A Human Tumor Lung Metastasis Model in Athymic Nude Rats

Inge Kjønniksen,1 Ritsa Storeng,1 Alexander Pihl, Theodore L. McLemore, and Øystein Fodstad2

ABSTRACT

Experimental lung metastases regularly developed in athymic Han×nu/rnu Rowett rats after i.v. injection of LOX human malignant melanoma cells. When 5 × 104 tumor cells were injected into 4-week-old rats, 89% of the animals died of lung tumors, with a mean survival time of 18 days. With 5- and 6-week-old rats, however, the fraction of animals that died decreased to 80 and 46%, with mean survival times of 35 and 38 days, respectively. The number of detectable lung colonies in each animal was about 35 in 5- and 6-week-old animals, compared to nearly 300 in 4-week-old rats. In the latter, a correlation was found between the number of tumor cells injected and the number of detectable lung colonies. The capacity of the LOX tumor to grow s.c. and to form experimental lung metastases was, by and large, similar in young nude rats and in nude mice, and no significant difference in morphology between the different tumors in the two species was seen. A high-resolution radiographic method was used to visualize lung colonies in the nude rats, and single tumors with diameters as small as 2–4 mm could be detected. By this method, for the first time, the effect of chemotherapy on a human tumor growing in a visceral organ of a rodent host could be followed by repeat X-ray examinations, mimicking a situation commonly faced in the clinic. This procedure may prove particularly useful for experimental chemotherapy studies, and may be extended to other human tumors that frequently metastasize to the lungs. Indications were obtained that some host-specific differences in tissue-preferred growth might exist, a possibility that will be further explored.

INTRODUCTION

Progress in metastasis research depends to a large extent on the use of experimental animal models that, as closely as possible, mimic the situation in humans. Previously, most metastasis studies were performed in syngeneic murine and rat tumor systems (1–4). The more recent development of human tumor metastasis models in athymic, nude mice (5–9) has provided improved possibilities for elucidating factors involved in the metastatic process.

For some types of experiments, i.e., those involving technically difficult procedures such as various types of cannulations and intracardial/intraarterial injections, it would be advantageous if larger animals could be used. One possibility is the athymic, nude rat, a mutant first described in 1978 (10). The effects of the nude mutation seem to be quite similar in rats and mice (11–13), but the nude rats are known to be more robust than the mice and can be kept free of disease also in conventional animal facilities (10). Despite this advantage, nude rats have been used only to a limited extent for experiments involving human cancers, and to our knowledge no detailed study on human tumor metastasis formation in nude rats has been reported.

Here we report on the establishment and usefulness of an experimental metastasis model in nude rats in which the animals die within 3–5 weeks after i.v. injection of the human melanoma cell line LOX. It was found that the survival time is dependent on the age of the rats at the time of tumor cell injection. We demonstrate also that the change in size of the lung tumors can be followed, in individual animals, by employing a radiographic method.

MATERIALS AND METHODS

Animals. Congenitally athymic, nude rats (Han×nu/rnu Rowett) were purchased germ free and gnotobiotic from Zentralinstitut für Versuchstiere, Hannover, West Germany, or bred in our own nude rodent facility. Animals of both sexes were used in the experiments, and after weaning at 3 weeks the rats were kept in positive pressure rooms with filtered and humidified air. All animals proved to be disease free throughout the experimental period.

Cell Line. The malignant amelanotic melanoma cell line LOX originates from a patient's lymph node metastasis (6). The cells were cultivated in monolayer in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% fetal calf serum at 37°C in a humidified mixture of 5% O2 and 5% CO2 in nitrogen. The cells were subcultured twice weekly after detachment with trypsin-EDTA and were found to be free of mycoplasma infections.

Morphological Studies. Tumor tissue was fixed in buffered formaldehyde, dehydrated, and embedded in paraffin. Sections were stained with H & E, and the morphology of s.c. and intrapulmonary tumors from rats was compared with that of corresponding tumors from mice and from the patient's lymph node metastasis.

