Establishment and Characterization of a Human Lung Cancer Cell Line NCI-H460-LNM35 with Consistent Lymphogenous Metastasis via Both Subcutaneous and Orthotopic Propagation

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ABSTRACT

Lymphogenous metastasis is a common feature of human lung cancers, but very little is known about the underlying mechanism. In the present study, in vivo selection was carried out to obtain a highly lymphogenous metastatic subline of a human large cell carcinoma of the lung, NCI-H460. The resulting subline, termed NCI-H460-LNM35 (LNM35), was shown to metastasize to regional lymph nodes with a 100% incidence not only as a result of orthotopic intrabronchial (i.b.) implantation, but also as a result of conventional s.c. implantation. LNM35 has a short latency period, allowing for the collection of experimental data within 28 days after i.b. inoculation and 45 days after s.c. inoculation. It was noted that orthotopically i.b.-propagated LNM35 closely mimicked the clinical manifestations of human lung cancer patients by infiltrating into lymphatic vessels and metastasizing to the mediastinal lymph nodes. The LNM35 cell line is, to the best of our knowledge, the first human lung cancer cell line to be reported as having lymphogenous metastatic properties, and the observed 100% incidence by s.c. inoculation gives LNM35 a significant advantage even over previously reported human cancer cell lines of other origins. Comparisons between LNM35 and its parental NCI-H460 cell lines were also made with regard to expression levels and/or activities of various molecules that are thought to play a part in the metastatic process. We show here that the expression of cyclooxygenase 2 is increased in LNM35 and that a specific cyclooxygenase 2 inhibitor, nimesulide, can inhibit the invasion of LNM35 in vitro through Matrigel containing basement membrane components.

INTRODUCTION

It has now been clearly established that lung cancer is a disease caused by the accumulation of multiple genetic alterations in both oncogenes and tumor suppressor genes (1). Despite considerable advances in the understanding of the molecular pathogenesis of lung cancer, only one in eight patients diagnosed as having lung cancer can be cured at present, while the rest of the cases eventually fail because of widespread metastases (2). The degree of lymphogenous spread is known to be an important parameter for the staging and assignment of treatment and useful for the assessment of patients’ prognoses (3). An inverse correlation between the extent of lymph node metastasis and postoperative survival of lung cancer patients (4) suggests that lymphogenous metastasis reflects the malignant potential of tumor cells and contributes to fatality.

The expression of certain molecules, such as adhesion receptors and ligands (5–17) as well as metalloproteinases (18–22), has been suggested to play a role in the development of metastatic lesions. Metastasis occurs via two distinct pathways, and tumor cells spread through blood and LVs (5). Although a large number of studies have been conducted, yielding considerable information about the metastatic processes, very little is known about how cancer cells propagate lymphogenous metastasis. Identification of molecules with a crucial role in the lymphogenous spread of cancer cells has been hampered by lack of an appropriate experimental model system. To date, a few cell lines derived from several types of human malignancies have been reported to have a high potential to metastasize regional lymph nodes when the tumor cells were injected at orthotopic sites (23–29), although they were found to metastasize to a much lesser extent when propagated s.c. However, no cell lines have been reported thus far as being useful for studies of the lymphogenous metastasis of human lung cancers.

In the present study, we describe an in vivo selection resulting in the establishment of a human lung cancer cell line, NCI-H460-LNM35 (LNM35), which consistently and spontaneously metastasizes to lymph nodes when injected either s.c. or orthotopically. Comparisons between LNM35 and its parental H460 cell lines are also made with regard to expression levels and/or activities of various molecules that are thought to play a part in the metastatic processes. We show that expression of COX-2 is increased in LNM35 and that a specific COX-2 inhibitor, nimesulide, can inhibit the invasion of LNM35 in vitro through Matrigel containing basement membrane components.

