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ANTIZYME DELAYS THE RESTORATION BY SPERMINE OF GROWTH OF POLYAMINE-DEFICIENT CELLS THROUGH ITS NEGATIVE REGULATION OF POLYAMINE TRANSPORT

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SUMMARY: Effects of antizyme on polyamine transport and spermine restoration of the growth of polyamine-deficient cells were examined by using mouse FM3A cells transfected with pMAMneoZ1 possessing rat antizyme cDNA under the control of glucocorticoid-inducible promoter. Treatments of the transfected cells with α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase (ODC), and dexamethasone, an inducer of antizyme, both caused a decrease in ODC activity and polyamine contents and inhibition of cell growth. However, spermine uptake of the transfected cells was repressed by dexamethasone but stimulated by DFMO. The decrease in the rate of spermine uptake in dexamethasone-treated cells was attributed to an increase in K_m value and a slight decrease in V_{max} value. Accordingly, restoration of cell growth by spermine was less effective in dexamethasone-treated cells than DFMO-treated cells. These results clearly indicate that antizyme has dual functions: one for ODC degradation and the other for negative regulation 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. Since excessive polyamines are toxic for cells (3–7), both polyamine biosynthesis and its transport are negatively regulated by polyamines. Antizyme is known to be induced by polyamines and to inhibit the activity of ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine biosynthesis, by forming an antizyme-ODC complex (8). It has also become clear that antizyme is involved in the rapid degradation of ODC by 26S proteasome (9–13). Therefore, antizyme plays an important role in the negative feedback regulation of polyamine biosynthesis.

Recently, it has been reported that ODC-overproducing cells easily accumulate polyamines under certain circumstances, and cell death occurs (5, 7, 14). Furthermore, it has been suggested that antizyme may function as a negative feedback regulator of polyamine transport, as the polyamine-stimulated and unstable protein is involved in the regulation (15). Soon after, it was also demonstrated that antizyme negatively regulates polyamine transport (14, 16), and the abnormal accumulation of polyamines, which is a specific character of ODC-overproducing cells, was restored to a normal level by antizyme (14). On the contrary, antizyme inhibited the

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growth of normal cells by making the cells polyamine-deficient through ODC degradation (17). In this study, we examined whether or not antizyme delays the restoration by spermine of polyamine-deficient cells through its negative regulation of polyamine transport.

MATERIALS AND METHODS

Cell Culture and Transfection — Established mouse mammary carcinoma FM3A cell lines (N1) were kindly donated by Dr. H. Matsuzaki (Saitama University, Japan). The N1 cells (1 x 10^4 cells/ml) were cultured in ES medium supplemented with 50 U/ml streptomycin, 100 U/ml penicillin G and 2% heat-inactivated fetal calf serum at 37°C in an atmosphere of 5% CO_2. pMAMneoZ1, possessing the majority (93%) of the antizyme coding region downstream from the dexamethasone-inducible mouse mammary tumor virus long terminal repeat promoter, was kindly supplied by Drs. Y. Murakami and S. Hayashi (Jikei University School of Medicine, Japan). Transfection of N1 cells with pMAMneoZ1 was performed according to the method described previously (14). The pMAMneoZ1 transfectant (FZ10) thus obtained was also cultured as described above except that 1 mg/ml G418 (geneticin) was added to the medium. When spermine was added to the medium, 1 mM aminoguanidine, an inhibitor of amine oxidase in serum (18), was also added. α-difluoromethylornithine (DFMO), an inhibitor of ODC, kindly provided by Marion Merrell Dow Inc., was used for making the cells polyamine-deficient.

Assays for Polyamine Transport, ODC and Antizyme — After washing FZ10 cells with NaCl buffer (135 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 20 mM Hepes/Tris, pH 7.2), the spermine transport activity was measured according to the previous report (19) using 2 x 10^6 cells and 5 μM [^{14}C]spermine. The amount of radioactivity in the cells was measured in 10 ml Triton/toluene scintillant after sonication with 1 ml 1% SDS. Initial rates of the transport were measured by incubation of the reaction mixture for 3, 6 and 10 min, and the K_m and V_{max} values were calculated from Lineweaver-Burk plots. ODC and antizyme activities were measured according to the methods of He *et al.* (6) and Matsufuji *et al.* (20), respectively. Protein was determined by the method of Lowry *et al.* (21).

