Extrapulmonary, Tissue-specific Metastasis Formation in Nude Mice Injected with FEMX-I Human Melanoma Cells

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ABSTRACT

FEMX-I human malignant melanoma cells, originating from a lymph node metastasis in a patient, uniquely and selectively produced extrapulmonary metastases after i.v. injection of cells prepared from xenografts into adult, nude mice. After a lag time of approximately 50 days, metastases were observed in s.c. sites at the back and front of the neck, and in axilla and inguinal regions. Tumor colony formation in lungs were never detected. The interscapular tumors showed a close relationship to brown fat, partly infiltrating this tissue, whereas the other s.c. tumors seemed to be localized to lymph nodes. Mesenterial and mediastinal lymph node metastases were frequently found, together with retroperitoneal tumors along the spine. The normal cells of the adrenal medulla were often replaced by melanoma cells, whereas the cortical tissue was not affected. The conclusion that FEMX-I cells possess an inherent ability for tissue-specific metastasis formation is supported by the metastatic pattern seen after i.p. and intrasplenic injection, as well as after inoculation of the cells in the footpads of the mice. The relatively slowly growing FEMX-I tumors showed the same differentiated morphology as the patient's tumor, independent of the site of growth and the number of passages in the animals. The FEMX-I tumor was easily established as a cell line in vitro. Such cells showed a strongly reduced metastatic capacity, indicating that the in vitro growth conditions had induced alterations in the FEMX-I cells influencing their ability to form site-specific metastases, changes that were shown to be reversible. It is suggested that structures on the surface of the tumor cells, as well as growth factors in the host tissues, may be of importance for the observed tissue specificity. The FEMX-I melanoma, which, as a human tumor in nude mice, has a unique metastatic pattern, offers possibilities for investigating mechanisms involved in site-specific metastasis formation, as well as for testing effects of antitumorigenic, chemotherapeutic, and immunotherapeutic agents against human extrapulmonary micro- and macrometastases.

INTRODUCTION

Many widely accepted concepts on mechanisms involved in metastasis formation are based on data obtained in experiments involving rodent tumors (1–7). Important biological differences may exist, however, between rodent and human tumors, and progress in this area has been hampered by the unavailability of suitable models involving human cancers.

The athymic, nude mouse has proved to be an excellent host for studying the biology and chemosensitivity of human tumors, usually grown as s.c. xenografts (8–16). However, a shortcoming of the model is that spontaneous metastasis is a rare event (17–19), most often seen in young, immunologically immature or immunosuppressed mice (18, 20–22), and often only after s.c. injection of the cells (13, 18), a procedure that may result in accidental inoculation of tumor cells directly into blood vessels.

Recently, however, several tumors reproducibly giving metastases in adult nude mice have been reported (20, 23–30). Many of these cases involve orthotopic injection of the tumor cells, i.e., injection at a site relevant for the tumor in question, such as the gut (colon cancer) and the kidney (renal cancer). In other cases the cells are injected i.s. or i.v., the latter method used to obtain lung colonies. Common to these new models is that the metastases usually appear in the tissues where circulating tumor cells encounter the first lymph node or blood capillary bed. It is possible, therefore, that some of the tumors merely represent growth of passively trapped, but invasively growing cells.

We have recently developed a human melanoma model in nude mice, LOX, which is suitable for studying factors involved in lung colony formation, and useful also for chemosensitivity studies (24, 25). Here we report a new metastasis model involving a human malignant melanoma that gives an entirely different metastatic pattern. After i.v. injection of FEMX-I tumor cells, extrapulmonary metastases develop in a reproducible and apparently tissue-specific manner within a period of about 50 days. The new model provides unique possibilities for studying mechanisms involved in homing and growth of metastatic human tumor cells in specific tissues.

MATERIALS AND METHODS

Mice. BALB/c athymic, nude mice were purchased from the Laboratory Breeding and Research Center, G1. Bomholtgaard, Ry, Denmark. NMRI and triple immune-defective NIH-III (Fodstad, 1987) nude mice were bred in our own nude mice facility. The animals were kept in laminar air-flow rooms at constant temperature (24–26°C) and humidity (30–50%). In some experiments the mice were placed in laminar air-flow benches in plastic cages with autoclaved filter tops. The food and bedding were sterilized and the animals were given tap water ad libitum in sterilized bottles.