MATERIALS AND METHODS

Animals and Cell Lines. Five-week-old female athymic nude mice and SCID mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. The NCI-H460 (H460) cell line at passage 136 (ATCC HTB 177), which was originally established by Carney et al. (30), was obtained from the American Type Culture Collection (Rockville, MD). NCI-H460 is a human large-cell lung carcinoma line with mutant K-ras and wild-type p53 (31, 32). Derivation by in vivo selection of the high-lung-metastatic LuM1 and low-lung-metastatic NM11 sublines derived from a murine colon adenocarcinoma 26 tumor cell line was described previously (22, 33, 34). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

In Vivo Selection. In vivo selection was carried out to establish a high-lung-metastatic subline of H460, using the procedures described by Fidler (35) and also in a previous study of ours (22). In brief, 1.0 × 10⁶ of the parental H460 cells in 100 µl of serum-free RPMI 1640 medium were injected in the s.c. tissue of the left abdominal wall of 7-week-old female SCID mice. Lung tissues containing the metastatic tumor cells were excised, minced, and reim- planted in the abdominal wall of new recipient mice for the selection of high-metastatic tumor cells. After two rounds of in vivo selection by means of sequential implantations, metastatic nodules in the lung tissues were harvested.

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to initiate *in vitro* culture of the metastatic tumor cells. Further selection was then carried out by injecting the resulting cell line into s.c. tissue, followed by *in vitro* propagation of tumor cells obtained from spontaneous ALN metastasis. A clonal cell line was established from the cultured cell mixture with the propagation of tumor cells obtained from spontaneous ALN metastasis. Then carried out by injecting the resulting cell line into s.c. tissue, followed by *in vivo* propagation of tumor cells obtained from spontaneous ALN metastasis. A clonal cell line was established from the cultured cell mixture with the propagation of tumor cells obtained from spontaneous ALN metastasis.

**Spontaneous Metastasis Assay.** Cells (1.0 × 10^3) in 100 μl of serum-free RPMI 1640 medium were implanted in the s.c. tissues of the left abdominal wall of 7-week-old female SCID mice or nude mice. At 45 days (SCID mice) or 55 days (nude mice) after s.c. implantation, mice were sacrificed by cervical dislocation under deep anesthesia, and internal organs, including lung, liver, kidney, and spleen as well as lymph nodes and s.c.T.s, were resected. The resected specimens were weighed, fixed with 4% paraformdehyde, and processed for light microscopic examination of the paraffin-embedded sections stained with H&E. The lung-metastatic nodules were examined and counted under a dissecting microscope.

**Orthotopic Implantation.** Mice were anesthetized by i.p. injection with 0.28 mg/kg body weight of 2,2,2-tribromoethanol (Aldrich Chemical Company, Milwaukee, WI). A 1-cm long ventral midline incision was made in the neck to expose the trachea for direct inspection of the orotracheal intubation of a 20-gauge catheter/needle unit (Terumo, Tokyo, Japan), which was advanced through the oral cavity to a depth of 2.1 cm from the incisor teeth under visual inspection through the exposed trachea. The needle was then pulled out, leaving only the outer 20-gauge catheter, through which a blunt-ended 25-gauge needle (Top, Tokyo, Japan) was inserted a depth of 4.4 cm. Next, 1.0 × 10^3 of the cultured LNM35 cells in 50 μl of serum-free RPMI 1640 medium were directly inoculated through the inserted needle into the bronchial artery or the left ventral aortic vein, and the skin incision was closed with two stitches. The mice were sacrificed as described above 28 days after orthotopic implantation, and lung and mediastinum were removed *en block* and fixed with 4% paraformaldehyde. Metastasis to the MLNs was examined under a dissecting microscope and confirmed by histological examination of paraffin-embedded sections stained with H&E.

**Growth Curves.** Cells (1.0 × 10^3) were inoculated onto 3.5-cm dishes. At daily intervals, triplicate samples were harvested and counted with a Coulter counter (Coulter Electronics, Luton, United Kingdom), and cell numbers were averaged for each time interval.

