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Arginine Deiminase Inhibits Cell Proliferation by Arresting Cell Cycle and Inducing Apoptosis

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We have previously demonstrated that arginine deiminase inhibits the proliferation of vascular endothelial cells, but the mechanisms leading to growth inhibition have remained unclear. We report here that low concentrations of arginine deiminase purified from *Mycoplasma arginini* inhibit proliferation of various cultured cells by arresting the cell cycle in G_1 and/or S phase with higher arginine deiminase concentrations leading to subsequent apoptosis. Our results demonstrate that arginine deiminase inhibits cell proliferation not only by depletion of arginine, but also by mechanisms involving the cell cycle and death signals.

Probably the best-known and clinically most important growth-inhibitory enzyme is asparaginase. It is used successfully for the treatment of acute lymphatic leukemia and certain solid malignancies. However, asparaginase treatment is accompanied by serious side effects including anaphylactic shock, coagulopathies as well as liver and pancreatic toxicity (1). These properties outline the need for alternative treatments.

We have previously found a growth-inhibitory protein in supernatants of cultured cells contaminated by mycoplasmas. By purifying and sequencing this protein, we were able to demonstrate that it was identical with mycoplasma-derived arginine deiminase. The purified enzyme inhibited proliferation of vascular endothelial cells in a potent manner (2). Our findings were confirmed by others which had shown that arginine deiminase inhibits the proliferation of various malignant cells in culture (3–5) and of malignant tumor growth in vivo (6). Arginine deiminase appears to have few systemic side effects (6) suggesting that it may have clinical potential as an inhibitor of tumor angiogenesis and tumor growth.

Arginine deiminase can act by converting the nonessential amino acid arginine into citrulline. The resulting depletion of arginine in cultured cells or in the living organism was thought to be solely responsible for its growth-inhibitory activity (4). However, data of our own preliminary experiments suggested that arginine deiminase may act through alternative mechanisms, i.e., by interfering with the cell cycle. The latter consists of the DNA synthesis (S) and mitotic (M) phases separated by the gap (G) phases where G_2 follows S phase. Following M phase, cells can either enter G_0 phase and then remain quiescent or they can enter G_1 and subsequently S phase. Within S phase, cells can chose either to proceed cycling or to stop proliferating without cell cycle completion. Thus, they become quiescent S_0 cells, i.e. cells with an S phase DNA content, but not incorporating 5-bromo-2′-deoxyuridine (BrdU) or other DNA precursors (7). Previous studies have shown that the cell cycle is intricately related to the induction of cell death (8). The present study was therefore initiated to determine whether arginine deiminase could modulate the cell cycle and/or signals associated with cell death.

MATERIALS AND METHODS

Cells. *Mycoplasma arginini* (ATCC 23838) was purchased from the American Type Culture Collection and cultured aerobically for 72 h at 37°C in 2 l of Bacto PPLO broth w/o CV (pH 7.0) (Difco) containing L-arginine (1%), horse serum (20%) (Biochrom, Germany), yeast extract (2.5%) (Difco) and Penicilline G (2000 U/ml). Sources and maintenance of cells and cell lines were as mentioned earlier (2, 9, 10).

Purification. Mycoplasmas were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.0), suspended in 24 ml of potassium phosphate buffer (10 mM, pH 7.0) and sonicated for 15 min in an ice bath. The suspension was centrifuged (1 h at 100,000 g and 4°C) and the supernatants were applied to an anion exchange column (Q-Sepharose Fast Flow, Pharmacia, Sweden) of 1 × 4 cm

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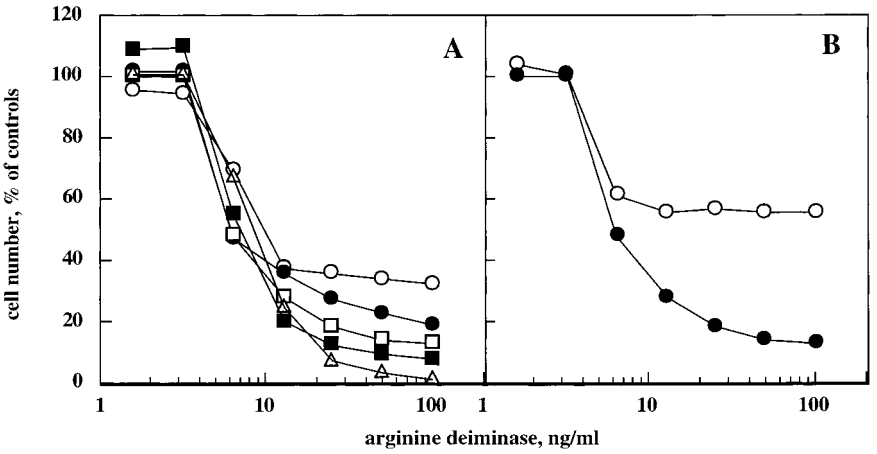