Tumor Lines. The FEMX tumor originates from an inguinal metastasis which appeared in a 52-year-old Caucasian female 2 years after the removal of a melanotic malignant melanoma from the patient's big toe. First a xenograft tumor line was obtained by implantation of tissue from the metastasis, and cells from an early xenograft passage were used to establish a continuous cell line in vitro (31). Such cells were later injected back s.c. into nude mice, resulting in a new xenograft line, denominated FEMX. After i.v. injection into nude mice of FEMX cells, s.c. metastases developed in one animal, and this tissue was used to establish the FEMX-I line in vivo and in vitro.

i.v. Injection of Tumor Cells. Single cell suspensions prepared (32) from s.c. tumors (8–12 mm), from EDTA-treated monolayer cultures, and from ascites fluid withdrawn from mice with i.p. tumors, were diluted in medium to appropriate concentrations of tumor cells. Viable cells, usually 1 x 10⁶, were injected i.v. in volumes of 0.2–0.3 ml into groups of athymic mice. The mice were checked 3–4 times weekly and observed for visible metastases and general health condition.

Cell Cultivation in Vitro. FEMX-I monolayer cultures were established by preparing single cell suspensions from s.c. xenografts. The cells were suspended in RPMI medium containing 10% fetal calf serum, seeded in Falcon flasks (25 cm²) and incubated at 37°C. Seeded FEMX-I cells rapidly attached to the plastic and a permanent cell line was established.

The abbreviations used are: i.s., intrasplenically; NK, natural killer.

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RESULTS

Establishment of the FEMX-I Metastatic Tumor Line. The first s.c. metastasis appeared in the interscapular region in one of three nude mice 58 days after i.v. injection of $3 \times 10^6$ FEMX cells obtained from s.c. xenografts (second passage). When the mouse was sacrificed 15 days later, metastases were observed also in the submandibular, the left inguinal, and the right hip regions (Fig. 1). Tissue from the large neck tumor was used for s.c. transplantation into nude mice, and for preparation of single cells used in attempts to establish a monolayer cell culture. The resulting s.c. and in vitro lines were denominated FEMX-I.

When i.v. injection of cells from FEMX xenografts were repeated several times, metastases developed in approximately 10% of the animals (data not shown). The FEMX-I xenograft line has shown a stable growth rate (volume doubling time of approximately 7 days) during serial transplantation in nude mice for more than 20 passages.

Frequency and Tissue Distribution of FEMX-I Metastases. In contrast to the situation with FEMX cells, metastases develop in nearly all animals injected i.v. with single cells obtained from FEMX-I xenografts. The results from four different experiments are shown in Table 1, together with data on the distribution of metastases in different tissues and the lag time from the day of injection until the appearance of measurable s.c. tumors.

In most animals metastases were first observed in the interscapular region of the mice, i.e., at a location where mice are not known to have lymph nodes. The s.c. metastases grew to a size of 5–7 mm within approximately 50 days, with a range of mean lag times varying from 41.9 to 63.0 days in different experiments (Table 1).

In some animals the tumors first appeared in the submandibular area or in the axilla/inguinal region. In a few animals metastases were seen in the shoulder/hip region, away from the site of known lymph nodes. At the time of autopsy (80–120 days after injection of the cells), metastases were frequently observed in mesenterial lymph nodes, often in the adrenals and in retroperitoneal lymph nodes. Mediastinal tumors occurred regularly, and metastases in the kidney (not shown) were seen in a few cases. The results in Table 1 demonstrate a high degree of reproducibility with regard both to tissue distribution of the metastases and the lag time before they could be observed.

Adrenal metastases varied in size from microscopic to a diameter of 6–7 mm. As shown in Fig. 2A, the tumor cells are present only in the adrenal medulla where they replace the normal cells, pushing the cortical cells outward without infiltrating the cortex.

A section of a representative interscapular metastasis (Fig. 2B) demonstrates a close association between the tumor cells and the brown fat tissue known to be present at this location in mice. As was seen also in some submandibular metastases, the tumor cells seem to infiltrate the brown fat. Usually, however, the submandibular metastases were located in lymph nodes where they disrupted the normal node structure. These tumors were found close to the mandibular salivary gland (Fig. 2C), but the tumor cells did not infiltrate this tissue.

Since, after i.v. injection, the first capillary bed encountered by the tumor cells is that of the lungs, it is particularly interesting that we have never observed experimental lung metastases with cells obtained directly from FEMX-I xenografts. This is not due to high sensitivity of the tumor cells to NK cells in the lungs of the animals, as the metastatic pattern was the same in adult BALB/c nude mice with high NK activity and in NIH-III (9) and beige-nude mice (24) with low NK levels (not shown).