**Antibodies.** SNH-3 (specific to sialyl Lewis X), 2F3–6 (specific to sialyl Lewis X-variant), and 2D-3 (specific to sialyl Lewis A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metalloproteinases A (MMP-2, MMP-9), and matrix metallog
noted that the number of lung-metastatic nodules in mice inoculated with LNM35 cells was markedly larger than that seen in mice injected with parental NCI-H460 cells (Table 1). No spontaneous metastasis in any other organ was observed in either parental H460 or LNM35 cells.

These experiments were carried out, and both macroscopic and histological examinations confirmed 100% occurrence of lymph node metastases of LNM35 in marked contrast to the complete absence of such occurrence in the case of parental H460 (Table 1). LNM35 cells have been maintained in culture for 1 year without noticeable changes in their lymphogenous metastatic potential.

Orthotopic Propagation of LNM35. We examined whether LNM35 exhibits regional lymph node metastases when propagated orthotopically by using the i.b. implantation technique with modification to the original one previously described by McLemore et al. (39). Our modified technique, which employs orotracheal intubation, is relatively easy and requires an average of 10 min for completion. Table 2 summarizes the observed frequencies of successful tumor propagation in the lung and those of histologically confirmed metastases to the MLNs in cases with i.b.-implanted tumors (Table 2 and Fig. 3). Histological examination also showed intravasation of LNM35 cells into the LVs (Fig. 3, i.b.T., arrows) and blood vessels (data not shown) of the lung as well as into the lymphatic afferent vessels of MLNs (LV in Fig. 3, MLN). These macroscopic and microscopic features of this orthotopic propagation model are similar to those seen in lung cancer cases, which suggests that the lymphogenous metastatic processes of LNM35 mimic those occurring in patients.

Expression of Gelatinases and Their Specific Inhibitor and of Adhesion Molecules in LNM35 and Parental H460. Zymographic examinations of the concentrated conditioned medium revealed that...
LNM35 and H460 cells did not secrete either gelatinase A or B at appreciable levels, whereas Western blot analysis of TIMP-2 showed that the amount of TIMP-2 secreted by LNM35 cells was similar to that secreted by parental H460 cells (data not shown). Control experiments using a murine colon cancer cell line (LuM1) with high lung-metastatic potential and its low metastatic counterpart, NM11, confirmed a high expression of gelatinase B in the former and secretion of a comparatively large amount of TIMP-2 in the latter. FACS analysis revealed that the α2, α3, α5, α6, αv, and β1 subunits of integrins were expressed in both LNM35 and H460 at similar levels, whereas neither expressed the α1, α4, β2, and β3 subunits (data not shown). Expression levels of CD44 were similar for LNM35 and H460, whereas neither cell line expressed E-cadherin or the ligands for E-selectin, sialyl Lewis X, sialyl Lewis X-variant, or sialyl Lewis A (data not shown). We conclude that the observed significant differences between the metastatic potentials of LNM35 and H460 could not be accounted for by the differences in expression patterns of either the gelatinases and their specific inhibitor or of the adhesion molecules and carbohydrate chains examined thus far.

**COX-2 Expression and Effect of Nimesulide on in Vitro Motility and Invasion.** Because our previous immunohistological studies of COX-2 expression in human lung cancer patients suggested a possible association of the increase in COX-2 expression with invasion and metastasis as well as with poor prognosis, expression of COX-2 was also examined in LNM35 and H460. Northern blot analysis showed that COX-2 expression was significantly increased in LNM35 when compared with that in H460 (Fig. 4A). As an initial step toward elucidation of the potential relationship of the increased expression of COX-2 with the highly metastatic phenotype of LNM35, we examined the effects of a specific COX-2 inhibitor, nimesulide, in LNM35 in vitro. Nimesulide was shown to be potent in the inhibition of invasion through Matrigel as well as of cell motility in a dose-dependent manner at concentrations significantly lower than that required for the inhibition of cell growth (Fig. 4B).