FIG. 1. Effect of increasing concentrations of arginine deiminase on the proliferation of cultured cells. (A) Cells derived from human umbilical vein (HUVE) (○), osteosarcoma (SaOS) (●), neuroblastoma (SH-EP, WAC2) (□, △), and retinoblastoma (Y-79) (■) were seeded in arginine-containing medium. (B) SH-EP cells were seeded in arginine-free (○) or arginine-containing (●) medium. (A and B) Cells received the indicated concentrations of arginine deiminase and were counted after 3 days as outlined under Materials and Methods. Results are expressed as a percentage of cells that had not received arginine deiminase and are the average of triplicate determinations which varied by less than 5% of the means.

size equilibrated with potassium phosphate buffer (10 mM, pH 7.0). The column was washed with the same buffer and then eluted with the buffer containing a linear gradient of 0.0 to 0.5 M NaCl using a flow rate of 20 ml/h. The active fractions were pooled and applied to an arginine-Sepharose 4B column (Pharmacia) of 1 × 4 cm size which had been equilibrated with the potassium phosphate buffer. The column was washed with the buffer and eluted with the buffer containing a linear 0.0 to 1.0 M NaCl gradient. All purification steps were carried out at 4°C. Analysis of the active fractions revealed a single protein band of 45 kDa as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining thereby confirming the purity of arginine deiminase.

Arginine deiminase determination. Fractions of interest were examined for arginine deiminase activity as described (3) where one unit of activity was defined as the amount of enzyme able to convert 1 μmole of L-arginine to citrulline per minute in the assay conditions.

Arginine determination. Arginine was quantified using an LC 3000 amino acid analyzer (Eppendorf, Germany).

Cell proliferation assay. Cells were seeded in triplicate into 24-well multi-well plates. Each well received 1×10^4 cells in 0.5 ml of the respective medium containing various concentrations of purified arginine deiminase or buffer only (controls). Human umbilical vein endothelial (HUVE) cells were stimulated to proliferate by adding basic fibroblast growth factor (bFGF, 1 ng/ml) every other day, while tumor cells did not receive bFGF. Cells were counted after 3 days in culture using a Coulter particle counter (2, 9, 10).

Cell cycle analysis. Cells were seeded at a density of 5×10^5 into 10-cm dishes. After 4 h, cells received purified arginine deiminase (50 ng/ml) or buffer only (controls). After 3 d, they were incubated with BrdU (10 μM, Sigma) for 30 min and harvested. Nuclei were prepared, incubated with mouse anti-BrdU IgG (Becton Dickinson) and subsequently with FITC-conjugated goat anti-mouse IgG (DuPont). They were then stained with propidium iodide. Green (FITC) and red (PI) fluorescence after laser excitation at 488 nm were recorded with a FACScan flow cytometer (Becton Dickinson) and plotted in two-parameter histograms. Cell cycle fractions were determined using windows in the scattergrams (7).

Apoptosis assays. For the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method, cells were seeded at a density of 1×10^4/well onto 4-well culture slides (Falcon). After 12 h, they received arginine deiminase (200 ng/ml) or buffer only (controls), and were incubated for 3 days. Dead cells in the supernatants were collected by centrifugation, air-dried and fixed on slides with paraformaldehyde (4% in PBS, pH 7.0). Slides were rinsed, permeabilized for 2 min at 21°C in 0.1% sodium citrate containing 0.1% Triton-X 100 and washed twice with PBS. Semi-dry slides were incubated in a humidified chamber for 1 h at 37°C with 40 μl of TUNEL mixture (Boehringer Mannheim, Germany).

For nuclear staining, cells labeled by the TUNEL assay were stained by incubation for 20 min at 21°C with 4′-6-diamidino-2-phenylindole (DAPI, 2 μg/ml (Sigma)), rinsed with PBS, exposed to 20 μl of anti-fade medium (DABCO (Sigma), 2.5% (w/v) in PBS containing 50% glycerol), sealed with a cover slip and evaluated under a fluorescence microscope.

RESULTS

Arginine deiminase from *Mycoplasma arginini* was purified to homogeneity, as revealed by a single protein band obtained after analysis of the final product by SDS-PAGE and Coomassie staining (data not shown). Figure 1A demonstrates that arginine deiminase inhibited the proliferation of various cultured cells, including normal vascular endothelial (HUVE), and neoplastic cells derived from neuroblastoma (SH-EP, WAC2), retinoblastoma (Y-79) and osteosarcoma (SaOS) in a potent manner with half-maximal inhibition achieved with concentrations of ~5 to 10 ng/ml.