Measurement of Polyamines — FM3A cells (6 x 10^6) were harvested and extracted with 0.3 ml of 0.2 M $HClO_4$. The supernatant thus obtained was used for the polyamine assay. Polyamines were analyzed with a Toyo Soda high-performance liquid chromatography system as described previously (22).

Western Blot Analysis of Antizyme and ODC — Cell lysate containing 40 μg protein was used for the analysis of antizyme. ODC purified according to the method of Kanamoto *et al.* (11) from cell lysate containing 2 mg protein was used for the analysis of ODC. Rabbit polyclonal antibodies for mouse FM3A ODC (11) and recombinant Z1 antizyme (20) were kind gifts from Drs. Y. Murakami and S. Hayashi. Western blot analysis was performed as described (23) using the ProBlot Western Blot AP system (Promega).

RESULTS

Degradation of ODC and Inhibition of Polyamine Uptake by Antizyme in FM3A Cells — We have recently found that antizyme protects against abnormal accumulation and toxicity of polyamines in mouse FM3A ODC-overproducing cells, in which the polyamine transport system is activated (14). Thus, we next examined whether or not antizyme can inhibit polyamine transport in normal FM3A cells. Eukaryotic cells generally contain an inducible and saturable transport system that incorporates all three polyamines (24). As shown in Fig. 1A, antizyme was induced by dexamethasone in antizyme cDNA-transfected (FZ10) cells. Under these conditions, ODC was completely degraded (Fig. 1B). Since 40 μg and 2 mg protein of

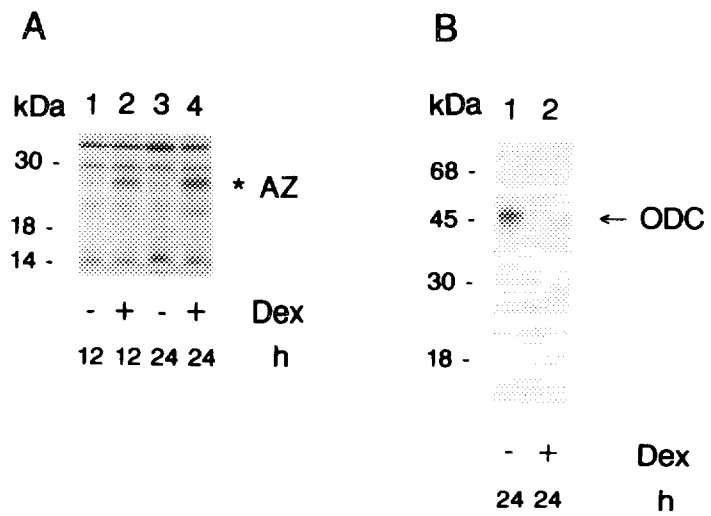

Fig. 1. Western blot analysis of antizyme (A) and ODC (B) in cell lysate. FZ10 cells were cultured in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of dexamethasone for 12 and 24 h, as indicated. Asterisk and arrow indicate antizyme and ODC, respectively. The numbers on the left represent molecular mass in kDa.

cell lysate were used for the detection of antizyme and ODC, respectively, antizyme probably exists in a larger amount than ODC.

As shown in Fig. 2, ODC activity was completely lost at 24 h after addition of dexamethasone, and spermine uptake decreased in parallel, but only by about 60%. Of particular interest here is that, although no significant amount of ODC existed in cells, the spermine transport was only partially inhibited. In the absence of dexamethasone, a very small amount of antizyme may be synthesized since a faint band was observed at the same position as antizyme (Fig. 1A), and ODC and spermine uptake activities were slightly decreased (Fig. 2).

ODC activity of FZ10 cells cultured without dexamethasone was about 8 units/mg protein, while antizyme activity of the cells cultured with dexamethasone was approximately 160 units/mg protein when these activities were defined according to the previous publication (20). The results also support the notion that antizyme exists in a larger amount than ODC in FZ10 cells.