**DISCUSSION**

In the present study, we successfully established a human lung cancer cell line, LNM35, which is capable of spontaneous metastasis to lymph nodes with a 100% incidence. A number of tumor cell lines have been shown to possess hematogenous metastatic potential and proved to be useful for studies of underlying mechanisms and in the search for new therapeutics (22, 35, 40–43). However, only a few human cancer cell lines have been described in the literature thus far as being useful for studies of lymphogenous metastasis (Refs. 23–29; Table 3). The LNM35 cell line is, to the best of our knowledge, the first human lung cancer cell line to be reported as having such a biological property. Moreover, LNM35 has significant advantages over previously reported cell lines. LNM35 spontaneously metastasizes at a 100% incidence not only as a result of orthotopic i.b. propagation but also as a result of conventional s.c. injection. This proven highly reproducible nature of lymphogenous metastases makes LNM35 very different from others because s.c. inoculation of other
introduction of the COX-2 gene may intensify invasiveness of colon and lung adenocarcinomas (45, 47, 48), it has also been reported that suggested to play a significant role in the carcinogenesis of colorectal cinomas (46). Although an increase in expression of COX-2 has been shown that COX-2 expression may be associated with a poor prognosis in lung cancer cells infiltrating into the surrounding stromal tissues and because we previously found that COX-2 is expressed intensely in significantly increased in LNM35. We could also show that motility and however, allowed us to identify that COX-2 expression was signifi-
can in vivo in a human esophageal cancer cell line. Cancer Res.,

**LYMPHOGENOUS METASTATIC LUNG CANCER LINE LNM35**

Table 3 Comparison of LNM35 with other human cancer cell lines previously reported as having lymphogenous metastatic potentials

<table>
<thead>
<tr>
<th>Human cell line</th>
<th>Tumor type</th>
<th>Implantation</th>
<th>Lymp node metastasis incidence (%)</th>
<th>Days to autopsy</th>
<th>References</th>
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</thead>
<tbody>
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<td>LNM35</td>
<td>Lung cancer</td>
<td>Ectopic (s.c.)</td>
<td>100</td>
<td>45</td>
<td>This study</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate cancer</td>
<td>Ectopic (s.c.)</td>
<td>100b</td>
<td>28</td>
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<tr>
<td>MDA-MB-435</td>
<td>Breast cancer</td>
<td>Ectopic (s.c.)</td>
<td>0</td>
<td>112</td>
<td>(26)</td>
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<tr>
<td>LNCaP</td>
<td>Prostate cancer</td>
<td>Ectopic (s.c.)</td>
<td>100</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>FEMX-1</td>
<td>Melanoma</td>
<td>Ectopic (s.c.)</td>
<td>100</td>
<td>90</td>
<td>(27)</td>
</tr>
<tr>
<td>MeWo</td>
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<td>80</td>
<td>50</td>
<td>(25)</td>
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<tr>
<td>SUIT-2</td>
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<td>Ectopic (s.c.)</td>
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<td>21</td>
<td>(23)</td>
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<tr>
<td>M4Be</td>
<td>Melanoma</td>
<td>Ectopic (s.c.)</td>
<td>50</td>
<td>112</td>
<td>(28)</td>
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<tr>
<td>SUIT-2</td>
<td>Melanoma</td>
<td>Ectopic (s.c.)</td>
<td>20</td>
<td>90</td>
<td>(24)</td>
</tr>
</tbody>
</table>

* pro., intraprostatic injection; m.f.p., injection into the mammary fatpad.

# ACKNOWLEDGMENTS

We thank Drs. H. Nakanishi and S. Shimizu for their helpful suggestions. We are also grateful to Drs. R. Kannagi, J. L. Strominger, and L. J. Old for their generous gifts of monoclonal antibodies.

**REFERENCES**


LYMPHOGENOUS METASTATIC LUNG CANCER LINE LNM3


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_Cancer Res_ 2000;60:2535-2540.

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