Characterization of Tumorigenicity, Mortality, Metastasis, and Splenomegaly of Two Cultured Murine Colon Lines

Noriyuki Sato, Maria C. Michaelides, and Marc K. Wallack

Section of Surgical Oncology, Department of Surgery, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

The murine colon tumors 26 and 36 have been adapted to culture in vitro from the parental, serially transplanted tumors. The biological activities of these cultured colon lines (C-C26 and C-C36) have been characterized in vivo by s.c. inoculation of serial doses of cells into syngeneic BALB/c mice. The C-C26 line is highly tumorigenic and has a low tendency (9.1%) for metastasis to the lungs. Moreover, mice inoculated with C-C26 exhibited high mortality and normal or atrophied spleens. In contrast, the C-C36 line is less tumorigenic and highly metastatic to the lungs (77.8%). Mortality was lower in animals inoculated with C-C36, and at autopsy, splenomegaly was frequently (72.2%) observed without any visible metastatic nodules in these spleens. Metastasis to the lungs was intimately associated with splenomegaly, and death followed closely. Our findings with the C-C26 and C-C36 lines agree with those reported for the parental tumors with respect to tumorigenicity, tumor growth, and mortality. However, they differ with respect to their metastatic potential, because previous reports showed this to be higher for the serially transplanted colon 26 than the colon 36 tumor cells. Furthermore, this work describes the remarkable splenomegaly observed in mice inoculated with C-C36 but not with C-C26. This finding may in turn provide us with the opportunity to compare the functional status of the spleen in these tumor-bearing animals.

INTRODUCTION

The importance of the appropriate animal tumor models which closely correspond to human cancers is generally appreciated (4, 7, 10). However, except for murine leukemias, the number of such models has been quite limited.

In the last few years, several chemically induced murine colon tumors have been described (1, 3, 9, 17). These tumors represent various levels of differentiation, growth rates, metastatic potentials, and sensitivities to chemotherapeutic agents. They should provide us with a better understanding of the biology of colon cancer (7, 14, 19).

The investigations reported to date with these colon tumors have usually used serially transplanted tumors (1, 9). These tumors were transplanted either as a single cell suspension or as a tumor fragment implant. Cultured murine colon lines would offer both practical and theoretical advantages for extending investigations with these tumors, and very recently, Brattain et al. (2) have described the establishment of murine colon carcinoma cell lines.

In this report, we have compared in vivo the biological activities of C-C26 and C-C36 which we adapted to in vitro culture from the parental, serially transplanted tumors of BALB/c mice (3). These in vitro tumors differ in the lag period between the s.c. inoculation of the cells and appearance of tumor, growth rate, tumorigenicity, metastatic tendency for the lung, and the effect on the host spleen.

While the 2 cultured colon lines differ from each other, they are quite similar to their parental lines in tumorigenicity, tumor growth, and mortality. However, their metastatic tendencies were nearly reversed from those observed for the parental colon tumors. Furthermore, a large number of C-C36 tumor-bearing animals developed splenomegaly, while the C-C26 tumor-bearing mice had normal or even atrophied spleens. This difference may provide us with the opportunity to investigate the immune responses to these tumors and, specifically, the functional status of the spleen in tumor-bearing hosts.

MATERIALS AND METHODS

Mice. Six- to 8-week-old male BALB/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Establishment of Cell Lines. The transplantable colon tumors 26 and 36 in BALB/c mice were kindly provided by Dr. T. H. Corbett and Dr. D. P. Griswold, Jr., of Southern Research Institute, Birmingham, Ala. Colon tumor 26 is a 1,2-dimethylhydrazine-1,2-dimethylurethane-induced tumor and by histopathology an undifferentiated carcinoma (3). Colon tumor 36 is a 1,2-dimethylhydrazine-di-HCl-induced tumor with the histopathology of a well-differentiated adenocarcinoma (3). Both tumors were established to grow in vitro by the spin-out technique of Leibovitz et al. (11) with slight modifications. Briefly, tumors obtained from mice were trimmed to remove necrotic and hemorrhagic portions and minced with scalpels to provide tissue fragments of about 1 cu mm. These minces were washed once, then resuspended in medium, and gently stirred for 30 min at 37°C. The cells obtained from the supernatant were washed twice, then resuspended in medium, and cultured with CO2. The C-C26 line is presently in its 30th passage in vitro, and the C-C36 line is presently in its 130th passage in vitro. These experiments utilized cells from passages 20 to 25 for C-C26 and 120 to 125 for C-C36. Both lines grow in Eagle's modified medium with 5% newborn bovine serum and L-glutamine (292 μg/ml), Fungizone (2.5 μg/ml), and Keflin (20 μg/ml). They are grown and maintained in plastic tissue culture flasks (Costar Models 3275 and 3150; Costar, Cambridge, Mass.).