Delay of the Restoration of the Growth of Antizyme-Caused Polyamine Deficient Cells by Spermine — We then compared the restoration by spermine of the growth of polyamine-deficient cells caused by antizyme and DFMO. As shown in Fig. 3, both 1 μM dexamethasone and 50 μM DFMO inhibited the growth of antizyme cDNA-transfected (FZ10) cells. Dexamethasone did not inhibit the growth of normal FM3A (N1) cells. When the growths of N1 and FZ10 cells were compared, that of the former was seen to be faster. This may be related to the synthesis of the small amount of antizyme in FZ10 cells cultured in the absence of dexamethasone.

The inhibition of cell growth occurred in parallel with the decrease in polyamine content. The addition of dexamethasone and DFMO caused a great decrease in putrescine and

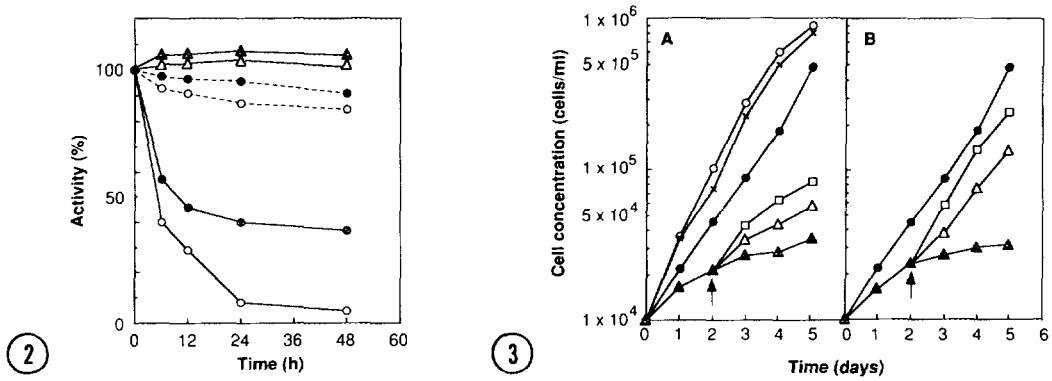

Fig. 2. Spermine transport and ODC activities of FM3A cells. Assays were performed as described in Materials and Methods. Each value is the average of duplicate determinations. ●—● And ●---●, spermine uptake activities of FZ10 cells cultured in the presence and absence of dexamethasone, respectively (100% activity, 42.1 pmol/min/mg protein); ○—○ and ○---○, ODC activities of FZ10 cells cultured in the presence and absence of dexamethasone, respectively (100% activity, 132 pmol/min/mg protein); ▲—▲ and Δ—Δ, spermine uptake (100%, 43.8 pmol/min/mg protein) and ODC (100%, 148 pmol/min/mg protein) activities of N1 cells, respectively.

Fig. 3. Recovery of cell growth by spermine of polyamine-deficient cells caused by dexamethasone (A) and DFMO (B). Each value is the average of three determinations. Standard error was within ±10% for each point. ○—○ and ×—×, growth curves of N1 cells cultured in the presence and absence of dexamethasone, respectively; ▲—▲ and ●—●, growth curves of FZ10 cells cultured in the presence and absence of dexamethasone (A) and DFMO (B), respectively; Δ—Δ and □—□, 1 and 3 μM spermine, respectively, were added together with 1 mM aminoguanidine on day 2 as shown by arrows.

spermidine and a slight decrease in spermine (Table I). Spermine rapidly restored the growth of DFMO-treated cells (Fig. 3), and spermine content in the cells increased by 1.6- and 2.1-fold at 24 h after the addition of 1 and 3 μM spermine, respectively (Table I). However, the addition of spermine to dexamethasone-treated cells only slowly restored the cell growth (Fig. 3), and the increase in spermine content in the cells at 24 h after the addition of 1 and 3 μM spermine was only 1.1- and 1.2-fold, respectively.

Spermine uptake activity was then measured. DFMO increased the uptake activity by 1.3-fold. Thus, the difference in the rate of spermine uptake between DFMO- and dexamethasone-treated cells was approximately 2.6-fold when 5 μM spermine was used as substrate (Fig. 4). However, the K_m values for spermine of DFMO- and dexamethasone-treated cells were 1.5 and 5.6 μM, respectively. It has been reported that the K_m value for spermine was not influenced by DFMO (19). The results suggest that antizyme decreases the affinity of spermine to polyamine transport protein.

