15.8	10.8	78.6	52.7
−	+	12	0.30	10.3	33.1	37.3	26.5
−	+	24	0.20	7.8	30.6	28.5	20.3
−	+	48	0.10	3.9	20.9	16.7	11.7
+	−	12	<0.1	13.6	10.7	44.9	26.2
+	+	12	0.10	12.2	18.3	29.4	20.8
+	+	24	0.10	6.8	17.8	25.5	18.2
+	+	48	0.10	3.5	15.4	14.5	10.7

Dexamethasone (Dex) "+" indicates that cells were pretreated with 1 μM dexamethasone for 24 h to induce antizyme synthesis. Where indicated, 10 μM spermine was added to the medium. Each value is the average of three determinations. The standard error was within ±10% for each value.

sequence of 159 amino acids in the COOH terminus (23, 31), since rat natural antizyme is synthesized by causing a frame shift at the termination codon existing in the open reading frame (32). If the amino acid sequences of rat and mouse antizymes have high homology, the amino acid residues in the NH_2 terminus of the 27-kDa antizyme would be more hydrophobic than those of the 24-kDa antizyme. Thus, the 27-kDa antizyme may have a stronger affinity for polyamine transport protein existing in membranes than for ODC.

There were time lags between the synthesis of antizyme, the decrease in polyamine transport activity, and the recovery of cell growth (Figs. 3 and 4 and Table 2). Since large amounts of ODC exist in AZ-2 cells, antizyme synthesized at the early period may necessarily bind to ODC, even though it has a stronger affinity for polyamine transport protein, and then bind to the transport protein later. It may also take a longer time period to synthesize proteins that are necessary for cell growth after the decrease of polyamine content in cells.

We have thus far discussed the findings that antizyme interacts directly with polyamine transport protein and affects the rate of polyamine uptake without influencing the K_m values of polyamines for the transport protein when the molar ratio of antizyme to ODC increases. However, the possibility that antizyme also acts in some indirect manner on polyamine transport cannot be ruled out. For example, antizyme may be involved in inactivation of a postulated intracellular polyamine-binding protein that holds polyamines in the cells once they have entered via the transporter.

Although the experiments were performed with AZ-2 cells cultured in the presence of DFMO, which provides stability and ensures reliable analysis of the amounts of ODC and antizymes, essentially the same results were obtained with AZ-2 cells cultured in the absence of DFMO (data not shown).

Our results clearly indicate that antizyme has dual functions—one for ODC degradation (9–13) and the other for negative regulation of polyamine transport.

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