Subcutaneous Tumor Growth. LOX tumor tissue cubes (2 × 2 mm) were prepared from s.c. growing xenografts and inoculated in the flanks of groups of rats of different ages (2–4, or 6-week-old at the time of transplantation). Two perpendicular diameters of the tumors were measured with calipers three times weekly. Tumor volumes were calculated according to the formula 0.5 × L × W² (14), and growth curves were constructed.

Experimental Metastasis Formation. Monolayer LOX cells were grown to near confluence, washed twice with phosphate buffered saline, detached with EDTA (6) and resuspended in RPMI 1640. Nude rats were anesthetized with halothane (Trofield Surgicals A.C., Zug, Switzerland) and N2O, and injected i.v., in a lateral tail vein, with different numbers of cells in 0.1–0.2 ml of RPMI. The rats were checked daily for up to 3 months. Animals with marked dyspnea were sacrificed by a lethal dose of halothane/N2O, and the time from the day of tumor cell injection was recorded. The lungs were inflated by intrabronchial injection of Bouin's solution, and the number of tumor colonies on the surface of the lungs was counted by visual inspection.

Radiographic Studies. The rats were anesthetized with a mixture of 2.5 mg/kg midazolam, 5 mg/kg flumazenil, and 0.1 mg/kg fentanyl i.p., and X-ray films were taken using a Senogenpick 500T mammogram device (Thomson-CGR Medical Corporation, Columbia, MD) employing molybdenum anode and filter, focus 0.3 mm, 25 kW exposure at 16–20 mAs and a distance of 55 cm, essentially as previously described (15, 16). To achieve good immobilization, anterior-posterior compression was applied. The film, Ortho M, Kodak, was exposed in a Kodak MinR-casette with Kodak MinR-screen.

Chemotherapy Experiments. Groups of 4-week-old rats carrying s.c. LOX xenografts were treated, when the tumors had reached a size of 16–20 mAs and a distance of 55 cm, essentially as previously described (15, 16). To achieve good immobilization, anterior-posterior compression was applied. The film, Ortho M, Kodak, was exposed in a Kodak MinR-casette with Kodak MinR-screen.

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RESULTS

Growth of s.c. LOX Xenografts. Tumor take was observed in almost all 6-week-old rats in about 7 days after transplantation of LOX tissue obtained from s.c. xenografts in nude mice. The tumors grew progressively and could thereafter be serially transplanted to other animals. The growth rate of the s.c. tumors was similar to that observed in nude mice, with a doubling time during exponential growth of approximately 1.8 days (data not shown).

Due to the variable success of previous investigators in growing human tumors in nude rats, and since young nude mice are reported to support xenograft growth better than adult animals (17), we studied whether the age of the rats at the time of transplantation would affect the growth of the s.c. LOX tumor. No clear differences in the time before tumor growth was observed, or in the growth rate of the established tumors, were found between animals transplanted at 2, 4, and 6 weeks of age.

Lung Colony Formation. In experiments in nude mice we have previously found (6) that after i.v. injection of $1 \times 10^6$ LOX cells from monolayer cultures the animals regularly die of progressively growing lung tumors in about 35 days. Using the same number of cells, the survival time of 5-week-old nude rats was also 35 days. Lungs removed from rats and mice killed when they started to become dyspneic, contained tumor colonies that seemed to have similar patterns of distribution, as well as size relative to the size of the host lung (Fig. 1, A and C).

As was found in mice (6), the number of rat lung colonies differed from animal to animal, and in lungs with larger tumors, the colonies tended to become confluent. Light microscopic studies of the rat tumors showed closely the same morphological picture as those of mouse lung colonies, s.c. xenografts in both species, and of the original patients’ tumor (not shown).

