Putrescine-dependent Invasive Capacity of Rat Ascites Hepatoma Cells

Yoshiyuki Ashida, Jun-ichi Kido, Fumiyo Kinoshita, Mizuho Nishino, Kiyoko Shinkai, Hitoshi Akedo, and Hideo Inoue

Departments of Biochemistry [Y. A., J.-I. K., H. I.] and Pedodontics [F. K., M. N.], School of Dentistry, University of Tokushima, 3 Kuramoto-cho, Tokushima 770, and Department of Tumor Biochemistry, Center for Adult Diseases, Osaka, 1 Nakamichi, Higashinari-ku, Osaka 537 [K. S., H. A.], Japan

ABSTRACT

The effects of inhibitors of polyamine synthesis on the invasive capacity of rat ascites hepatoma (LC-AH) cells were examined by in vitro assay of penetration of the LC-AH cells through a monolayer of calf pulmonary arterial endothelial (CPAE) cells. Pretreatment of LC-AH cells with 2-fluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, before seeding them onto a CPAE cell monolayer and culturing them for 24 h in the absence of DFMO decreased the number of penetrating tumor cells time and dose dependently (about 35% of the maximal inhibition) without affecting their viability or proliferative activity. DFMO treatment caused a marked decrease in the intracellular level of putrescine but not of spermidine or spermine. The DFMO-induced decreases in invasive capacity and putrescine level were almost completely reversed by the addition of putrescine to the medium during pretreatment with DFMO or invasion assay but were not affected by exogenous spermidine or spermine. No change in the invasive capacity was observed when the CPAE cells were treated with DFMO and the LC-AH cells with methylglyoxal-bis(guanylhydrazone), an inhibitor of S-adenosylmethionine decarboxylase, which depressed the spermidine and spermine levels but increased the putrescine level in the LC-AH cells.

These results suggest that intracellular putrescine modulates the in vitro invasive capacity of LC-AH cells.

INTRODUCTION

There are reports that the invasive capacity of tumor cells is determined genetically and is markedly modulated by a variety of cellular factors and drugs (1–7). Previously we reported that inhibitors of phospholipid methylation lower the in vitro invasiveness of tumor cells by decreasing the membrane fluidity by causing hypomethylation of membrane phospholipids (6). This finding and reports from other laboratories (8–12) suggest that the tumor cell membrane plays crucial roles in the invasiveness of tumor cells.

In general, tumor cells contain high concentrations of polyamines (13) putrescine, spermidine, and spermine, which are reported to participate not only in cell proliferation but also in a variety of cellular events including functions of the cell membrane (14). Therefore, cellular polyamines have been suggested to be related to the invasive capacity of tumor cells. In fact, there are several reports of significant inhibition of visually detectable pulmonary metastases in tumor-bearing mice after the administration of DFMO (15–22), an irreversible inhibitor of ODC which is a key enzyme in polyamine synthesis. However, in vivo assay methods involve long-term (more than 2 weeks) treatment of tumor-bearing mice with DFMO, making it difficult to distinguish the antiproliferative and antimetastatic effects of DFMO.

In this study, we examined the effects of polyamines on the invasive capacity of tumor cells using an in vitro assay system in which LC-AH cells were seeded onto a monolayer of CPAE cells, and the number of LC-AH cells that penetrated the monolayer was counted 24 h later (23). As reported previously (1, 4, 24), the invasive capacities of different sublines of rat ascites hepatoma cells measured by this in vitro assay correspond well with their respective in vivo invasive potentials. Thus, this system provides a rapid and quantitative method for the detection of alterations in the invasiveness of tumor cells.

MATERIALS AND METHODS

Materials. LC-AH cells, a subline of AH cells which show high invasiveness into endothelial cells, were obtained by successive passage in culture of the parent cells (23). CPAE cells were a gift from the Japanese Cancer Research Resources Bank. FCS and horse serum were purchased from Flow Laboratories (McLean, VA). The sources of chemicals were as follows: 1,3-diaminopropane and cadaverine were from Wako Pure Chemical Industries (Osaka, Japan); putrescine, spermidine, and spermine were from Sigma Chemical Co. (St. Louis, MO); DFMO was from Merrell Dow Pharmaceuticals, Inc. (Cincinnati, OH); HAVA was from Dainippon Pharmaceutical Co. (Osaka, Japan); MGBG was from Aldrich Chemical Co. (Milwaukee, WI); and (methyl-3H)thyminidine (2.4 TBq/mmol) was from American Radiolabeled Chemicals (St. Louis, MO).

