In Vivo Invasion Assay of Low Passage Cultured Cells Derived from Human Lung Cancer Using Deepithelialized Rat Tracheas Xenotransplanted into Nude Mice

Hisami Yamakawa, Masayuki Baba, and Yutaka Yamaguchi

Department of Surgery, Institute of Pulmonary Cancer Research, School of Medicine, Chiba University, 1-8-1 Inohana, Chiba 280, Japan

ABSTRACT

Human lung tumor-derived cell lines with low passage generation were transplanted into nude mice to determine their growth behavior and invasive potential. Six cell lines (HKT-2, HKT-3, HKT-5, HKT-6, HKT-7, HKT-8) were inoculated into deepithelialized rat tracheas (5 × 10⁴ cells/trachea). After cell inoculation, the tracheas were sealed and transplanted into the subcutis of nude mice. In a parallel experiment, these cell lines (1 × 10⁴ cells) were injected s.c. In the subcutis, the tumor rate of HKT-3, the lowest of all, was only 13% with a long latency period of 18 weeks, and 3 cell lines (HKT-2, HKT-3, HKT-7) did not show any invasive growth to the surrounding tissue. In rat tracheas, all cell lines proliferated within 3 weeks, and 4 of them (HKT-2, HKT-5, HKT-6, HKT-8) showed invasive growth to the tracheal wall within 1–2 weeks. Cells growing in the tracheal wall showed higher [³H]thymidine labeling indexes and greater atypia, such as larger nuclei and prominent nucleoli, than those in the tracheal lumen. The s.c. tumor rate correlated with the incidence of invasive growth to the tracheal wall. The survival of the patients originally bearing the six tumors also correlated closely with the invasive potential of this system. These results indicate that the system using low passage cell lines can evaluate the invasive potential shortly after the inoculation of a relatively small number of cells and can be used as a clinically reliable biological invasion assay.

INTRODUCTION

The investigation of human tumors using nude mouse xenograft models (1–6) is capable of taking into account the host reactions, whereas this is impossible in in vitro systems (7–9). Nude mice offer an invasion environment similar to that of humans; however, s.c. injection procedures for conventional xenotransplantation into nude mice have encountered problems with regard to long latency periods and low tumor take rates (10). Other problems are the difficulties in observing the initial proliferation of cells and in evaluating the invasive potential, since human tissues and cells implanted s.c. into nude mice grow expansively, generally without invading the surrounding tissues (6).

Recently, several new transplantation procedures with satisfactory tumor take rates have been reported (1–5). These models, using deepithelialized rat tracheas transplanted s.c. into nude mice (11–16) or silicone chambers transplanted onto the dorsal muscle fascia of mice (17–20), allow observation of not only normal epithelial cell proliferation but also tumor cell proliferation from early to late invasive stages.

However, the growth behavior of cloned, uniform cells with established high passage cell lines, does not accurately reflect that of the primary tumor in the host. Cancer cells with low passage generation obtained from resected human lung cancer tissues have a heterogeneity (21–23) similar to that of the primary tumor, in contrast to established high passage cell lines consisting of uniform cells as a result of selection.

In the present study, we compared in vivo proliferation of low passage cell lines injected s.c. into nude mice with those inoculated into deepithelialized rat tracheas subsequently xenotransplanted into nude mice. In addition, the invasive potentials of the cell lines as well as the histological differences between invasive and noninvasive sites were examined.

MATERIALS AND METHODS

Animals. Specific-pathogen-free, 4–6-week-old female BALB/c nude mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and used as recipients for cell injection and tracheal xenotransplantation. They were maintained on γ-irradiated feed, and water and cages were autoclaved. The prices were kept in a laminar flow chamber.

Preparation of Tracheas for Cell Inoculation. Tracheas of Fischer 344 rats (Charles River Japan, Inc., Tokyo, Japan) were aseptically removed from anesthetized male rats weighing over 250 g. As described previously (14), after removal, the tracheas were mounted on polyethylene tubing. Deepithelialization was performed by repetitive freezing (~80°C) and thawing (37°C) in HBSS (24) containing gentamicin (50 µg/ml; Sigma, St. Louis, MO) four times.

