Establishment of Murine Endothelial Cell Lines That Develop Angiosarcomas in Vivo: Brief Demonstration of a Proposed Animal Model for Kaposi's Sarcoma

Noriyuki Sato, Takashi Sato, Shuji Takahashi, and Kokichi Kikuchi

Department of Pathology, Sapporo Medical College, S.1, W.17, Chuo-ku, 060 Sapporo, Japan

ABSTRACT

We have reported previously that 100% of the incidence of malignant endotheliomas and angiosarcomas was developed in specific-pathogen-free BALB/c mice given s.c. injections of 1,2-dimethylhydrazine dihydrochloride. These tumors appeared selectively in the liver. In the present study, we have successfully established two cell lines in vitro (D10 and D14) from some of these tumors and have characterized the biological features of these lines. D10 and D14 have a 24- to 26-h doubling time and show a spindle formation in confluent monolayer cultures. The D10 line has a low capability of anchorage-independent growth in soft agar as well as less tumorigenicity in syngeneic mice. When as many as $10^7$ D10 cells were injected s.c. into syngeneic mice, only one-half of the mice developed tumors composed of neoplastic endothelial cells with ample vascular lumina of various sizes. The histological appearance of this tumor resembled that of Kaposi’s sarcoma as a human counterpart. The D14 line, in contrast, has a relatively high capability to grow in the soft agar culture. However, the tumor growth in vivo was less tumorigenic. None of the mice given injections of less than $10^6$ D14 cells developed tumors. Although all of mice given injections of $10^7$ D14 cells developed tumors, these tumors regressed completely until the 6th week after inoculation, suggesting that these cells were immunogenic in the transplantation assays using syngeneic mice. These lines may provide useful information for the study of cytological features of vascular tumors.

INTRODUCTION

There are still no clues for the histogenesis of vascular tumors. Animal models for these tumors as well as established cell lines available for the experiments also have been extremely limited in the past (1–3). In our previous report (4), it was demonstrated that SPF mice given s.c. injections of DMH for 30 weeks developed a 100% tumor incidence of malignant endotheliomas and angiosarcomas of the blood vessels. The tumors appeared selectively in the liver. Although DMH is one of the most reliable carcinogens for experimental colonic carcinogenesis (5–7), the data showed that the colon tumor developed in only 2% of these specific mice. This model reveals that some bacterial flora, missing in these SPF mice specifically, could have a definitive effect on carcinogenesis. Thus the target organ selectivity of carcinogenic activity by DMH may be determined.

On the other hand, it is obvious that this tumor model provides an opportunity to study vascular tumorigenesis by DMH. Our initial efforts were to establish tumor lines in vitro. Of all 50 vascular tumors of the liver, only 2 lines (D10 and D14) were successfully established in vitro. We have characterized some of the biological features of these lines. Both of these lines are less tumorigenic in vivo than was a syngeneic colon tumor C-C38, and only mice given s.c. injections of as many as $10^7$ of these cells developed tumors. The D10 line showed the potential for creating fine blood channels and provided the tumor with histological features resembling those of human Kaposi’s sarcoma (8–11).

These carcinogen-induced vascular tumor cell lines derived from the liver give us an opportunity to understand the biological characteristics of malignant endothelial cells and may suggest the etiological aspects for developing angiogenic sarcomas, including Kaposi’s sarcoma.

MATERIALS AND METHODS

Mice. Male BALB/c SPF mice, 6 weeks old, were obtained from CLEA Japan Co., Shizuoka, Japan, and housed at the facility of SPF animals of Sapporo Medical College. These mice have already been evaluated by CLEA Japan Co. to be free of Pasteurella pneumotropica, Escherichia coli 0115 a,c,k, Pseudomonas Aeruginosa, Salmonella spp., Mycoplasma pulmonis, Giardia muris, Spironucleus muris, and Syphacia spp.

Tumors. Tumor induction by DMH was reported previously (4). All 50 SPF mice receiving the carcinogen developed vascular tumors selectively in the liver and showed hypercellular foci histologically with marked proliferation of endothelial-like cells, consistent with the histological features of angiosarcomas and hemangioendotheliomas. Of 50 mice, 27 were used for trials to establish the in vitro tumor lines.