Cell Culture. LC-AH and CPAE cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in air. The medium used was DMEM supplemented with NaHCO3 (3.7 mg/ml), penicillin (100 units/ml), streptomycin sulfate (0.1 mg/ml), and 10% (LC-AH cells) or 20% (CPAE cells) FCS. The culture medium for LC-AH cells was changed every 3 days.

Assay of Invasive Capacity. In vitro invasion of LC-AH cells was assayed as reported previously (23). Briefly, tumor cells (1.4–1.6 x 106 cells/ml) which had been cultured for 3 days after inoculation (1.2 x 103 cells/ml) were collected by centrifugation and suspended (1 x 106 cells/ml) in fresh 10% FCS-DMEM. The cells were treated with inhibitors of polyamine synthesis or polyamines for various times as indicated and then washed with DMEM to remove the drugs. A cell suspension (2 x 104 cells/ml) in 10% FCS-DMEM was prepared using a Coulter counter to determine the cell number and the trypan blue-exclusion test to determine cell viability. Then 1 ml of the suspension was seeded onto a confluent CPAE cell monolayer grown on a 12-well plate (3.8 cm2 well; Corning). After coculture for 24 h in 10% FCS-DMEM, the number of tumor cells that had penetrated the endothelial cell monolayer was counted under a phase-contrast microscope. Under these assay conditions, 40–45% of seeded cells that had not been treated with inhibitors penetrated the monolayer. When spermidine or spermine was added to the medium for invasion assay, FCS was replaced by horse serum, which contains no polyamine oxidase (25), because polyamine oxidase in FCS produces cytotoxic aldehydes from spermidine and spermine (26). This replacement did not affect the invasive capacity of untreated or DFMO-treated LC-AH cells.

Proliferative Activity. The LC-AH cells (2 x 103 cells/ml) used for invasion assay were cultured in 10% FCS-DMEM for 23 h, and then (methyl-3H)thyminidine (37 kBq/ml) was added. After 1 h, the labeling was stopped by the addition of cold 2 µl (final concentration) thymidine, and the cells were washed three times with phosphate-buffered saline. The packed cells were solubilized in 0.3 M NaOH, and DNA was

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: DFMO, N-(2-fluoromethyl)ornithine; ODC, ornithine decarboxylase; LC-AH, a subline of rat ascites hepatoma; CPAE, calf pulmonary arterial endothelial; FCS, fetal calf serum; HAVA, N-2-hydrizin-5-aminovaleric acid; MGBG, methylglyoxal-bisguanylhydrazone; DMEM, Dulbecco’s modified Eagle’s medium.
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RESULTS

Effects of DFMO and MGBG on the Invasive Capacity of LC-AH Cells. LC-AH cells were treated with DFMO, an ODC inhibitor, or MGBG, an inhibitor of S-adenosylmethionine decarboxylase, and then their invasive capacity was measured in the absence of inhibitors. Time- and dose-dependent inhibition of the invasiveness was observed with DFMO (Fig. 1). Because treatment with 0.5 mM DFMO for 5 h caused about 35% of maximal inhibition without causing any decrease in cell viability (more than 97%), these conditions were used for DFMO treatment in subsequent experiments. No significant decrease in invasive capacity or cell viability was detected on treatment of the tumor cells with 10 μM MGBG for 8 h. Treatment with more than 20 μM MGBG for 10 h resulted in a dose-dependent inhibition of the invasion but also a dose-dependent decrease in cell viability (data not shown), suggesting that its inhibition was due to its cytotoxic effects. Treatment of CPAE cells with 0.5 mM DFMO for 5 h did not influence the invasiveness of LC-AH cells (data not shown), indicating that DFMO incorporated into LC-AH cells did not increase the susceptibility of CPAE cells to the invasion by acting on the latter during coculture for invasion assay.