Cell Lines and Culture Conditions. Six newly established lung cancer cell lines were used: two epidermoid carcinomas (HKT-2, HKT-8); three adenocarcinomas (HKT-3, HKT-5, HKT-6); and one large cell carcinoma (HKT-7) (Table I). They were established as follows. Lung tumors freshly removed from primary lung cancer patients without prior radiotherapy or chemotherapy were minced and connective tissues were removed by nylon mesh filtration in RPMI 1640 (GIBCO, Grand Island, NY) containing gentamicin (50 µg/ml). Throughout these procedures, no digestion by trypsin or cell separation by EDTA was performed. Cell suspensions were cultured in ACB-1 medium (13) containing gentamicin (50 µg/ml) to inhibit the growth of fibroblasts in polystyrene 60-mm tissue culture dishes (Corning Glass Works, Corning, NY). The cells were incubated at 37°C in humidified gassed (95% air, 5% CO₂) incubators (Forma Scientific, Inc., Marietta, OH). After 3 or 4 days the culture medium was changed to remove cellular debris. After the first week, the medium was changed to RPMI 1640 supplemented with 10% fetal calf serum (GIBCO) for continued tumor growth. In the present study, we compared in vivo proliferation of low passage cell lines injected s.c. into nude mice with those inoculated into deepithelialized rat tracheas subsequently xenotransplanted into nude mice. In addition, the invasive potentials of the cell lines as well as the histological differences between invasive and noninvasive sites were examined.

In Vitro Growth Rate Determinations. Cells from the same passage generation used for both the i.t. inoculation and s.c. injection procedures were plated at a density of 1 × 10⁴ cells/polystyrene 60-mm tissue culture dish. Cell counts were performed every second day up to day 20 from triplicate plates. A growth curve was constructed for each cell line, and the population-doubling time was determined from the growth curve prior to reaching a plateau. The abbreviations used are: HBS, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid-buffered saline; LI, labeling index; IT, intratracheal.
Cell Inoculation and Transplantation. The cultured cells of each cell line were harvested by incubation in HBS containing 0.025% trypsin and 0.01% EDTA, washed, and resuspended in RPMI 1640 supplemented with 10% fetal calf serum and gentamicin. The cells (5 x 10^5 cells in 0.1 ml of medium) were inoculated into deepithelialized rat tracheas. The open end of each trachea was then sealed before grafting it subdermally into the back of nude mice (two tracheas/mouse).

Cell Injection s.c. In a parallel experiment, cells (1 x 10^6 cells in 0.1 ml of medium) from each of the lines were injected s.c. into the back of nontransplanted nude mice (two injection sites/mouse).

Observation and Histological Evaluation of Transplanted Tracheas and s.c. Cell Injection Sites. Repopulation and growth were investigated in the tumors of the tracheal transplants. Two to seven tracheal transplants per cell line were removed at random at 4 days and 1, 2, 3, 4, 6, and 8 weeks after xenotransplantation and cell inoculation. They were then fixed in neutral buffered formalin, cut into 10–13 2-mm-thick blocks, and embedded in paraffin. As for the histological evaluation of the transplants, the observation of inoculated cells constituting simple or stratified epithelia on the basement membrane of the trachea was an indication of positive cell growth.

Cell injection sites were palpated, and the latency period until tumor nodules became palpable as well as the number of tumors were recorded weekly. Tumors at injection sites 5 mm in diameter or larger were excised together with surrounding skin and muscles and processed for histological analysis.

Autoradiographic Analysis. At each time point of tracheal excision, the mice were given i.p. injections of 100 µCi ['H]thymidine (22 Ci/mmol; Amersham International Plc, Amersham, Buckinghamshire, England) 1 h before sacrifice. After fixation and embedding in paraffin, sections 3 µm thick were dipped in NR-M2 emulsion (Konika, Tokyo, Japan). After 14 days of exposure at 4°C, the autoradiographs were developed, fixed, and stained with toluidine blue. The ['H]thymidine LI were expressed as numbers of labeled cells/100 total viable cells in the same area. Cells with nuclei showing 5 or more grains were considered to be labeled. Labeled cells among 1000 or more viable cells in any 3 areas or more were counted in the tissues of each cell line removed 4 weeks after xenotransplantation.