The mediastinal and most abdominal tumors probably represent lymph node metastases. This was, however, difficult to prove, as the tumors usually contained no normal lymph node structures. The retroperitoneal tumors also may represent metastatic growth in lymph nodes, but, as will be discussed below, the location of the tumors is close to sympathetic ganglia.

In one mouse with few, relatively large metastases in the lower abdomen, ascites fluid was abundantly present in the peritoneal cavity. This fluid contained a high concentration of tumor cells, with no contamination of erythrocytes and only a few leukocytes. Upon i.p. injection of the fluid into nude mice, an ascitic tumor developed in all animals, and this subline has later been passaged as a continuous ascitic tumor line.

Morphological Characteristics. Histological examination of
Table 1 Frequency, tissue distribution, lag time, and reproducibility of FEMX-I metastases after i.v. injection of tumor cells in nude mice

<table>
<thead>
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<th>Site of metastases (no. of mice)</th>
<th>Mice with metastases/mice injected</th>
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<tr>
<td></td>
<td>Interscapular region</td>
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<td>----------------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>1</td>
<td>7/8</td>
</tr>
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<td>2</td>
<td>8/8</td>
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<tr>
<td>3</td>
<td>8/8</td>
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<tr>
<td>4</td>
<td>9/10</td>
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\(^a\) Metastases in NIH-III nude mice within 120 days after injection of \(1 \times 10^6\) FEMX-I cells.

\(^b\) Time until s.c. metastases had reached a size of 5–7 mm.

Fig. 2. Tissue sections of FEMX-I metastases in the adrenal gland (A), in soft tissue parts of the neck (B), and in a submandibular lymph node (C). In A, the normal cells in the adrenal medulla are replaced by the tumor, but the malignant cells do not infiltrate the cortical tissue (× 140); B, infiltration of tumor cells into brown fat (× 280); C, metastasis in a lymph node next to the mandibular salivary gland. A well-defined area of brown fat tissue is seen between the tumor and the gland (× 280).

the original patient's biopsy demonstrated the typical picture of a malignant melanoma lymph node metastasis, with lymphocytes surrounding the tumor cells (Fig. 3A). Tumor specimens of FEMX-I s.c. xenografts (Fig. 3B) at different passages, and of various FEMX-I metastases consistently showed the same tumor morphology, with no indications of significant changes in cell characteristics or of any selection of tumor cell subpopulations (Figs. 2 and 3). An example of the ultrastructural features found in all tumors examined is demonstrated in Fig. 4. Several premelanosomes can be seen in the cytoplasm.

The monolayer cultures consist of polygonal to spindle-shaped cells (Fig. 5). During exponential growth the cell doubling time is approximately 30–35 h.

Chromosome Analysis. FEMX-I cells obtained from either short term in vitro cultures of xenograft cells or from permanent monolayer cultures were examined. On the whole the same findings were made in the different cell preparations (data not shown). The chromosome number ranged from 39 to 68, with most of the cells in the near triploid state. Chromosomes 7 and 15 were almost absent, but material from these appeared in...
Tissue-specific human melanoma metastasis in nude mice

The results indicate that in vitro cultivation had changed cell characteristics important for the metastatic process, or selected a cell population with a reduced metastatic potential. To examine these possibilities, monolayer cells were inoculated s.c. in nude mice and cells from the resulting tumors were injected i.v. into other animals. This time metastases developed at the same frequency and localization, and after the same lag time as when cells from the FEMX-I xenograft line or from ascites were used (not shown). The data indicate that the in vitro conditions induce reversible changes in the metastatic capacity of the FEMX-I cells. In other experiments it was found that even after long term in vitro cultivation the metastatic ability of the cells was rapidly regained when they were again grown as xenografts.

To see how rapidly the in vitro conditions impair the metastatic capacity, FEMX-I xenograft cells were seeded into culture flasks and kept in culture for 1 week only, before being detached with EDTA and injected i.v. into mice. The results showed that with these cells as many as 60% of the animals developed metastases, but the lag time was similar to that seen with cells from long term cultures (data not shown), indicating that the metastatic capacity was reduced already after 1 week of incubation.