Effects of DFMO and MGBG on Polyamine Levels of LC-AH Cells. On inoculation of 1.2 × 10⁵ LC-AH cells/ml in 10% FCS-DMEM, the growth rate was very rapid initially but decreased markedly 3 days later when the cell population reached about 1.5 × 10⁶ cells/ml. The polyamine pattern of these slowly growing LC-AH cells (Fig. 2A, O h) was quite different from those of many animal tissues (28) and a variety of tumor cells such as Ehrlich ascites tumor cells (29), B16 melanoma (19), Lewis lung carcinoma (15, 16, 21, 22), and leukemia cells (22) in which the cellular putrescine level is less than 30% of the spermidine level. In the LC-AH cells, the putrescine levels were high, being about 85 and 110% of the spermidine concentrations at 0 and 8 h, respectively (Fig. 2A). The elevation of the putrescine level at 5 and 8 h was due to an increase in ODC activity induced by about 1.5-fold dilution of the cells with fresh medium at 0 h (see Fig. 1); the ODC activities were 1.9 and 11.2 nmol/mg protein at 0 and 5 h, respectively, measured as reported previously (27). No significant increases in spermidine and spermine levels were observed within 8 h (Fig. 2A).

The putrescine level of LC-AH cells was markedly decreased by DFMO (Fig. 2B). This decrease was dose dependent, and 5 h after the addition of 0.5 mM DFMO the level was less than one-third of the control level, indicating strong inhibition of ODC by this inhibitor. A higher concentration of 1 mM DFMO caused no further decrease in the putrescine level at 5 or 8 h (data not shown). DFMO had no significant effect on the levels of spermidine and spermine within 8 h (Fig. 2B). On the other hand, on addition of 10 μM MGBG the cellular levels of spermidine and spermine were significantly depressed after 8 h, and conversely, the putrescine level was significantly increased after 5 h (Fig. 2C), indicating the inhibition of S-adenosylmethionine decarboxylase activity by MGBG in LC-AH cells.

Restoration by Putrescine of Invasive Capacity Depressed by DFMO. The results shown in Fig. 1 and 2 suggested a close relationship between putrescine depletion and a decrease in invasive capacity. Next we examined the effects of exogenous polyamines on the depressed invasive capacity of DFMO-treated cells. The naturally occurring diamines 1,3-diaminopropane and cadaverine were also tested for comparison, although these diamines were not detectable in LC-AH cells. In this experiment, LC-AH cells were treated with 0.5 mM DFMO for 5 h in the absence or presence of various concentrations of diamines or polyamines, and then their invasive capacity was measured in the absence of DFMO and the amines. Fig. 3 shows that putrescine dose-dependently restored the invasive capacity of DFMO-treated cells, causing complete recovery at 20 μM. Cadaverine had a slight effect, but 1,3-diaminopropane, spermidine, and spermine did not overcome the suppressive effect of DFMO. Putrescine stimulated the invasive capacity of only DFMO-treated cells, and treatment of LC-AH cells with putrescine (Fig. 3), spermidine, or spermine alone (data not shown) had no effect on their invasiveness.
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Fig. 3. Influences of diamines and polyamines on DFMO-induced suppression of invasive capacity of LC-AH cells. LC-AH cells (1 x 10⁶ cells/ml) were treated with 0.5 mM DFMO and various concentrations of amines for 5 h, and then their invasive capacity was determined in the absence of DFMO and amines. The invasive capacities of the cells treated with putrescine alone in the absence of DFMO are also shown for comparison. Points, mean for 2-4 experiments. •, putrescine; ◊, cadaverine; Δ, 1,3-diaminopropane; □, spermidine; ▲, spermine; ○, putrescine alone.

The intracellular polyamine levels and proliferative activities of LC-AH cells treated with DFMO and polyamines for 5 h were next examined. The effects of another inhibitor of ODC, HAVA (30), and MBGB were also examined. As shown in Experiment A of Table 1, complete restoration of the putrescine level by exogenous putrescine was observed in both DFMO- and HAVA-treated cells. Exogenous spermidine and spermine were also incorporated into LC-AH cells in the absence or presence of the inhibitors, and the intracellular concentrations of these amines were higher than those of control cells. None of these treatments with inhibitors and polyamines affected the proliferative activity or cell viability (data not shown), suggesting that the inhibitors and polyamines were not cytotoxic under the conditions used.

Complete restoration by putrescine of the invasive activity of cells suppressed by DFMO or HAVA was also observed when putrescine was added to the medium for invasion assay (Experiment B of Table 1). Again spermidine and spermine were ineffective, and polyamines did not alter the invasive capacity of control cells that had not been treated with the inhibitors.