When LOX cells were injected i.v. into animals of different ages, it was found that the survival time and lung colony formation differed with the age of the rats (Table 1), in contrast to the situation when the LOX tumor was grown s.c. The lifespan of 4-week-old rats after injection of $5 \times 10^5$ cells was 19 ± 2 days, compared to 35 ± 10 and 38 ± 10 days for 5- and 6-week-old rats, respectively. It is also seen that after injection of $1 \times 10^6$ LOX cells, 19/20 (95%) of 4-week-old rats died of lung colonies, whereas only 72% (13/18) of 5-week-old and 47% (9/19) of 6-week-old animals died. Similar results were obtained when 5 $\times 10^5$ cells were injected, and with $1 \times 10^5$ cells the percentage of animals that died was 100% in 4-week-old rats and only 13% in 5-week-old animals. Also, the life-span of the rats was clearly shorter for animals injected at 4 weeks of age compared to those in other groups (Table 1).

In agreement with these data, the number of lung colonies in animals that died of their metastases was much higher in young rats, where a correlation was found between the number of colonies and the number of tumor cells injected. The effect of age is also evident from the pictures demonstrated in Fig. 1. It is seen that the lungs of a rat injected at 4 weeks of age (Fig. 1B) contained numerous small, partly confluent colonies, most of them less than 2–3 mm in diameter, whereas the lungs of a 5-week-old rat (Fig. 1A) had much fewer, but larger colonies. Altogether these results show a strong effect of animal age on lung colony formation, suggesting that some host defense mechanisms against tumor cells may be less well developed in the young rats.

Extrapulmonary metastases were observed in a few cases. Thus, in one case a tumor was found on the back of the animal, and some animals developed hind leg paralysis (6/39 of 5-week-old, and 5/40 of 6-week-old rats). These rats were killed and not included in the survival studies. Autopsy and preliminary histological examination have revealed that these animals all had lung colonies, and in addition metastases closely associated with the spine were detected in the lumbar region of the rats. This finding may explain the paralyses (18), and the phenomenon is now being studied in more detail. Importantly, similar metastases have never been observed in nude mice. Of the rats that survived to the end of the 3-month observation period, approximately 1/3 had lung colonies.

Radiography of Lung Tumor Colonies. In attempts to simulate the situation in patients with lung metastases, we used a technique employing the equipment used for clinical mammography to monitor the growth of LOX lung colonies in rats. After i.v. injection of $1 \times 10^6$ tumor cells into 5-week-old animals, lung tumors became visible on X-ray films taken about 17 days later. In Fig. 2 pictures of films taken 1 week (left) and 3 weeks (right) after tumor cell injection are shown. Whereas in the first case no lung tumors could be detected, after 3 weeks massive tumor involvement of the lungs was visible. The minimum size of tumors that could be detected by this method was dependent on the localization of the tumor in the lung, as well as on the total number of tumor colonies. In most cases, single colonies with a diameter down to 2–4 mm were detectable, and as revealed on autopsy, tumors larger than 7 mm were never missed. A higher resolution was not obtained, partly because of the breathing of the anesthetized animals.

Chemotherapy Experiments. We have previously shown (6, 19) that s.c. LOX xenografts in nude mice are extremely sensitive to the investigational drug mitozolomide, and marginally sensitive to cis-dichlorodiammineplatinum(II). In comparable experiments in rats, the s.c. xenografts showed a similar response pattern (Fig. 3), indicating that the pharmacology of the drugs used and the local tumor environment are comparable in the two different hosts.

To see whether consecutive X-ray examinations could be used for assessing the effect of chemotherapy in our rat lung colony model, we monitored the lungs of three 4-week-old rats injected with $1 \times 10^6$ LOX cells. On Day 13, the examination gave pictures that seemed to represent a high number of small, partly distinguishable tumors in the lungs (not shown). Three days later, when the mitozolomide treatment was started, the colonies had become more manifest (Fig. 4). A second dose of chemotherapy was given on Day 23, and the effect of the treatment is illustrated by a representative picture taken on Day
Table 1 Lung colony formation in nude rats injected i.v. with LOX cells. Importance of animal age and number of tumor cells injected

<table>
<thead>
<tr>
<th>No. of cells injected</th>
<th>Deaths/total</th>
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<td>$1 \times 10^6$</td>
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<td>19 $\pm$ 2</td>
<td>272 $\pm$ 101</td>
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<tr>
<td>$1 \times 10^7$</td>
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<td>29 $\pm$ 6</td>
<td>52 $\pm$ 29</td>
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*Mean ± SD.