1 This work was supported in part by NIH Grant 2SO7RR05389-19.
2 To whom requests for reprints should be addressed, at Department of Surgery, Washington University School of Medicine, 4960 Audubon Avenue, St. Louis, Mo. 63110.

Received November 17, 1980; accepted March 9, 1981.
Characterization of Growth in Vitro. Doubling time, adherence to plastics, morphology, and presence or absence of cytoplasmic granules were investigated. The doubling time was determined as follows. One thousand cells/well were seeded into microtiter plates (Falcon Model 3040; Falcon Plastic, Oxnard, Calif.), and the number of cells adherent to these wells was established 24 hr later. Nonadherent cells were removed, the medium was replenished, and the amount of time necessary to increase by 2-fold the number of adherent cells was determined. The adherent properties were determined in a similar manner by counting the number of cells adhering to the bottom of wells of Falcon Model 3040 microtiter plates 24 hr after seeding them with 1 x 10^3 cells/well. In both cases, the wells were fixed and stained with Giemsa for counting.

Preparation of Tumor Cell Suspensions. Single cell suspensions of the tumor cells were obtained by trypsinization (0.05% trypsin-0.02% EDTA) of confluent monolayers (1 to 2 min for C-C36 and 3 to 4 min for C-C26). The cells were washed twice with PBS and resuspended in the appropriate volume of PBS for inoculation. Cell viabilities were usually greater than 90%.

Determination of Transplantation Characteristics. To investigate the tumorgenicity of the cultured colon lines, C-C26 or C-C36 cells were resuspended in PBS as described above and injected s.c. into the back of groups of BALB/c mice (9 to 10 mice in each group) in concentrations ranging from 10^3 to 10^7 viable cells/mouse. Animals were observed daily for up to 4 months for tumor growth and mortality. At weekly intervals, tumor growth was assessed by measuring the width and length of the tumor in 0.1 mm with a caliper and by calculating their average. The values provided in the data are corrected for the average thickness of the skin of uninoculated mice. Autopsies were performed on dead animals for macropathological examination for macroscopic evidence of metastases. The status of the spleens in the tumor-bearing mice was compared macropathologically to spleens from normal animals, and their weights were recorded.

RESULTS

Characteristics of C-C26 and C-C36 in Vitro. Some characteristics of C-C26 and C-C36 in vitro are described in Table 1. The doubling time for C-C26 cells was 15 to 21 hr, and its cellular morphology was fibroblastic, but epithelioid characteristics were often suggested, and these cells were less adherent than were C-C26 cells.

Determination of Transplantation Characteristics of C-C26 and C-C36. For this experiment, groups of BALB/c mice, 10 mice/group, were inoculated s.c. with 10^3 to 10^7 cells of C-C26 or C-C36. Within 2 weeks of inoculation with 10^3 C-C26 cells, all the mice developed tumors about 10 mm in diameter. Animals inoculated with the same dose of C-C36 tumors developed in that time tumors smaller than 5 mm. Thereafter, in each group of mice, both tumors grew rapidly without any regressions (Chart 1, a and b). This shorter latency period for the development of C-C26 tumors compared to C-C36 tumors at the 10^3 dose was also consistently observed at all the other doses as well. For example, 3 weeks after inoculation with 10^4 cells of C-C26, 10 mice had developed palpable tumors, whereas 5 weeks were required for all the mice inoculated with 10^4 cells of C-C36 to develop tumors. It should be noted that none of the mice in this latter case developed tumors within the first 2 weeks. All animals receiving C-C26 cells (ranging from 10^3 to 10^7) developed tumors within 4 weeks of inoculation. One of the mice inoculated with 10^3 C-C36 cells failed to develop a tumor; the rest of the animals receiving C-C36 cells developed tumors within 7 weeks of inoculation (Table 2; Chart 1). Mortality is indicated in Chart 2, and for mice given injection of C-C26, there was a clear dose-response effect; i.e., there was an inverse correlation between the number of cells inoculated and mean animal survival time. This effect held true for the first death noted in each group. This dose-response effect was generally observed with the C-C36 tumor as well, with an interesting exception in the case of the group receiving 10^4...
Table 2