Tissue specificity of FEMX-I metastases. In attempts to examine to what extent the rather unique metastatic pattern seen with FEMX-I reflects a true tissue specificity of the tumor cells for lymph nodes, brown fat, and the adrenals, we injected FEMX-I cells i.s., i.p. (cells obtained from the ascites line), and into the hind footpad of the mice. In the latter case, the resulting tumor was removed by amputation when it reached a size of about 5–6 mm.

After i.s. injection metastases developed in the liver of some of the mice, and other intraperitoneal tumor deposits such as in the adrenals and lymph nodes were also observed (data not shown), but lung colonies or other metastases outside the abdominal cavity were never detected. When the tumor was grown as ascites, intra-abdominal and sometimes also extrapulmonary mediastinal tumors were found. In the experiments with footpad inoculation of the cells, metastases developed in regional lymph nodes, but further spread was not observed. The liver metastases after i.s. injection can be explained by the ready access to the portal venous system, and altogether the data indicate that the FEMX-I cells possess a tissue-specific metastatic ability independent of the route of injection.

Discussion

It is a well-known experience in experimental and clinical cancer that different tumor types, as well as various subgroups of the same type, show different organ preferences for metastasis. The mechanism underlying this phenomenon is not well understood, and very little experimental data is available on the specific interaction between human metastatic cancer cells and the host organs most commonly involved. This has to a large extent been due to the general lack of suitable in vivo models for human cancer metastasis, particularly models in which site-specific metastasis formation cannot be explained by simple hemodynamic or mechanical factors.

Here we report a unique new metastasis model involving a human tumor. Thus, after i.v. injection into nude mice of FEMX-I human melanoma cells metastases are selectively and reproducibly formed in lymph nodes, brown fat, and in the adrenal medulla, whereas experimental lung metastases are not seen. The latter observation is particularly interesting in view of the fact that after i.v. injection the cells first encounter the lung capillary net. Moreover, we have previously shown that i.v. injection into nude mice of LOX human melanoma cells results in progressively growing lung tumors (23, 25), as also has been reported to occur with a few other human tumors (26, 37). The absence of FEMX-I lung colonies in mice with large

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Fig. 4. Electron micrograph of a FEMX-I tumor cell with several premelanosomes in the cytoplasm (× 26,400). Inset, high magnification of a characteristic premelanosome (× 61,600).

Fig. 5. Monolayer culture of FEMX-I cells (× 175).

Table 2 Metastatic capacity of FEMX-I human melanoma cells obtained from xenografts, ascites, and from monolayer cultures

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Mice with metastases/mice injected</th>
<th>Lag timea (days ± SE)</th>
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<tr>
<td>Xenografts</td>
<td>38/40</td>
<td>53.6 ± 4.6</td>
</tr>
<tr>
<td>Ascites</td>
<td>8/8</td>
<td>50.4 ± 2.0</td>
</tr>
<tr>
<td>Monolayers</td>
<td>3/21</td>
<td>97.0 ± 10.0</td>
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a Metastases in NIH-III nude mice within 120 days after injection of 1 × 10⁶ FEMX-I cells.

metastatic tumors elsewhere, seems to rule out the possibility that the metastatic pattern seen actually represents secondary metastases from (lung) metastases.

That FEMX-I cells, as well as LOX cells, possess inherent tissue specificity is supported by results obtained after cell injection i.p. and i.s. Thus, it was found here that with both procedures the FEMX-I cells metastasized to liver, adrenals, and to abdominal lymph nodes, but never to the lungs, whereas the LOX cells in addition to intraabdominal tumors give lung metastases that eventually kill the animals (25, 38).

The tissue specificity of the FEMX-I cells is further supported by the results obtained after injection of cells in the footpads of the mice. Thus, whereas lung metastases developed when LOX cells were used (25), only lymph node tumors appeared with FEMX-I cells. Since the FEMX-I line originates from a lymph node metastasis in the patient, the cells have apparently retained metastatic characteristics of the parent tumor.

The localization of the metastases to adrenals and brown fat is of particular interest. In rodents, the interscapular brown fat pad seems to be a relatively frequent site for metastasis after intracardial injection of tumor cells (1), but to our knowledge metastasis formation of human cells to brown fat has not been described previously. Metastases to the adrenal cortex have been found after i.c. injection of rat and murine tumor cells (1, 39), but has never been reported to occur with human cancers in nude mice. In our model, the FEMX-I adrenal tumors develop after i.v. injection and are specifically located in the medulla, replacing completely the normal cells, but the tumor cells do not infiltrate the cortical tissue.

