Establishment of a Human Ovarian Cancer Cell Line Capable of Forming Ascites in Nude Mice and Effects of Tranexamic Acid on Cell Proliferation and Ascites Formation

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ABSTRACT

The present study was designed to obtain an experimental tumor model as similar as possible to human ovarian cancer which often had a large amount of ascites and to assess the therapeutic value of tranexamic acid. Human tumor cell lines which form ascites in nude mice were established from ascites of patients with serous cystadenocarcinoma of the ovary. Two cloned cell lines designated HRA and HR-1 were obtained from the parent cell line designated HR. All of these cultured cell lines had about 2.5-3.5 times higher lactate dehydrogenase activities than the original tumor. The original tumor and the tumor grown in nude mice had all 5 bands of lactate dehydrogenase isoenzymes, while all cultured cell lines had only a marked lactate dehydrogenase-3 in addition to a faint lactate dehydrogenase-2. Modal chromosome numbers of HR cells ranged from 50-76, while that of HRA cells ranged widely from 40-140. The DNA histograms of HR and HRA cells were similar to each other, showing predominately G1 and S phases. Although these cell lines had ability to produce ascites in the nude mice when the cells were inoculated i.p., the HRA cell inoculation made ascites most rapidly and brought about the shortest median survival (39 days). The proliferations of all three cell lines were dose-dependently inhibited by tranexamic acid. However, the concentration of this drug required for 50% inhibition of the proliferation of HRA cells was about one-half of that of HR and HR-1 cells. In addition, i.p. injections of tranexamic acid to nude mice treated with cisplatin resulted in a significant inhibition of the ascites formation and prolongation of 50% survival.

INTRODUCTION

Patients with advanced ovarian cancer have often large amounts of ascites. Mechanisms of ascites formation in patients with advanced ovarian cancer are not yet fully understood. Accordingly, establishment of ovarian cancer cell lines capable of forming ascites was considered to be useful to elucidate the mechanism of ascites production in ovarian cancer patients. Survival rates of patients with large quantities of ascites were less than one-half of those of patients with no ascites (1). It has also been reported that a fibrinolytic inhibitor, tranexamic acid, given to animals with certain experimental tumors prolongs survival and inhibits ascites production (2, 3). In a recent study, we described that tranexamic acid inhibited growth of human ovarian cancer cell lines and i.p. injections of this drug resulted in a significant decrease of ascites in patients with large amounts of ascites (4, 5).

We now report establishment of human ovarian cancer cell lines capable of producing ascites in nude mice and ability of tranexamic acid to inhibit not only ascites formation but also cell proliferation, subsequently bringing about prolongation of survival of the nude mice where HRA cells were inoculated i.p.

MATERIALS AND METHODS

Agent. Tranexamic acid was obtained from Daiichi Pharmaceutical Co., Tokyo, Japan.

Cells and Cell Culture. HR cells were established from ascites of a patient with serous cystadenocarcinoma of the ovary on January 1, 1983. Preoperative laboratory findings of this patient showed abnormally high levels of LDH3 (506 units/liter), HBD (240 units/liter) and LDH-4 (20%). HR-1 and HRA cloned cells were isolated by a single-cell plating technique (6) from the HR cells. The passage number of the HR, HR-1, and HRA cells was 106, 43, and 36, respectively. The cells were incubated in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin (100 units/ml and 100 μg/ml, respectively; Grand Island Biological Co.) in 5% CO2 at 37°C. The medium was changed every 3 days, and the cells were passed when confluency was achieved. For the determination of doubling time, 104 cells were seeded in 24-well Nunc multidisks (Nunc, Roskilde, Denmark) and incubated at 37°C. Cells of each well were harvested after 24, 48, 72, and 96 h and counted using a hemocytometer. The doubling time of the HR, HR-1, and HRA cells was 13.2, 17.6, and 15.6, respectively (Fig. 1).

Chromosome Analysis. Two h after addition of Colcemid (0.5 μg/ml; Grand Island Biological Co.) cells were removed by vigorous pipetting and incubated in 0.075 M KCl at room temperature. Ten min later they were fixed with 25% glacial acetic acid in anhydrous methanol. Chromosome analysis was performed by sequential staining of the metaphases with 10% Giemsa (7).