Establishment and Characterization in Vitro of Cell Lines. Tumors were successfully established in vitro by the spin-out technique (14). Briefly tumors obtained from mice were trimmed to remove necrotic portions and minced with scalpels to provide tissue fragments of about 1 mm$^3$. 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 in 5% CO$_2$ incubator at 37°C. The D10 line is presently in its 80th passage in vitro, and the D14 line is presently in its 70th passage in vitro. These experiments utilized cells from passages 50 to 60 for D10 and 50 to 55 for D14. Both lines grow in Eagle’s modified medium with 5% newborn calf serum and L-glutamine (292 µg/ml). They are grown and maintained in plastic tissue culture flasks (Costar Models 3275 and 3150; Costar, Cambridge, MA). These cells have been tested by Dr. Yoichi Minamijima, Department of Microbiology, Miyazaki Medical College, Kiyotake, Miyazaki, Japan, and were found to be free of murine cytomegalovirus antigens. We studied several of the growth characteristics of these cells in vitro such as doubling time and anchorage-independent growth in soft agar as well as morphological observations. The doubling time was determined as follows. One thousand cells/well were seeded into microtitr plates (Falcon Model 3040; Falcon Plastics, Oxnard, CA), and the number of cells adherent to these wells was established 24 h 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.

Anchorage-independent cell growth in 0.27% soft agar culture was

Received 4/8/85; revised 7/10/85, 9/12/85; accepted 9/16/85.

1 To whom requests for reprints should be addressed.

2 The abbreviations used are: SPF, specific pathogen free; DMH, 1,2-dimethylhydrazine dihydrochloride.
Determined for inocula ranging from $10^2$ to $10^5$ cells of lines, as described previously by several investigators (12, 13). The cell growth was scored microscopically at the 3rd week after plating, and the colony formation was expressed as:

\[
\text{% of plating efficiency} = \frac{\text{No. of clusters} - \text{no. of original cell aggregates}}{\text{No. of viable nucleated cells plated}} \times 100
\]

C-C36 cells were used for soft agar culture controls. The cells have been shown previously to be highly tumorigenic in vivo and in vitro (14).

**Determination of Transplantation Characteristics of Lines.** In order to characterize the tumorigenicity in vivo of these lines, D10 and D14 cells were resuspended in 0.15 M NaCl-10 mM KPO4 (pH 7.2) and were inoculated s.c. into the backs of BALB/c mice (5–6 for each group) in concentrations ranging from $10^6$ to $10^7$ viable cells/mouse. C-C36 cells ($10^5$) were also injected s.c. into more than 10 mice and were utilized to compare tumorigenicity in vivo with those of D10 and D14. At weekly intervals, the tumor development was assessed. In a separate experiment, mice were inoculated with $10^7$ D10 or D14 cells, and the histological features of tumors developed in vivo were also investigated. Mice that had developed tumors from these cells were sacrificed at the 3rd week after inoculation for this experiment.

**RESULTS**

**Establishment of Tumor Lines.** Twenty-seven SPF BALB/c mice given s.c. injections of DMH for 30 weeks were sacrificed, and their vascular liver tumors were processed to grow in vitro. Only 2 tumors of the 27 vascular tumors were adapted successfully to grow in vitro; these were designated as D10 and D14, respectively. The histology of carcinogen-induced original tumors was shown in Fig. 1 (D10) and Fig. 2 (D14). Both of these tumors showed hypercellular foci composed of proliferated endothelial cells with dilated vascular lumina of various sizes. Electron microscopically, so-called Weibel-Palade bodies were observed in the cytoplasm of some tumor cells (data not shown).

**Characteristics of D10 and D14 in Vitro.** Some characteristics of D10 and D14 lines in vitro are described in Table 1. The doubling time of D10 cells was 24 h, and the cellular morphology of these cells was spindle-like without any characteristics of epithelial cells (Fig. 3). The cells adhered well to the plastic surfaces. D14 cells had a doubling time of 25.7 h, and their morphology was also spindle-like and fibroblastic (Fig. 4). They also adhered moderately well to the plastic surfaces. The D10 and D14 lines showed a similar growth in vitro but were different in the anchorage-independent growth of the soft agar culture.

**Anchorage-independent colony growth at the 3rd week of culture of D10 and D14 cells inoculated in soft agar**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Inoculum (cells/9.6-cm² dish)</th>
<th>Plating efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10</td>
<td>$10^7$</td>
<td>4.0 ± 1.2(^a)</td>
</tr>
<tr>
<td>D10</td>
<td>$10^6$</td>
<td>9.6 ± 3.6(^b)</td>
</tr>
<tr>
<td>D14</td>
<td>$10^7$</td>
<td>36.0 ± 4.4(^b)</td>
</tr>
<tr>
<td>D14</td>
<td>$10^6$</td>
<td>21.8 ± 9.0(^b)</td>
</tr>
<tr>
<td>C-C36</td>
<td>$10^7$</td>
<td>87.0 ± 6.2(^b)</td>
</tr>
<tr>
<td>C-C36</td>
<td>$10^6$</td>
<td>55.6 ± 8.0(^b)</td>
</tr>
</tbody>
</table>

\(^a\) See "Materials and Methods."
\(^b\) Mean ± SE.