DISCUSSION

Several reports (15-22) have shown that oral administration of DFMO to tumor-bearing animals for 2 weeks or more reduces the frequency and development of pulmonary metastases and concomitantly inhibits growth of the primary tumor. On the other hand, Zirvi et al. (31) reported that DFMO significantly inhibits liver metastasis but has little or no effect on the growth of primary tumors in nude mice 12-14 weeks after intrasplenic injection of human tumor cells. Their results do not, however, necessarily indicate that DFMO inhibits only metastatic processes and not the growth of tumor cells settled in metastatic regions, because DFMO-induced growth inhibition of tumor cells has been suggested to depend on the size of the tumor burden, being much greater with a low tumor burden, such as an early metastatic tumor (32). Concerning DFMO suppression of tumor growth, Takigawa et al. (33) suggested that polyamine depletion by DFMO inhibits the proliferation of endothelial cells, resulting in the depression of angiogenesis, which is required for the expansion of both the primary tumor mass and metastatic colonies. Therefore, it is possible that the DFMO-induced suppression of metastases observed by the long-term in vivo assay method may reflect only the antiproliferative effects of this inhibitor on tumor cells and/or endothelial cells.

Although the in vitro invasion assay system used in this study does not contain a basement membrane composed of the extracellular matrix, which is thought to be an important barrier against tumor metastases (34), it provides a rapid method for the estimation of the capacity of tumor cells to penetrate an endothelial cell layer, which is essential for intravasation and extravasation during hematogenous metastasis.

Here we show for the first time the dissociation of the anti-proliferative and antimetastatic effects of ODC inhibitors (DFMO and HAVA) on tumor cells. Treatment of LC-AH cells with the inhibitors for 5 h resulted in decreases in both their invasive capacity (Fig. 1; Table 1) and their putrescine level (Fig. 2; Table 1) without alterations in their viability (data not shown). The intracellular polyamine levels and proliferative activities of LC-AH cells treated with DFMO and polyamines for 5 h were next examined. The effects of another inhibitor of ODC, HAVA (30), and MBGB were also examined. As shown in Experiment A of Table 1, complete restoration of the putrescine level by exogenous putrescine was observed in both DFMO- and HAVA-treated cells. Exogenous spermidine and spermine were also incorporated into LC-AH cells in the absence or presence of the inhibitors, and the intracellular concentrations of these amines were higher than those of control cells. None of these treatments with inhibitors and polyamines affected the proliferative activity or cell viability (data not shown), suggesting that the inhibitors and polyamines were not cytotoxic under the conditions used.

Complete restoration by putrescine of the invasive activity of cells suppressed by DFMO or HAVA was also observed when putrescine was added to the medium for invasion assay (Experiment B of Table 1). Again spermidine and spermine were ineffective, and polyamines did not alter the invasive capacity of control cells that had not been treated with the inhibitors.

Table 1 Effects of inhibitors of polyamine synthesis and polyamines on polyamine levels, proliferative activity, and invasive capacity of LC-AH cells