Latent period for palpable tumor development in mice inoculated with varying doses of C-C26 or C-C36 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Dose</th>
<th>1 wk</th>
<th>2 wk</th>
<th>3 wk</th>
<th>4 wk</th>
<th>5 wk</th>
<th>6 wk</th>
<th>7 wk</th>
<th>8 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C26</td>
<td>$10^3$</td>
<td>0</td>
<td>10.0</td>
<td>80.0</td>
<td>100.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>0</td>
<td>50.0</td>
<td>100.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>10.0</td>
<td>90.0</td>
<td>100.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>90.0</td>
<td>100.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>C-C36</td>
<td>$10^3$</td>
<td>0</td>
<td>0</td>
<td>11.1</td>
<td>55.6</td>
<td>77.2</td>
<td>88.9</td>
<td>88.9</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>0</td>
<td>0</td>
<td>40.0</td>
<td>70.0</td>
<td>100.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>0</td>
<td>20.0</td>
<td>100.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>20.0</td>
<td>90.0</td>
<td>100.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>$10^7$</td>
<td>77.2</td>
<td>100.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>

* One hundred palpable tumors.

Development of Metastases and Splenomegaly. To investigate the development of metastasis and splenomegaly as a function of time after tumor inoculation, 2 groups of mice with 30 mice in each group were inoculated with C-C26 ($10^5$ cells/mouse) or C-C36 ($10^6$ cells/mouse). The difference in the number of tumor cells used in each case was determined by the results from the dose-response experiment, which showed C-C26 cells to be more tumorigenic than C-C36 cells. The experimental plan was to sacrifice at weekly intervals 5 mice from each group for pathological examination. The tumor incidence was 100% in both groups, and the first group of C-C26 tumor-bearing mice was sacrificed 2 weeks following tumor inoculation. Since C-C36 is a slower-growing tumor, the first group of 5 mice was sacrificed 4 weeks after tumor inoculation. Animal death was encountered toward the end of this experiment, and the number of mice sacrificed was reduced on 2 occasions (fifth and sixth week) to 3 C-C26-bearing mice; by the 7th week, all animals in this group were dead, so we were unable to continue with this experiment. Since mortality was milder for the C-C36-bearing mice, 5 mice were always sacrificed in this group, except for a single occasion when 6 animals were sacrificed. The results are summarized in Chart 4 in which data from the appropriate groups from the previous experiment are included to determine the relationship between the development of metastasis and mortality. This chart shows the C-C36 cells to be highly metastatic and a positive correlation between metastases and mortality. Death of the C-C36 tumor-
Table 3

Autopsy findings on the frequency of metastasis to the lung and the spleen status of mice bearing C-C26 or C-C36 tumors

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of cases autopsied</th>
<th>No. of cases metastasis</th>
<th>Relation to inoculation dose</th>
<th>No. of cases autopsied</th>
<th>No. of cases of normal or atrophied spleen</th>
<th>No. of cases of enlarged spleen</th>
<th>% of splenomegaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C26</td>
<td>11</td>
<td>1 (9.1) *</td>
<td>10^7</td>
<td>1</td>
<td>0</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^8</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^9</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^10</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^11</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C36</td>
<td>18</td>
<td>14 (77.8)</td>
<td>10^7</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^8</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^9</td>
<td>5</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^10</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^11</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of metastasis.

Table 4

Correlation of pulmonary metastasis to splenomegaly in C-C36 tumor-bearing mice sacrificed weekly

<table>
<thead>
<tr>
<th>Metastasis (+)</th>
<th>No. of cases</th>
<th>% of splenomegaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenomegaly (+)</td>
<td>13</td>
<td>92.3</td>
</tr>
<tr>
<td>Splenomegaly (–)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Metastasis (–)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Splenomegaly (+)</td>
<td>6</td>
<td>50.0</td>
</tr>
<tr>
<td>Splenomegaly (–)</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

The pathological findings on the spleens of all the tumor-bearing mice are summarized in Chart 5. The spleens of the animals inoculated with C-C26 were found to be normal or atrophied. However, the spleens of the C-C36 tumor-bearing mice were enlarged, and there was a direct correlation of the degree of splenomegaly with the time allowed to elapse between tumor cell inoculation and sacrifice. There was no macroscopic evidence of tumor metastasis in these enlarged spleens, and the etiology of the splenomegaly is presently unclear. The correlation between the development of splenomegaly and the development of pulmonary metastases was analyzed, and the results are summarized in Table 4. Upon examination of the C-C36 tumor-bearing animals without any metastases, 50% had enlarged spleens while 92.3% of the animals with metastasis had developed splenomegaly, indicating a positive correlation between these 2 phenomena. Whether cause and effect relationships underlie this positive correlation remains to be determined by future investigations.