Statistical Analysis. The results of the in vivo invasion study were analyzed by the χ² test, and the correlation between s.c. tumor take rate and the incidence of invasive growth to the tracheal wall was determined.

RESULTS

Characterization of Primary Tumors and In Vitro Growth Rate. The degree of differentiation of HKT-5 tumor was poor and those of four other cell lines, except HKT-7, were moderate. None of the tumors had distant hematogenous metastasis, while five of six had several lymph node metastases. At each passage of the cells used for both inoculation and injection, the population-doubling time in vitro varied from 40.0 to 95.3 h (Table 1).

Tumorigenicity in s.c. Cell-injected Sites. All cell lines expressed malignant growth potential by tumor formation in s.c. cell-injected sites. However, the cumulative tumor incidences varied considerably, from 13 to 100%, and the latency period ranged from 1 to 18 weeks (Fig. 1). Three cell lines (HKT-5, HKT-6, HKT-8) showed invasive growth to the surrounding tissues of nude mice after 4, 5, and 3 weeks, respectively. The remaining three (HKT-2, HKT-3, HKT-7) showed no invasive growth up to 12, 25, and 14 weeks after cell injection, respectively. Histological appearances of the tumors induced by s.c. injection were similar to those observed in their primary sites, whereas some differences in the degree of differentiation were observed.

Repopulation and Growth in Tracheal Transplants. All cell lines yielded 100% positive cell growth in the tracheal transplants from 3 weeks after cell inoculation. In every cell line, the repopulation and growth of inoculated cells were initially detected within 1 week, followed then by individually typical growth patterns (Table 2; Fig. 2).

Invasive Growth to the Tracheal Wall and s.c. Connective Tissue. In four cell lines (HKT-2, HKT-5, HKT-6, HKT-8), invasive growth to the tracheal wall was observed within 2 weeks after cell inoculation; tumors invading the surrounding s.c. connective tissue were seen in HKT-6 and HKT-8, but 2 other cell lines (HKT-3, HKT-7) failed to show any invasive behavior even in an 8-week observation period. The cumulative incidences of invasive growth of each cell line varied from 0 to 60%. In the studies of the relationship between the incidence of invasive growth of the cell lines and the patients’ survival after operation, the patients from whom 2 noninvasive cell lines (HKT-3, HKT-7) were derived showed better prognoses than the others, regardless of the extent of primary tumors (Table 3). A higher tumor incidence in the subcutis tended to correlate with a higher incidence of invasive growth in tracheal transplants (Fig. 3). In all 4 cell lines with invasive growth, the cells invading the tracheal wall presented more highly malignant features such as enlargement of nuclei and nucleoli as well as
IN VIVO INVASION ASSAY USING RAT TRACHEAL TRANSPLANTS

higher nuclear:cytoplasmic ratios, and also formed less differentiated nests, than those observed in the tracheal lumen (Fig. 4).

[3H]Thymidine Labeling Index. There was no difference in the LI of noninvasive sites between the cell lines that were not invasive and those that were invasive. HKT-5 and HKT-8 had relatively high (LI (10.97 and 15.57) in the invasive sites reflecting the high tumor incidence (100%) in the subcutis. In most cases (except HKT-2), LI was significantly higher than that of noninvasive sites (Table 4; Fig. 5).