Flow Cytometry. Cell cycle phase distribution analyses were performed with a Jasco Model FCS-1 (Japan Spectroscopic Co., Ltd., Tokyo, Japan) multiparameter cell sorter using propidium iodide-stained nuclei of the cells. Briefly, control and treated cells were washed twice with ice-cold 0.85% sodium chloride solution and the cell pellet resuspended in hypotonic propidium iodide staining solution (8). The nuclei were maintained at 4°C for 24 h in the staining solution prior to analysis for cell cycle phase perturbation. At least 104 nuclei were analyzed in each sample, and the fraction of cells in G1, Go, G2, and G2 + M of the cell cycle was determined as reported previously (9).

Assay for LDH and HBD Activities. Culture cells were washed with phosphate-buffered saline solution, packed by centrifugation at 1400 × g for 5 min, and resuspended in 1 ml of cold 0.1 M Tris-HCl buffer (pH 7.4). The original tumor and the tumor grown in nude mice were suspended in an equal volume of the same buffer used for culture cells. These suspensions were homogenized with a VirTis 45 homogenizer (The VirTis Co., Inc., Gardiner, NY) operating at 25,000 rpm for 4 min. The homogenates were centrifuged at 105,000 × g for 60 min with a Hitachi ultracentrifuge (Hitachi, Ltd., Tokyo, Japan), and the resultant supernatants were used for enzyme assay. LDH activity was assayed spectrophotometrically at 30°C by measuring the rate of NADH decrease as described previously (10). HBD was measured by using the HBD-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described previously (11). Protein was determined by the method of Lowry et al. (12). Separation of LDH isoenzymes was performed by using cellulose acetate membrane electrophoresis as reported previously (13).

In Vitro Treatment. For determining effects of tranexamic acid on proliferations of HR, HR-1, and HRA cells in vitro, viable 104 cells

Received 10/21/85; revised 6/25/86, 9/16/86; accepted 10/7/86.

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2 Supported in part by a grant from the Special Scientific Research Program of the Defense Agency in Japan.

3 To whom requests for reprints should be addressed.

2 The abbreviations used are: LDH, lactate dehydrogenase; HBD, α-hydroxybutyrate dehydrogenase.
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Fig. 1. Growth curves of HR, HR-1, and HRA cell lines. Viable \(10^4\) cells were seeded in 24-well Nunc multidishes and incubated at 37°C. Cells of each well were harvested after hours indicated and counted using a hematocytometer. The doubling times of HR, HR-1, and HRA cell lines were 13.2, 17.6, and 15.6 h, respectively.

were seeded into 24-well Nunc multidishes, and incubated in a humidified atmosphere of 5% CO₂ at 37°C. After 24 h of culture, various concentrations of tranexamic acid were added to the medium. After 72 h of additional culture, cells in each well were harvested and counted using a hematocytometer. All counts were done in quadruplicate and the viability was assessed by trypan blue dye exclusion.

Nude Mice. Six-week-old female BALB/c nude mice were obtained from Japan Clea Laboratories, Tokyo, Japan, and maintained in a pathogen-free environment. When necessary, the animals were killed and dissected. The tumor tissues were fixed in formalin for histological examination and stained with hematoxylin and eosin.

Ascites Formation in Nude Mice. For comparing ability of HR, HR-1, and HRA cell lines to form ascites in nude mice, viable \(2 \times 10^7\) cells of each cell line were i.p. inoculated and the formation of ascites (>0.5 ml) was confirmed by palpation and needle aspiration of the abdominal cavity every week. Date of ascites formation and death was recorded.

In Vivo Treatment. For determining the adjuvant effect of tranexamic acid on cisplatin, \(2 \times 10^7\) HRA cells were inoculated i.p. into nude mice and they were divided into 3 groups: an untreated group; a cisplatin-treated group; and a cisplatin and tranexamic acid-treated group. Each group consisted of 5 mice. After 2 weeks of HRA cell inoculation, cisplatin (2 mg/kg) was administered i.p. every week for 4 weeks. I.p. injections of tranexamic acid (0.8 g/kg) were performed twice a week for 4 weeks after 2 weeks of cell inoculation. After cell inoculation, nude mice were bled every week from the tail vein, and the blood was collected in hematocrit tubes. Hematocrit and body weight were measured to examine side effects of the drug.

Statistical Analysis. Results are presented as mean ± SD. The statistical analysis was performed by Mann-Whitney U test.