Fig. 2. X-ray pictures of a nude rat injected at 5 weeks of age, taken 1 week (left) and 3 weeks (right) after i.v. injection of $1 \times 10^5$ LOX cells.

Fig. 3. Subcutaneous tumor growth of LOX xenografts in groups of nude rats treated with mitozolomide, cis-dichlorodiammineplatinum(II) (CDDP) or left as untreated controls. The drugs were given i.v. as a single dose when the tumors had reached a size of approximately 4 x 4 mm. The tumor diameters were measured three times weekly with calipers, and tumor volume (mean ± SD) was calculated.

DISCUSSION

In the present study, it was found that the LOX human melanoma formed experimental lung metastasis after i.v. injection into athymic rats, in a similar way as previously observed (6) in nude mice. Moreover, we demonstrate that repeat X-ray examination can be used in rats for monitoring the size of the lung tumors. This method may be particularly useful in therapy experiments. Importantly, the number of lung colonies and the life-span of the rats were influenced by the age of the animals at the time of injection of the LOX cells, a situation not seen in mice.

For several purposes the size of the nude rat makes it an attractive host for tumor xenograft studies. However, a reduced malignancy of xenografted tumors in nude rats was indicated by reports that murine tumors, which are highly metastatic from s.c. sites in syngeneic mice, grew noninvasively and did not produce spontaneous metastases in nude rats (20, 21). Moreover, some authors have reported poor take rates of human cancers transplanted s.c. in nude rats, and other authors have observed spontaneous regression of such tumors (13, 22-24). In contrast, Bastert et al. (25) and Salomon et al. (21) reported similar and high take rates for several human cancers in nude rats and in nude mice.

The apparent inconsistency in the published results of work with xenografts in nude rats may have several explanations: The success in obtaining tumor take may differ with the rat strain used, as it is possible that the effect of the nude mutation to some extent may differ with the genetic background of the animals; the health status of the rats may influence the results; and the limited number of experiments performed, each involving relatively few animals, may not give a representative picture
of the usefulness of the nude rat for such research. In addition, it is well known (26-28) that the take rate of human cancers in nude mice differ with tumor type, as well as with individual characteristics of the tumor.

In the present study, the take rate and the volume doubling time of s.c. LOX tumors were the same in rats as those previously found in nude mice. The tumors could be passaged serially with no change in important characteristics. The morphological pictures of the s.c. and lung tumors in rats and mice were closely similar, and the mean life-span of 5-6-week-old rats injected i.v. with 5 x 10^5-1 x 10^6 cells varied between 28 and 35 days, similar to what was previously found in mice (6). The data suggest, therefore, that healthy Rowett nude rats as used here, have a promising potential for use in studies involving human tumors. Together with the findings in mice (6), the present data show that the LOX cells have a preference for growth in the lungs of the host, in agreement with the seed and soil theory originally proposed by Paget (29).

A clear relationship between colonization of the lungs and the age of the animals was found. Thus, in 4-week-old rats the number of lung colonies was higher and the life-span was shorter than in the older animals receiving the same number of cells. In the young rats, a correlation was found between the number of cells injected and the number of detectable lung colonies, whereas this relationship was less clear in older animals.

The pronounced differences between 4- and 5-6-week-old rats in lung colony formation are surprising. However, other investigators have observed variation in s.c. tumor growth with age of nude rats. Thus, Maruo et al. (30) and Partridge et al. (31) showed that human tumors implanted in 4-week-old rats grew more rapidly, and consequently higher tumor weights were obtained within the observation time, than in 6-10-week-old animals. It is noteworthy that in the present study the s.c. growth of LOX xenografts did not differ with the age of the animals.

In nude mice, age-dependent variation both in s.c. growth and metastasis formation of human cancers has been reported. Sordat et al. (32) obtained higher take rates in newborn nude mice than in adult animals, and Hanna and Fidler (17) observed increased metastatic potential of several tumor lines in 3-week-old compared to 6-week-old mice. These findings may be explained by immunological factors, as it is known (17, 33) that very young mice are less immunologically mature than adult animals. However, in a previous study in our laboratory we could not demonstrate any difference in the life-span of young and adult nude mice injected i.v. with B16F10 and LOX cells (33).