To investigate whether arginine deiminase inhibited cell proliferation by simple depletion of arginine from the culture medium or whether there were other mechanisms involved, we prepared medium that either contained or completely lacked arginine, respectively, as verified by amino acid analysis of the medium. Figure 1B demonstrates that arginine deiminase inhibited SH-EP cell proliferation in arginine-containing medium, but

TABLE 1

Effect of Arginine Deiminase on Cell Cycle Distribution in SH-EP, HUVE, WAC2, SaOS, and Y-79 Cells

	Controls				Treatment with arginine deiminase			
Cell line	S (%)	G_1 (%)	G_2 (%)	S_0 (%)	S (%)	G_1 (%)	G_2 (%)	S_0 (%)
SH-EP*	8.07	62.56	14.57	14.80	0.92	86.90	9.12	3.06
SH-EP	32.88	44.58	18.61	3.93	1.44	88.94	6.25	3.37
HUVE	20.15	47.76	24.28	7.81	1.36	58.92	34.39	5.31
WAC2	21.95	57.92	17.73	2.40	6.10	57.13	14.05	22.73
SaOS	20.15	38.43	21.75	5.45	22.71	37.71	14.71	24.86
Y-79	51.44	15.93	23.11	9.53	1.91	59.17	17.34	21.58

Note. Cells were either not treated (controls) or treated with 50 ng/ml of arginine deiminase for 3 days in arginine-containing medium (except for SH-EP cells marked with an asterisk which were treated in arginine-free medium). Results are expressed as a percentage of the total cell population in different compartments of the cell cycle and are the average of triplicate determinations which varied by less than 5% of the means.

also, though somewhat weaker, in the arginine-free medium. This supported our hypothesis, that arginine depletion was not solely responsible for growth inhibition, but that other mechanisms could contribute.

To determine whether arginine deiminase could inhibit cell proliferation by arresting cell cycle, we examined its effect on the distribution of cells in various phases of proliferation. Indeed, arginine deiminase arrested neuroblastoma (SH-EP) cells in the G_1 phase in both arginine-containing and arginine-free medium (Table 1). Although the arginine-free condition by itself induced a significant G_1 arrest, arginine deiminase could potentiate this effect. Interestingly, arginine deiminase's effect on the cell cycle varied somewhat between the cell lines used. As shown in Table 1, cells of HUVE were arrested only in G_1 like SH-EP, cells of WAC2 and SaOS were delayed or became inactive in S (S_0), and both phenomena occurred in Y-79. These S_0 cells were identified by their S phase DNA content and their concomitant inability to incorporate the DNA precursor BrdU.

As modulation of the cell cycle can often precede cell death, we investigated the effect of arginine deiminase on apoptosis. When arginine deiminase was applied to the cultured cells at high concentrations (200 ng/ml), signs of apoptosis like cell shrinkage, nuclear fragmentation and chromatin condensation were observed (Figs. 2A & B). That arginine deiminase could indeed induce apoptosis was verified by its ability to generate DNA breaks in a significant fraction of the cells (Figs. 2C & D). This fraction varied between various cell species examined, including vascular endothelial and a total of 4 tumor cell lines, but ranged between 30 to 95% of the treated cells as compared to only 5% in the untreated controls.

DISCUSSION

In immune-competent or -deficient organisms, infection with mycoplasmas can produce various diseases including Crohn's disease, rheumatoid arthritis, pneumonia and sepsis (11). The mechanisms responsible for mycoplasma pathogenesis are not well understood, but they could be the result of a close interaction of the microorganism with host cells (11). For example, mycoplasmas might secrete humoral factors able to impair host-defense mechanisms including inflammation and tissue regeneration. The latter mechanisms are contingent on a rapid and effective proliferation of cells originating from the immune system or from local tissue. It was therefore conceivable that mycoplasma-derived arginine deiminase could deplete arginine from the local tissue and thereby inhibit proliferation of host cells including immune (12) and vascular endothelial (2) cells. When present in high concentrations, arginine deiminase could even induce host cell death.

Indeed, we have demonstrated here that arginine deiminase inhibits the proliferation of vascular endothelial and various established cell lines in culture. The growth inhibitory activity of arginine deiminase had previously been thought to be due to the depletion of arginine (4). However, our present data indicate that in addition to simple depletion of arginine from the cellular microenvironment, arginine deiminase can modulate cell cycle in a specific manner. Arginine deiminase may arrest cells in G_1, but it may also allow them to progress into S phase inhibiting their progression into G_2 phase. In the latter case, the cells do not complete replication and they become quiescent S_0 cells. Similar observations were made with cultured human tumor cell lines exposed to hypoxia, hypoglycemia and low pH (Zölzer and Streffer, submitted for publication). It is usually assumed that apoptosis and G_1 arrest are related (8). Indeed, we find apoptosis in HUVE, SH-EP and Y-79 cells treated by arginine deiminase, where the G_1 arrest was observed. On the other hand, arginine deiminase also induces apoptosis in WAC2 and SaOS, which obviously lack the G_1/S check point. This is contrary to expectation and points to the existance of apoptotic mechanism independent of