DISCUSSION
The most important feature of the nude mouse xenograft model is that the transplanted tumor cells or tissues have a sufficiently high tumor take rate to be analyzed. Indeed, one major advantage of the present transplantation system com-
IN VIVO INVASION ASSAY USING RAT TRACHEAL TRANSPLANTS

Table 3 Patients' survival and incidence of invasive growth of tumors induced by inoculation of cancer cell lines into deepithelialized rat tracheas

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Outcome (^a)</th>
<th>4 days</th>
<th>1 wk</th>
<th>2 wk</th>
<th>3 wk</th>
<th>4 wk</th>
<th>6 wk</th>
<th>8 wk</th>
<th>%</th>
<th>Total incidence of invasive growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKT-2</td>
<td>5 mo dead</td>
<td>0/2</td>
<td>0/4</td>
<td>2/6</td>
<td>0/4</td>
<td>2/4</td>
<td>1/2</td>
<td>0/2</td>
<td>21</td>
<td>5/24</td>
</tr>
<tr>
<td>HKT-3</td>
<td>28 mo alive</td>
<td>0/4</td>
<td>0/4</td>
<td>0/6</td>
<td>0/7</td>
<td>0/4</td>
<td>0/4</td>
<td>0/2</td>
<td>0</td>
<td>0/29</td>
</tr>
<tr>
<td>HKT-5</td>
<td>18 mo dead</td>
<td>0/2</td>
<td>1/4</td>
<td>3/6</td>
<td>2/4</td>
<td>2/4</td>
<td>1/2</td>
<td>1/2</td>
<td>42</td>
<td>10/24</td>
</tr>
<tr>
<td>HKT-6</td>
<td>3 mo dead</td>
<td>0/2</td>
<td>1/6</td>
<td>5/5</td>
<td>2/4</td>
<td>3/4</td>
<td>2/2</td>
<td>2/2</td>
<td>60</td>
<td>15/25</td>
</tr>
<tr>
<td>HKT-7</td>
<td>23 mo alive</td>
<td>0/3</td>
<td>0/4</td>
<td>0/6</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/2</td>
<td>0</td>
<td>0/25</td>
</tr>
<tr>
<td>HKT-8</td>
<td>3 mo dead</td>
<td>0/2</td>
<td>0/4</td>
<td>2/6</td>
<td>3/4</td>
<td>4/4</td>
<td>2/2</td>
<td>1/1</td>
<td>52</td>
<td>12/23</td>
</tr>
</tbody>
</table>

\(^a\) Patients' survival after operations.

\(^b\) Number of positive tracheas/number of inoculated tracheas.

Table 4 Number of \(^3\)Hthymidine-labeled cells in tumors growing in rat tracheas

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Invasive site</th>
<th>Noninvasive site</th>
<th>(P) ((x^2) test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKT-2</td>
<td>5.78</td>
<td>3.99</td>
<td>NS(^a)</td>
</tr>
<tr>
<td>HKT-3</td>
<td>NI</td>
<td>2.37</td>
<td></td>
</tr>
<tr>
<td>HKT-5</td>
<td>10.97</td>
<td>2.91</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HKT-6</td>
<td>4.28</td>
<td>1.69</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HKT-7</td>
<td>NI</td>
<td>4.02</td>
<td></td>
</tr>
<tr>
<td>HKT-8</td>
<td>15.57</td>
<td>11.87</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\(^a\) NS, not significant; NI, did not invade.

Fig. 3. Correlation between s.c. tumor take rate and the incidence of invasive growth to the rat tracheal wall. Coefficient of correlation \((r)\) was 0.8458. Symbols are the same as for Fig. 1.

Fig. 4. Histological cross-sections of invasively growing cells 2 (A) and 6 (B) weeks after i.t. inoculation. Arrowhead, luminal side. N, I, and S, noninvasive, invasive, and stromal cells, respectively. A, HKT-5. Note the enlargement of nuclei and prominent nucleoli in tumor cells of invasive site. H & E, \(\times 66\). B, HKT-6. Note the difference between the tumor cells in the tracheal lumen (noninvasive site) and those in the tracheal wall (invasive site). Goblet-like cells grew in cribriform pattern in the tracheal lumen; in contrast, cells with large nuclei and dark cytoplasm grew in the tracheal wall. H & E, \(\times 66\).

Fig. 5. Cells proliferating in the noninvasive site (A) and invasive site (B) 4 weeks after i.t. inoculation of HKT-5 cells. Note the comparatively many \(^3\)Hthymidine-labeled cells (arrowhead) in the invasive site. Toluidine blue, \(\times 132\) (A), \(\times 264\) (B).