The present data obtained with LOX cells in rats suggest that in this species immunological differences between 4- and 5-6-week-old animals exist that may explain the higher take rate of i.v. injected cells in the younger animals. This notion was further supported by the finding that a few 2-week-old rats injected successfully i.v. with 1 x 10^6 LOX cells, died even earlier (after 13-16 days) than 4-week-old animals (data not shown). The effector cells conceivably responsible for the observed age-dependent differences may include NK cells (30) and cytotoxic macrophages (31, 34), as well as B-lymphocytes and even T-cell-like cells. Because of the complexity involved, elucidation of this issue may require rather comprehensive immunological studies.

The fact that athymic rats seem to be less susceptible to infections than nude mice may indicate that the nude mutation has a somewhat less pronounced effect on the immune system in rats than in mice. The difference between the two species in susceptibility to growth of human tumor cells would then be expected to increase during the first weeks after birth, reflecting the maturation of the immune effector mechanisms presumably taking place in this period. Altogether, the results obtained with the LOX cells in nude mice and rats seem to be consistent with this view, which, if correct, also would suggest that young athymic rats may be used to obtain metastatic models involving other human cancers.

Human tumor xenograft models in mice have proved particularly useful for experimental chemo- and radiotherapy (19, 26, 27), and it is clear that the transplants by and large retain the sensitivity of the parent tumors (26, 27). It has been argued that chemotherapy studies involving s.c. xenografts may not be relevant for most human cancers which usually grow at other sites, implying that the local growth conditions may differ from those in the patients, and that the pharmacology of the drugs tested may also be different (27). With the LOX melanoma, tested in rats (as here), or in mice (6, 19), the response to the compounds examined did not differ whether the tumor was growing s.c. or in the lungs. It seems clear, however, that for
several purposes a lung colony model may be more clinically relevant than studies on s.c. xenografts.

The objective with all models for chemosenstivity testing is that the model shall mimic, as closely as possible, the situation in the patient, in whom change in tumor size during treatment is most commonly assessed by repeat X-ray examinations. Moreover, it is important to avoid experiments in which the animals suffer from advanced tumor involvement of any major organ system, particularly experiments which entail survival as the end point. The radiographic method described meets both of these requirements, and here we have demonstrated for the first time how the effect of chemotherapy on human tumors in a visceral organ can be followed by the use of X-ray examinations. Since many human cancers frequently metastasize to the lungs, this method may prove particularly useful for therapy studies in those of such tumors that can be grown in the lungs of nude rats. Although the sensitivity of the method is still limited, the approach may be used to rapidly screen for significant drug effects.

The use of radiography to assess the growth of xenografted human tumors was first studied in nude mice injected intra- bronchially or intrathoracically with human lung carcinoma cells (15, 16). The advantage of the present model is that the cells are not deposited directly into the lung tissue and that the tumors develop more randomly in both lungs. Moreover, the size of the rats may be advantageous for monitoring tumor growth, particularly for the purpose of evaluating the effect of therapy.

Since the formation of manifest tumor metastases is the result of an interaction between the tumor cells and the normal host cells, comparison of the metastatic abilities of human tumors in nude mice and rats may provide a new means for elucidating factors involved in this interaction. The data obtained here with the LOX cells demonstrated a similar pattern of metastasis formation in the two species. However, preliminary experiments where we have compared the metastatic patterns of various human tumors, suggest that in some cases the tissue preferences may indeed be different in the two species, as indicated here by the hind leg paralysis observed in rats but not in mice. This may open new possibilities for identifying factors involved in this interaction between the tumor cells and the normal host system, particularly experiments which entail survival as the end point. The radiographic method described meets both of these requirements, and here we have demonstrated for the first time how the effect of chemotherapy on human tumors in a visceral organ can be followed by the use of X-ray examinations.

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