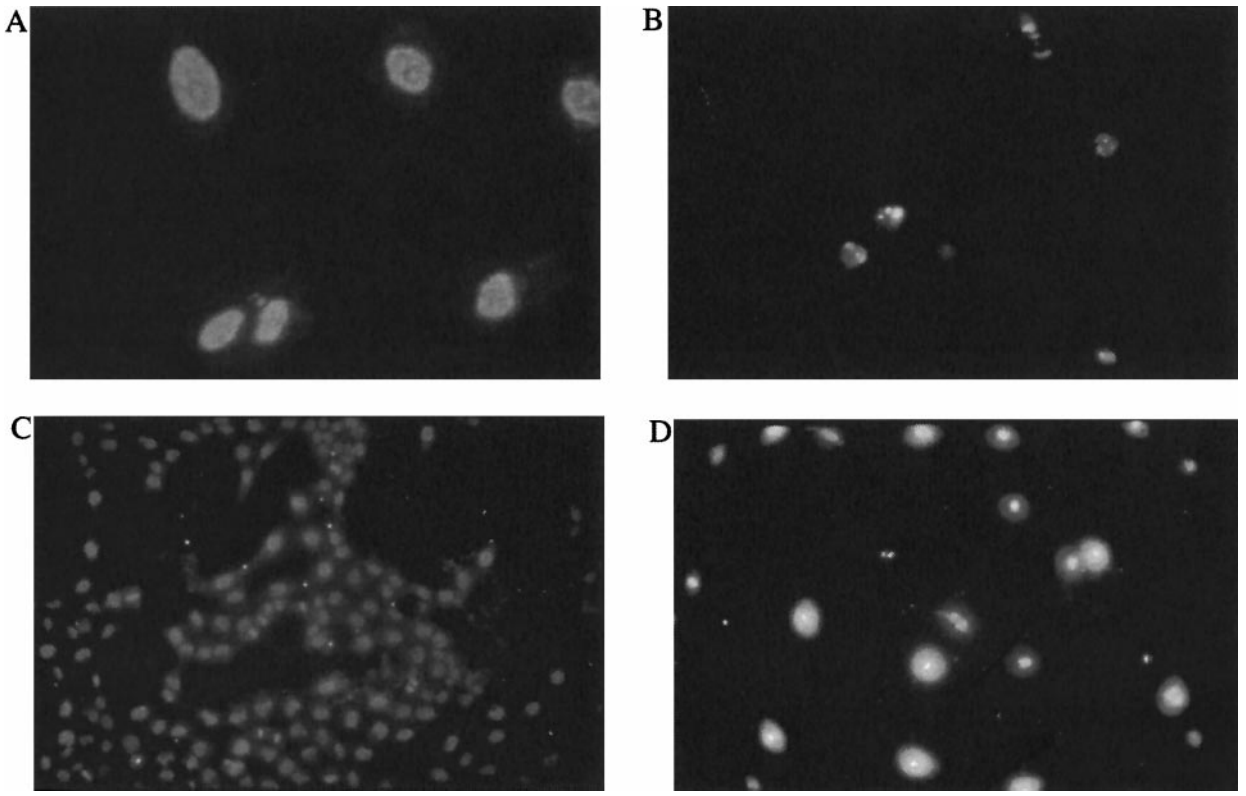

FIG. 2. Effect of arginine deiminase on nuclear morphology and DNA strand breaks induction in HUVE (A, B) and SH-EP (C, D) cells. Cells were either not treated (A, C; controls) or treated (B, D) with 200 ng/ml of arginine deiminase for 3 days. The cells were cytocentrifuged, stained with DAPI (A, B) (magnification, ×400), and end labeled with fluorescein-dUTP by use of TDT (C, D) (magnification, ×100). The treated cells appear smaller (B) and are labeled more intense (B, D) compared to controls (A, C).

the G_1 arrest. A similar lack of correlation between two endpoints has been found after irradiation of humor tumor cells (13).

Our demonstration that arginine deiminase inhibits cell cycle progression and induces apoptosis of both tumor and vascular endothelial cells could explain the effects of arginine deiminase on tumor growth in vivo (6). It appears possible that arginine deiminase acts by both inhibiting tumor growth and tumor angiogenesis and may therefore have clinical potential as an anti-tumor and anti-angiogenic agent. Arginine deiminase has been reported to have few side effects in animal studies (6) meriting its further clinical evaluation. Should arginine deiminase indeed prove to be devoid of significant side effects, it could also be a candidate to replace asparaginase in situations where the latter has produced adverse reactions (1). Thus, arginine deiminase might be of future use to improve the therapy of leukemia and solid malignancies either as a single agent or in combination with other agents (14).

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