RESULTS

Biological Characteristics of the Cell Line HRA. The parent cell line, designated HR, was established in vitro about 40 days after several passages from primary cultures (Fig. 2). HRA and HR-1 cells were isolated by a single-cell plating technique (6) from the parent HR cells. The morphological features of the cultured HRA cells are shown in Fig. 3. The HRA cells consist of larger and polygonal cells with enlarged nuclei and prominent nucleoli compared to the parent HR cells while those of HR-1 cells were similar to those of the parent HR cells. Typical features of a tumor obtained by inoculating s.c. HRA cells into nude mice were compared to those of the original tumor (Figs. 4 and 5). The HRA tumor that formed in athymic nude mice showed large areas of necrosis and consisted of sheets of elongated or polygonal cells with prominent nucleoli. The morphological appearance was partly similar to that of the original tumor. In addition, the mode of chromosome number in the parent HR cells (passage 106) was 68 (range, 50–78) while that of the cloned HRA cells (passage 36) was 65 (range, 40–140) (Fig. 6). Flow cytometric analysis of both these cell lines revealed that cell cycle phase distribution in both cells was similar,
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Fig. 5. Morphological features of HRA tumor grown in nude mice. The tumor was obtained by inoculating HRA cells s.c. into nude mice. The tumor showed solid growth in a large area and consisted of cells with numerous mitoses and prominent nucleoli. H & E, x 200.

Fig. 6. Distribution of chromosome counts in HR cells from passage 106 and HRA cells from passage 36.

Fig. 7. Representative DNA histograms of HR cells from passage 106 and HRA cells from passage 36.

Table 1 Comparison of specific LDH and HBD activity in HR cell lines

<table>
<thead>
<tr>
<th>Activity (IU/g protein)</th>
<th>Original tumor</th>
<th>Tumor grown in nude mice (HR)</th>
<th>Cultured cells (HR)</th>
<th>Cultured cells (HR-1)</th>
<th>Cultured cells (HRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH</td>
<td>2156.7</td>
<td>6425.0</td>
<td>5727.0</td>
<td>6060.2</td>
<td>7014.9</td>
</tr>
<tr>
<td>HBD</td>
<td>3130.6</td>
<td>1061.8</td>
<td>1105.0</td>
<td>1160.0</td>
<td>1319.3</td>
</tr>
</tbody>
</table>

* Assay methods of LDH and HBD are given in "Materials and Methods."
* Average of 3 experiments.

Effect of Tranexamic Acid on HRA Cell Proliferation in Vitro. The 3 days' exposure of cells with tranexamic acid resulted in a dose-dependent inhibition with regard to proliferation of all cell lines used in this study between concentrations of 2.5 and 20.0 mg/ml of this drug. The degree of inhibition was more marked in HRA cell proliferation, compared to those of HR and HR-1 cells. Namely, the 50% inhibitory concentration values for HRA, HR, and HR-1 were 5.5, 11.0, and 10.8 mg/ml, respectively (Fig. 9).

Adjuvant Effect of Tranexamic Acid on Cisplatin in Nude Mice Bearing HRA Cells. In the untreated group, ascites was formed in one of 5 nude mice at 14 days after inoculation and in all nude mice at 28 days. The hematocrit was significantly lower in nude mice after 21 days than in nude mice before inoculation. At 42 days, all nude mice were dead. In the cisplatin-treated group, ascites formation was observed in one of 5 nude mice at 21 days and in all nude mice at 35 days. At 42 days, 4 of 5 nude mice were still alive. In the cisplatin and tranexamic acid-treated group, ascites formation was markedly delayed, being observed in only one of 5 nude mice at 35 days. One of these mice did not form ascites but survived during the experimental period. Even at 49 days, 3 nude mice were alive, in contrast with none in the untreated and one in the cisplatin-treated group (Table 3). Regarding side effects, concentrations of cisplatin and tranexamic acid used in this study did not seem to affect body weight and hematocrit, when compared to those days after HR-1 cell inoculation all nude mice had ascites (Table 2). Median survival of HRA cell-inoculated nude mice was shortest (39 days) followed by 47 days of HR and 73 days of HR-1. However, when 10^5 cells were inoculated s.c. but not i.p., no difference between these cell lines was observed with regard to the survival time after s.c. inoculation of tumor and tumorigenicity (data not shown).
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Fig. 8. Densitometric tracings of LDH isoenzyme bands. These isoenzymes are called LDH-1, -2, -3, -4, and -5 in order of mobility from anode toward cathode on electrophoresis. Densitometry of LDH isoenzyme bands on membrane was performed with a Chromoscan 200 (Joyce-Loebl, Team Valley, Gateshead, England) with a 520-nm filter.