---

**DISCUSSION**

We have characterized some biological features in vitro and in vivo of the cultured murine endothelial lines, D10 and D14, which were successfully established from hepatic vascular tumors induced by DMH using SPF BALB/c mice (4). These 2 cultured cell lines exhibited a 24–26-h doubling time and a spindle formation on the confluent monolayer culture. The D14 line demonstrated a relatively high capability for anchorage-independent cellular growth in vitro. However, when inoculated into mice at
less than $10^6$ cells, D14 cells could not develop tumors. Even though all mice given injections of as many as $10^7$ cells developed tumors at the 2nd week after inoculation, the tumors regressed until the 5th week. These results may suggest that D14 cells were immunogenic in the syngeneic hosts. In contrast, the D10 line showed a reduced capability for anchorage-independent cellular growth in soft agar as well as less tumorigenicity in vivo in transplantation assays. When inoculated with $10^7$ D10 cells, only one-half of the mice developed tumors at the 2nd week after injections were administered. The histology of D10 and D14 tumors developed in vivo exhibited quite different characteristics. D10 tumor showed a proliferation of bundles of spindle-formed tumor cells, and these intermingled with each other, having fine capillary networks. Some of the capillaries contained a few red blood cells. These histological features of the D10 tumors closely resembled those of the so-called Kaposi’s sarcoma. On the other hand, D14 tumors demonstrated a histologically irregular proliferation of neoplastic endothelial cells, and the tumor cells were arranged in a diffuse pattern and were undifferentiated.

The etiological aspects and histogenesis of vascular tumors are not yet clarified (8, 15). The tissue cultures of endothelial neoplastic cells had limited success in the past (2, 16), and murine endothelial cell lines have been reported rarely in the literature. SPF mice used in the experiment are shown to be free of several microorganisms and showed a 100% incidence of vascular neoplasms selectively in the liver when injected with symmetrical DMH for 30 weeks. The D10 line is very interesting in that this tumor builds up a histological architecture that is extremely similar to that of Kaposi’s sarcoma as a human counterpart. The etiology of Kaposi’s sarcoma remains obscure at this time. It is shown that these patients had a 20-fold increased risk of lymphoreticular neoplasms as compared to the general public. An underlying immunosuppression and immunodeficiency was suggested. Among several infectious agents concomitantly and frequently found in the patients, cytomegalovirus is supposed to be one of the etiological viruses for the development of endothelial neoplasms (3, 17, 18). In fact, the ability of cytomegalovirus to induce neoplastic transformation in cultures of lung tissues from human embryo has been demonstrated (19). In our preliminary electron microscopic study, however, no virus particles have been shown in both the D10 and the D14 cells (data not shown). Furthermore no viral antigens were detected by antimurine cytomegalovirus serum in these cells.

Levy (20), on the other hand, suggested that the high incidence of Kaposi’s sarcoma observed in immunocompromised acquired immunodeficiency syndrome patients is not due to a decreased immunosurveillance but rather to a reactive secretion of immunomodulating factors with angiogenic promoting activity. These factors may enhance capillary proliferation and subsequent transformation of endothelial cells, possibly via cytomegalovirus. Cytomegalovirus-free D10 cells develop by themselves the endothelial neoplasms resembling Kaposi’s sarcoma of a human being, when inoculated into syngeneic mice. This fact may support the theory of autonomous proliferation of neoplastic endothelial cells rather than the possibility mentioned above. Comparison may be inadequate since the D10 line is not a human cell line. It was clearly demonstrated, however, that established D10 and D14 cell lines in the present study may provide us an opportunity to study the cytological characteristics of neoplastic endothelial cells.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Yoichi Minamijima for testing murine cytomegalovirus antigens in these cells.

REFERENCES

Fig. 5. D10 tumor developed in mice. Note that spindle-formed tumor cells proliferated and bundles of tumor cells are intermingled with each other. H & E, x 200.

Fig. 6. High power view of Fig. 5, showing the fine capillary network (arrow) created by neoplastic endothelial cells. A small number of red blood cells are contained in some areas of the lumen. H & E, x 400.

Fig. 7. D14 tumor developed in vivo, demonstrating a relatively diffuse growth of tumor cells. H & E, x 200.
Establishment of Murine Endothelial Cell Lines That Develop Angiosarcomas in Vivo: Brief Demonstration of a Proposed Animal Model for Kaposi's Sarcoma

Noriyuki Sato, Takashi Sato, Shuji Takahashi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/1/362

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.