The development of metastases depends on blood flow, on mechanical factors related to cell entrapment in the first capillary bed encountered, and also on the properties of both the tumor cells and host tissues (1, 4, 5, 7, 20, 25, 30, 40, 41), as was originally suggested by Paget (42). Differences in entrapment in lung capillaries cannot explain the differences in met-
TISSUE-SPECIFIC HUMAN MELANOMA METASTASIS IN NUDE MICE

The metastatic pattern displayed by FEMX-I cells can be explained only by specific interactions between the cancer cells and the microenvironment provided by the host tissues. The nature of the interaction between FEMX-I cells and the various host tissues is obscure. Lymphatic cells utilize specific recognition markers for homing to different lymphoid tissues (44), but no strong evidence has been presented supporting the existence of specific binding sites on the surface of the metastatic cells allowing them to adhere to similar markers expressed on endothelial cells in specific tissues. Nevertheless, the present finding that in vitro cultured FEMX-I cells had a strongly reduced metastatic capacity compared to cells obtained from the in vivo tumors, supports the view (4, 6, 7, 20, 41, 45) that cellular structures, probably exposed on the cell surface, are involved in the metastatic process. Since in vitro growth conditions can induce alterations in the expression of cell membrane antigens (35, 46), the changes in the cell membrane affecting the metastatic capacity of FEMX-I cells were probably induced by the in vitro growth conditions. That these changes are reversible became evident when monolayer cells were grown as s.c. xenografts before i.v. injection.

In addition to factors related to cell adhesion and extravasation, also factors stimulating or inhibiting growth probably influence metastasis formation (4, 20, 47). However, no growth factors are known to be specifically present in the tissues where FEMX-I metastases develop, and although metastasis to lymph nodes is one of the most frequent occurrences in clinical as well as in experimental cancer, such metastases are usually explained by mechanical entrapment of tumor cells circulating in the lymph.

One possible connection between brown fat and adrenal medulla tissue might be their relationship to the sympathetic nerve system. It is known that brown fat in rodents has a close association to sympathetic nerves, and epinephrine, which is produced in the adrenal medulla, is a sympathetic transmitter. Moreover, the possibility cannot be excluded that the retroperitoneal metastases frequently found might represent tumor growth in close association to sympathetic nerve ganglia. A biochemical explanation for this hypothetical relationship is not at hand, but experiments to explore the existence of a possible growth stimulatory effect of these tissues will be carried out. The absence of FEMX-I lung colony formation may suggest either a lack of stimulation or the presence of growth-inhibitory factors in the lung tissue.

Most metastatic human lines form rapidly growing and rather undifferentiated tumors in the mice. In contrast, the FEMX-I tumors are relatively slowly growing and have a more differentiated morphology with premelanosomes found in all tumors (Figs. 1 and 2). MeWo melanoma cells also produce melanin, but MeWo cells capable of producing experimental metastases were selected through a lengthy adaptation and selection procedure, and i.v. injection of such cells give a quite different pattern of metastasis (26).

No indications of permanent changes in FEMX-I cell characteristics, or of a selection of tumor cell subpopulations were obtained. Thus, all tumors and metastases had similar morphology, and chromosome studies performed on cells prepared from different tumors at different times showed the same karyotypic characteristics. The possibility that the FEMX-I line is homogenous and does not contain subpopulations of cells with different characteristics must be considered. However, preliminary data (not shown) indicate that this is not the case.

Most metastatic models involving human tumors have been developed by using established cell lines, often after very long term cultivation in vitro, or different manipulations that might alter the cells phenotypically have been performed. However, different growth conditions, and particularly cultivation in vitro (35, 36, 46), can induce changes in important cell characteristics. We have therefore used cells prepared from in vivo tumors for studying their metastatic capacity. This was of importance in the case of FEMX-I cells, but not when LOX cells were used (25).

The differentiated phenotype of the FEMX-I tumor, its relatively slow growth rate, and moderate to low sensitivity to conventional chemotherapeutic drugs (32, 48), indicate that this tumor may be representative of human melanomas. The metastasis model shows promise for further studies on mechanisms involved in site-specific metastasis formation, as well as for testing the effect of antimetastatic agents and of chemotherapy directed against extravascular micrometastases. Since the FEMX-I cells express abundant amounts of the high molecular weight melanoma-associated antigen (49), the potential role of immunotoxins in the therapy of minimal disease will also be examined.

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