Table 2 Characterization of HR, HR-1, and HRA cells in vivo

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of mice</th>
<th>Days of ascites formation*</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>10</td>
<td>41</td>
<td>47</td>
</tr>
<tr>
<td>HR-1</td>
<td>10</td>
<td>64</td>
<td>73</td>
</tr>
<tr>
<td>HRA</td>
<td>10</td>
<td>25</td>
<td>39</td>
</tr>
</tbody>
</table>

* Days when all nude mice had ascites after inoculation.

Table 3 Effects of cisplatin and tranexamic acid on ascites formation in nude mice after i.p. inoculation of tumor

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Untreated group</th>
<th>Cisplatin treated</th>
<th>Cisplatin and tranexamic acid treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0/5 (0)*</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>14</td>
<td>1/5 (20)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>21</td>
<td>3/5 (60)</td>
<td>1/5 (20)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>28</td>
<td>5/5 (100)</td>
<td>2/5 (40)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>35</td>
<td>4/4 (100)</td>
<td>5/5 (100)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>4/4 (100)</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>49</td>
<td>1/1 (100)</td>
<td>2/3 (67)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of mice with ascites. Presence of more than 0.5 ml ascitic fluids in abdominal cavity was confirmed by needle aspiration.

Fig. 10. Adjuvant effect of tranexamic acid on cisplatin survival of HRA cell-inoculated nude mice. After 2 weeks of i.p. injection of 2 × 10⁵ cells into nude mice, cisplatin (2 mg/kg) and tranexamic acid (0.8 g/kg) were administered i.p. every week and twice a week for 4 weeks, respectively. The 50% survivals of untreated, cisplatin-treated, and cisplatin and tranexamic acid-treated groups were 38, 48, and 64 days, respectively. The Mann-Whitney U test was used for statistical analysis.

DISCUSSION

In the present study, we established ascitic cell lines which have the ability to form ascites in nude mice from ascitic fluid of an ovarian cancer patient with a large amount of ascites. The cloned HRA cell line isolated from the parent HR cells formed ascites most rapidly when 2 × 10⁵ cells were i.p. inoculated into nude mice, and all these nude mice were dead within 42 days after inoculation unless therapy was administered.

Advanced ovarian cancer patients have often large quantities of ascitic fluid and their prognoses are poor (1). This fluid is highly fibrinolytic (3, 14). Furthermore, it has been shown that in organ cultures of normal human ovarian tissue only trace amounts of plasminogen activator are released, while malignant tumors originating from the same organ release large amounts in such cultures (15). Such activators have been found in the advancing front of invasive carcinoma (16-18) and are thought to be of importance for the fibrinolytic process necessary for the proliferation of tumor vessels (19). Thus, we attempted to determine the effect of a fibrinolytic inhibitor, tranexamic acid, on these ascitic cell lines in vitro as well as in vivo. Although proliferations of all 3 ascitic cell lines used in this study were dose-dependently inhibited between 2.5 and 20.0 mg/ml of tranexamic acid, the degree of inhibition was most marked in HRA cells. In addition, HRA cells had the most dominant...
capacity to form ascites in nude mice. We have demonstrated that tranexamic acid resulted in not only inhibition of cell proliferation but also morphological changes characterized as enlarged cells with abundant cytoplasm and prominent nucleoli (4).

Recently, cisplatin-based combination chemotherapy has been administered to advanced ovarian cancer patients (20–22). However, patients who have responded to such therapy often relapse and become resistant to drug therapy. Previous preliminary studies have reported that tranexamic acid resulted in elimination of both ascitic fluids in advanced ovarian cancer and pleural effusions in advanced breast cancer (5, 19, 23, 24). In order to further extend these observations, we attempted to determine the adjuvant effect on cisplatin by using the HRA cell-inoculated animal model. A combination of tranexamic acid and cisplatin resulted in a significant inhibition of ascites formation and prolongation of 50% survival in the HRA cell-inoculated nude mice, compared to those of nude mice treated with cisplatin alone or untreated nude mice. Adjuvant effects of tranexamic acid on cisplatin seemed to bring about inhibition of cell proliferation in ascitic fluid and subsequently to result in a decrease of ascitic fluid.

In conclusion, we have demonstrated that the HRA cell line can be used as an experimental therapeutic model for human ascitic ovarian cancers and that tranexamic acid inhibits directly HRA cell proliferation, subsequently inhibiting ascites formation and prolonging survival in HRA cell-inoculated nude mice.

REFERENCES

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Cancer Res 1987;47:592-596.

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