DISCUSSION

Negative regulation of polyamine transport by antizyme was previously demonstrated with mouse FM3A ODC-overproducing cells (14) and DFMO-treated rat hepatoma cells (16) transfected with antizyme cDNA. Furthermore, correlation between a decrease in polyamine

Table I. Polyamine contents in antizyme cDNA transfectant, FZ10 cells

Culture conditions		Polyamine content		
Treatment (μM)	Time (h)	Putrescine	Spermidine (nmol/min/mg protein)	Spermine
Control	48	3.95 ± 0.41	11.9 ± 1.4	11.1 ± 1.8
	72	3.09 ± 0.38	7.23 ± 0.74	7.67 ± 0.79
DFMO (50)	48	<0.1	1.05 ± 0.38	7.21 ± 0.95
	72	<0.1	0.78 ± 0.22	6.12 ± 0.42
DFMO (50) + Spermine (1)	72	<0.1	0.60 ± 0.14	11.2 ± 1.1
DFMO (50) + Spermine (3)	72	<0.1	0.52 ± 0.12	14.8 ± 1.8
DEX (1)	48	0.78 ± 0.15	3.25 ± 0.34	7.53 ± 0.57
	72	<0.1	1.52 ± 0.12	6.03 ± 0.31
DEX (1) + Spermine (1)	72	<0.1	1.82 ± 0.25	6.58 ± 0.85
DEX (1) + Spermine (3)	72	<0.1	1.47 ± 0.23	7.12 ± 0.91

The values are expressed as the mean ± S.D. for three determinations. Spermine was added 48 h after the addition of DFMO and dexamethasone. DEX, dexamethasone.

transport activity and the induction of antizyme by spermidine was shown using stable-ODC producing rat hepatoma (HMOA) cells and ODC-deficient Chinese-hamster ovary (C55.7) cells (16). In the latter case, however, we cannot judge whether the decrease in polyamine transport activity by the accumulation of spermidine in cells totally depends on the induction of antizyme or not.

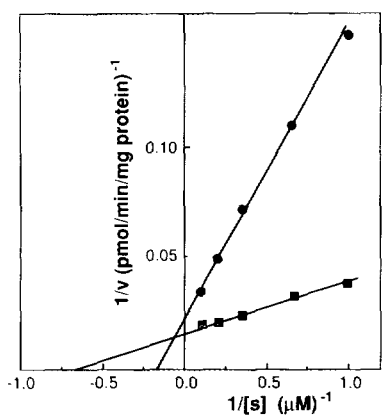

Fig. 4. Lineweaver-Burk plots of spermine uptake by FZ10 cells. Each value is the average of duplicate determinations. ■ , + DFMO (1.5 μM for K_m and 66.7 pmol/min/mg protein for V_{max}); ● , + dexamethasone (5.6 μM for K_m and 43.5 pmol/min/mg protein for V_{max}).

In this study, we tried to clarify whether antizyme functions as a negative regulator of polyamine transport in "normal' cells, in which ODC exists at an ordinary level. Thus, antizyme cDNA was transfected to FM3A cells. Although the amount of antizyme induced was less in FM3A cells than in ODC-overproducing FM3A cells, antizyme probably exists in a larger amount than ODC (Fig. 1). Under these conditions, the induction of antizyme by dexamethasone caused the inhibition of cell growth through the degradation of ODC, which confirmed the results reported by Murakami *et al.* (17). In addition, spermine transport activity was depressed by antizyme in spite of the fact that polyamine contents were decreased significantly. Accordingly, restoration of cell growth by spermine was less effective in dexamethasone-treated cells than in DFMO-treated cells.

We also examined whether antizyme protects against overaccumulation and toxicity of polyamines in normal FM3A cells like in ODC-overproducing cells (14). The concentration of spermine in the medium necessary to cause toxicity in normal and ODC-overproducing cells was 2 mM and 10 μM spermine, respectively (6, 14). The toxicity of normal FM3A cells by spermine was also reduced by antizyme (data not shown). The results taken together indicate that antizyme has dual functions: one for ODC degradation and the other for negative regulation of polyamine transport, regardless of the conditions. The mechanism of the inhibition of polyamine transport by antizyme is now under investigation. Since the decrease in spermine transport by antizyme was mainly due to the increase of K_m value for spermine, the possibility that the degradation of polyamine transport protein by antizyme is accelerated is minimal.

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