<table>
<thead>
<tr>
<th>Treatment (5 h)</th>
<th>Polymine level (% of control)</th>
<th>Proliferative activity (% of control)</th>
<th>Invasive activity (Cells/mm²)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100</td>
<td>100</td>
<td>22.0 ± 1.7</td>
<td>100</td>
</tr>
<tr>
<td>20 mM Put.</td>
<td>147±   1%</td>
<td>110  10%</td>
<td>23.6 ± 2.3</td>
<td>107</td>
</tr>
<tr>
<td>20 mM Spd.</td>
<td>92    ± 1%</td>
<td>163±  1%</td>
<td>21.8 ± 2.3</td>
<td>99</td>
</tr>
<tr>
<td>20 mM Spm.</td>
<td>90    ± 1%</td>
<td>171±  1%</td>
<td>22.7 ± 1.8</td>
<td>103</td>
</tr>
<tr>
<td>0.5 mM DFMO</td>
<td>36±  ± 1%</td>
<td>94  ± 1%</td>
<td>13.7 ± 1.2</td>
<td>62</td>
</tr>
<tr>
<td>0.5 mM DFMO + 20 mM Put.</td>
<td>116 ± 1%</td>
<td>107 ± 1%</td>
<td>23.3 ± 1.3</td>
<td>101</td>
</tr>
<tr>
<td>0.5 mM DFMO + 20 mM Spd.</td>
<td>42±  ± 1%</td>
<td>145±  1%</td>
<td>13.7 ± 0.8</td>
<td>63</td>
</tr>
<tr>
<td>0.5 mM DFMO + 20 mM Spm.</td>
<td>34±  ± 1%</td>
<td>182±  1%</td>
<td>12.8 ± 1.2</td>
<td>59</td>
</tr>
<tr>
<td>1 mM HAVA</td>
<td>32±  ± 1%</td>
<td>93  ± 1%</td>
<td>13.2 ± 1.1</td>
<td>60</td>
</tr>
<tr>
<td>1 mM HAVA + 20 mM Put.</td>
<td>105 ± 1%</td>
<td>103 ± 1%</td>
<td>21.6 ± 1.9</td>
<td>98</td>
</tr>
<tr>
<td>10 mM MGBG</td>
<td>128±  ± 1%</td>
<td>81±  ± 1%</td>
<td>21.4 ± 1.9</td>
<td>97</td>
</tr>
<tr>
<td>10 mM MGBG + 20 mM Put.</td>
<td>134±  ± 1%</td>
<td>125±  1%</td>
<td>20.7 ± 1.7</td>
<td>94</td>
</tr>
</tbody>
</table>

* In Experiment A, LC-AH cells (1 x 10⁶ cells/ml) were treated with an inhibitor and a polyamine for 5 h. Polyamine levels were measured just after treatment. For measuring proliferative activity, the treated cells were cultured for 23 h in the absence of an inhibitor or polyamine, and the incorporation of [3H]thymidine into DNA was determined as described in "Materials and Methods." Values are means for 3-5 experiments. Control values (means ± SD) were as follows: putrescine (Put.), 72.5 ± 8.4 nmol/mg DNA; spermidine (Spd.), 69.6 ± 7.0 nmol/mg DNA; spermine (Spm.), 53.1 ± 5.6 nmol/mg DNA; proliferative activity, 3.18 ± 0.31 dpm/mg DNA.

* In Experiment B, LC-AH cells were treated with or without an inhibitor alone for 5 h. After removal of the inhibitor, invasive capacity was determined in 10% horse serum-DMEM in the absence or presence of a polyamine. Values are means ± SD for 3-5 experiments.

* Significantly different from control value.
shown), proliferative activity (Table 1), or levels of spermidine and spermine (Fig. 2; Table 1). These DFMO- or HAVAl-induced decreases were completely reversed by the presence of exogenous putrescine during treatment with the inhibitors (Table 1). The recovery of the invasive capacity was not due to the inhibition of incorporation of DFMO into LC-AH cells by extracellular putrescine because putrescine was also effective when added to the medium during invasion assay, in which no DFMO was added (Table 1). Exogenous spermidine and spermine were also incorporated into LC-AH cells, increasing their respective intracellular levels significantly (Table 1), but DFMO-depressed invasive capacity was not restored by increases in the levels of these polyamines (Fig. 3; Table 1). Moreover, MGBG-induced decreases in spermidine and spermine levels did not affect the invasive capacity of the cells (Figs. 1 and 2; Table 1). All of these results strongly suggest that putrescine, but not spermidine or spermine, is related to the capacity of LC-AH cells to penetrate a monolayer of CPAE cells.

The precise role of putrescine in the penetration processes is unknown. Preliminary experiments showed that under the present conditions, the adhesion of LC-AH cells to the endothelial cell monolayer was complete within 2 h after seeding and was not affected by DFMO treatment. These findings suggest that putrescine participates in either tumor cell locomotion or the morphological change to a flattened configuration of penetrating tumor cells (35). In this connection, it is noteworthy that Kierszenbaum et al. (36) showed that treatment of mouse peritoneal macrophages with ODC inhibitors reduces their phagocytic capacity and that this capacity is restored by exogenous putrescine. Pohjanpelto et al. (37) reported that putrescine starvation causes the disappearance of actin filaments and microtubules in polyamine-auxotrophic Chinese hamster ovary cells. Although these groups did not identify the functional polyamine, their results and our findings suggest that putrescine is involved in the functions of cytoskeletons. This possibility is now under investigation in our laboratories.

REFERENCES


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