Compared to the s.c. injection method is that tumor cell proliferation in the rat trachea, confirmed of its human nature by in situ hybridization on the same transplantation system (25, 26), can be observed with 100% certainty within 4 days to 3 weeks after
i.t. cell inoculation. A further advantage of this system was that the number of cells required for proliferation in the rat trachea is less than one-half of that generally used in s.c. injection; our results with low passage cell lines were identical to those previously reported with established high passage cell lines (14).

No clear correlation was found between the s.c. tumor take rate and the incidence of positive growth in the rat tracheal lumina or in vitro growth rate. The s.c. tumor take rate, however, seemed to depend on the incidence of invasive growth into the tracheal wall. This suggests that cells with a low s.c. tumor take rate have a low invasive growth potential in the present i.t. inoculation system.

A comparison of the four invasive cell lines showed evidence of invasion into the rat tracheal wall within 1 or 2 weeks after cell inoculation, with the total positive invasion rates varying from 21 to 60%. Thus, the present system permits early detection of invasive potentials. In addition, quantitative analysis of the invasive potential of cell lines can also be performed through prolonged observation. Of 63 tumors that we initially attempted to culture in vitro, only 6 tumors were able to give rise to cell lines and they were successfully studied in this invasion assay. To clarify the application of this invasion assay to all operated cases of lung cancer, we are now directly inoculating the fresh tumor cell suspensions, without in vitro culture, surgically removed from lung cancer patients into deep epithelialized rat tracheas xenotransplanted into nude mice.

Another advantage of this system is that it permits a comparison of invasive and noninvasive sites. Tumors growing in invasive and noninvasive sites not only have morphological differences, but also autoradiographic examinations using [3H] thymidine have demonstrated that cells growing in the former have greater proliferative activities.

It is generally accepted that highly malignant cell populations are selected during long term culture both in vivo and in vitro. It is speculated that only the highly malignant cells capable of proliferating in the subcutis will be selected even when cells with in vitro low passage generation are injected s.c. into nude mice. In the rat tracheal lumina, however, cells which cannot proliferate in the subcutis might also proliferate.

Histological examination of the epithelia formed on the basement membrane of the rat trachea in the early stage revealed uniform cells with mild to moderate atypia. Thereafter, however, cells invading the tracheal wall displayed a higher degree of atypia than those proliferating on the basement membrane. This indicates that the differences in cellular morphology among noninvasive sites in the rat tracheal lumen, invasive sites in the rat tracheal wall, and the subcutis in nude mice may be due to the differences in environment. Cellular differentiation and morphological change have been reported to be affected by environmental factors in vivo, such as the implantation site (27-30). Since the tracheal lumen possesses free space, it offers the least mechanical restrictions against tumor growth. Conversely, in the subcutis and the tracheal wall, growth potential is expressed in an expansive or invasive manner, meaning that cells proliferating in these sites undergo genotypic and/or phenotypic changes to attain their optimum growth potential.

In conclusion, the present system is considered to be useful because it permits tumor cell proliferation with small cell numbers, a comparison between cells in invasive and noninvasive sites on tissue sections, and studies of invasive potential taking into account host factors. Further investigations with fresh tumor cell suspensions surgically removed from patients are necessary to clarify its reliability in predicting the clinical outcome in humans.

ACKNOWLEDGMENTS

The authors wish to thank Professor Y. Hayashi, Department of Pathology, Institute of Pulmonary Cancer Research, Chiba University, for fruitful discussion. We wish to thank T. Umemiya and M. Nemoto for excellent technical support and H. Oikawa for secretarial help.

REFERENCES


IN VIVO INVASION ASSAY USING RAT TRACHEAL TRANSPLANTS

In Vivo Invasion Assay of Low Passage Cultured Cells Derived from Human Lung Cancer Using Deepithelialized Rat Tracheas Xenotransplanted into Nude Mice

Hisami Yamakawa, Masayuki Baba and Yutaka Yamaguchi


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/50/22/7358