DISCUSSION

We have characterized in vivo some biological activities of the cultured murine colon tumors C-C26 and C-C36 which were established from serially transplanted tumors. These 2 cultured colon lines exhibited quite different characteristics when inoculated into mice. The C-C26 cells were very tumorigenic, but their metastatic potential was low. Animals inoculated with these cells exhibited high mortality and normal or atrophied spleens. In contrast, the C-C36 tumor cells were less tumorigenic and highly metastatic to the lungs. In these animals, mortality was milder, and a high incidence of splenomegaly was observed at autopsy without any evidence for metastases to the spleen. The development of splenomegaly and metastases to the lung depended, in both cases, on the time...
Comparison of Two Cultured Colon Lines in Vivo

allowed for tumor development after inoculation. In the C-C36-inoculated mice, death followed closely the development of metastases to the lung, suggesting a causal relationship. This was not true for the C-C26 tumor where metastases were rarely observed.

When the parental, serially transplanted colon tumors were investigated by Griswold and Corbett (9), colon tumor 26 was found to be highly metastatic to the lungs (70 to 100%), while colon tumor 36 was not (<5%). However, our results with the cultured tumors (C-C26 and C-C36) derived from these parental tumors indicated reversed metastatic potentials for these 2 lines, while the other biological parameters in vivo, such as tumorigenicity and mortality, remained the same for each of the cultured lines and its parental tumor. It is not clear how C-C36 has developed such a high metastatic potential. One possible explanation is that, during the adaptation to in vitro culture, cells with a high potential for metastases were selected to grow out from the parental tumor. The reverse may be true for C-C26 which lost its metastatic potential during adaptation. Poste and Fidler (16) have demonstrated that tumor cell populations are heterogeneous in their metastatic potential, even among cell lines which have been established in culture for many years, such as the B-16 murine melanoma cells. Our findings on the metastatic potential of C-C26 and C-C36 are also to be contrasted with those of Brattain et al. (2), who very recently reported the adaptation of the same parental tumors to in vitro culture and found the metastatic properties of their lines to be similar to that of the parental tumors.

We cannot offer an explanation for the differences in metastatic potential observed among these cultured colon lines. However, these findings present us with an opportunity to investigate and compare at the cellular and molecular level the basis for differences in metastatic potential of cultured murine colon lines derived from the same parental tumor. One of many possible explanations for the observed differences in metastatic potentials comes from the observations of Murray et al. (13) that cells with strong adherence characteristics to plastic substrates were prone to metastasize less often than cells characterized as less adherent. It would be interesting to compare our C-C26 and C-C36 with those of Brattain et al. (2) for adherence and other characteristics. From our own data, it can be seen in Table 1 that the highly metastatic C-C36 cells are less adherent than the poorly metastatic C-C26.

These investigations have presented us with another interesting finding, which is the high incidence of splenomegaly found only in the animals bearing the C-C36 tumor. Presently, the significance of splenomegaly in the tumor-bearing host is unclear (6, 12, 20, 21). However, Elgert and Farrar (4), using mice with methylcholanthrene-induced tumors, have reported recently the splenomegaly observed in these animals to be functionally correlated with diminished lymphocyte blastogenesis in vitro. Belnap et al. (1) have shown recently that colon tumor 36 was more immunogenic than was colon tumor 26. While we have not studied the immunogenicity of C-C26 and C-C36 cells, it may be reasonable to consider the high incidence of splenomegaly in C-C36 tumor-bearing mice to be related to the immunogenicity of the tumor cells and to the host responses to this tumor. Furthermore, the fact that the development of tumors and mortality in mice given injection of C-C36 cells were delayed when compared to mice given injection of C-C26 suggests the presence of host responses against the C-C36 tumor cells in these tumor-bearing animals. Moreover, there was a close correlation between the incidence of metastases and splenomegaly in mice given injection of C-C36 tumor cells. It is therefore possible that differences observed in the characteristics of the 2 cultured murine colon cells in vivo may indeed reflect differences in the response of the host to each tumor (5, 7, 8, 15, 18).

In conclusion, the 2 murine lines C-C26 and C-C36 may provide us with useful models for studying human colon cancer and for future immunogenicity and immunotherapy experiments. In addition, host-immune responses to these tumors will be investigated to determine whether they may be responsible for the splenomegaly observed in the C-C36 tumor-bearing animals. Furthermore, the differences in metastatic potential between these colon tumors and the tumors described by Brattain et al. (2) may provide us with the appropriate model for investigation of metastasis.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Suzanne Brennan and Nancy Brada in the care and establishment of the in vitro lines of C-C26 and C-C36 from the parental transplanted colon tumors. We also wish to thank Diane England for her assistance in preparing this manuscript.

REFERENCES


JUNE 1981

2271
Characterization of Tumorigenicity, Mortality, Metastasis, and Splenomegaly of Two Cultured Murine Colon Lines

Noriyuki Sato, Maria C. Michaelides and Marc K. Wallack


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/41/6/2267