

A Nod Scid mouse model to study human prostate cancer

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Prostate cancer is the second cause of cancer mortality in men in Western countries. To study new therapeutic approaches such as gene therapy, animal models of human prostate cancer with metastatic behavior are mandatory. We used the Nod Scid mouse strain to develop an orthotopic animal model. Two androgen-independent cell lines (PC-3 and DU 145) were used. Local tumor growth and metastases were analyzed. The tumor take rates were close to those reported in the literature. However, a high frequency of various metastatic sites has been observed (liver, lung, spleen, adrenal, kidney, lymph node, and diaphragm). It can be concluded that the Nod Scid mouse is a relevant preclinical animal model to study human prostate cancer. Metastatic sites seem more numerous in comparison to other orthotopic mice models described.

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Introduction

Prostatic carcinoma is the most frequent cancer in men and the second cause of cancer mortality in men in Western countries.¹ Forty to 50% of prostate cancer patients have a locally advanced or metastatic disease at presentation. Twenty to 35% of patients treated with curative intention will recur after surgery or radiotherapy.^{2,3} For these patients, there is no consensual adjuvant or salvage therapy but androgenic suppression as palliative treatment. Insights into new therapies as gene therapy for metastatic or persistent disease are mandatory.

In order to validate these new therapies, animal models are needed. The ideal animal model for prostate cancer does not exist as it should mimic the clinical situation observed in man: tumour should be of human origin, have a doubling time fast enough to allow studies in reasonable time, be androgen-dependent or androgen-sensitive, produce prostate specific antigen (PSA), create lymph node and bone metastasis and develop an androgen-independence status after castration.⁴

Animals used to study human prostate cancer are immunodeficient. They allow a tumor growth after implantation of human xenografts or cell lines in different locations. The production of metastases is dependent on

the site of implantation. Subcutaneous implantation rarely produces distant metastases, when orthotopic (intra-prostatic) implantation does allow metastatic spread. Orthotopic implantation in an immunodeficient rodent is a relevant model⁵ and the first description was made in 1992 by Stephenson *et al.*⁶

Immunodeficient rodent models generally used for prostate cancer studies are the nude mice model described in 1966 by Flanagan⁷ and the Scid mice model (Severe Combined ImmunoDeficiency) described in 1983 by Bosma *et al.*⁸ The nude mutation results in thymus aplasy with quantitative and functional T-lymphocyte defects. The Scid mutation results in a lack of T- and B-lymphocyte function. However, normal NK cell and myeloid function are present that may influence initial tumor growth and metastatic spread after implantation.⁹

In 1995 Shultz *et al.*¹⁰ described a new immunodeficient mouse model obtained by crossing the Scid and Nod mouse strains. The Nod strain (non obese diabetic) is characterized by a functional deficit in NK cells, an absence of circulating complement and defects in the differentiation and function of APCs (antigen-presenting cells). The Nod Scid model combines multiple functional defects of adaptive and innate immunity. It is very suitable for xenografts of human tumoral lines. A comparative study of tumor growth rate of various hematopoietic cancer cell lines in different animal models (Nude, Rag1, Scid and Nod Scid mice) has shown a better growth rate in the Nod Scid model.¹¹

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To our knowledge, the Nod Scid mouse has been used once as a preclinical model for prostate cancer but without orthotopic implantation.¹² In the present study, the Nod Scid mouse has been used for subcutaneous and orthotopic implantation of human prostate carcinoma cell lines in order to validate a new relevant model for gene therapy studies and to link therapeutic efficiency with transgene expression without implying the own animal immunity.

Materials and methods

Prostate cancer cell lines and culture conditions

PC-3 and DU 145, two human prostate carcinoma cell lines established from a bone and brain metastasis respectively, were used in this study.^{13–15} Both cell lines are androgen-independent and do not produce PSA. *In vitro*, the doubling time is 1.5 to 2 days. Both cell lines were provided by ATCC.

The PC-3 cell line was cultured in F12.K medium complemented with 1% L-glutamine (200 mM) + 1% penicillin (10 000 UI/ml)/streptomycin (10 000 UI/ml) + 2% sodium bicarbonate (7.5%) + 10% fetal calf serum.

The DU 145 cell line was cultured in MEM (minimum essential medium) complemented with 1% sodium pyruvate + 1% non essential amino acids + 1% penicillin (10 000 UI/ml)/streptomycin (10 000 UI/ml) + 2% sodium bicarbonate (7.5%) + 10% fetal calf serum.

The cells were seeded in a 100-mm culture plate and maintained at 37°C in a humidified incubator (85%) in 5% CO₂ atmosphere. They were divided once or twice a week according to growth rate.

In order to calibrate the cell suspension for subcutaneous or orthotopic implantation, cell counting was made using a Mallasez counting cell after vital stain coloration (Tryptan blue exclusion). Harvested tumors and metastases were sliced using a scalpel in a dissociation medium [specific medium PC-3 or DU 145 + 1/50 trypsin (125 mg/ml) + 1/100 collagenase (collagenase II 50 mg/ml)] in a 100 mm culture plate. Culture plates were placed in an incubator for 1 h at 37°C. Cell suspensions were sifted through a nylon sieve (70 µm in diameter). The filtrate obtained was centrifuged at 1600 rpm for 10 min. After aspiration of supernatant, the cells were cultured with their respective medium.

Animals

Nod Scid mice were provided by John E Dick (Toronto, Canada). Eight- to 10-week-old male mice were used and maintained in laminar flow cabinets under specific pathogen-free conditions. All food, water and litter were sterilised prior to use. Temperature (20–21°C) and humidity (50–60%) were controlled. Daily light cycles were 12 h light and 12 h dark. Cages were changed fully once or twice a week. Animals were manipulated under sterile conditions.

Subcutaneous implantation

Subcutaneous implantation of cell suspension was made without anaesthesia in the thoracic postero-lateral wound.

Tumor cell suspensions were injected using a 30-gauge needle and a 1-ml disposable syringe. The volume of inoculation was 100 µl (2 × 10⁶ tumor cells suspended in 100 µl of PBS).

Orthotopic implantation

Animals were anaesthetised with intraperitoneal injection of Xylasin-Ketamin. The implantation was made under surgical sterile conditions. The abdomen was cleaned with iodine solution and a 1 cm midline incision was made to expose the prostate gland. One million cells suspended in 50 µl of PBS were injected into a dorsal prostatic lobe using a 30-gauge needle and a 1 ml disposable syringe. The abdominal wound was closed in two layers with 6/0 nylon surgical suture.

Follow-up and necropsy procedures

Tumor progression was monitored by palpation twice a week by the same investigator (CB). Subcutaneous tumor size was measured using a calliper.

Animals were euthanized according to tumor size or clinical status during the observation period. Mice were killed by cervical dislocation between the fifth and eleventh week after implantation.

An autopsy was performed in orthotopic animals to assess the distribution of metastases. Prostatic tumors and metastases were harvested and fixed in 10% formalin for histological analysis. Subcutaneous and orthotopic tumors were weighed.

Results

Subcutaneous model

Seventeen Nod Scid mice have had subcutaneous implantation: five mice with PC-3 cell suspension and 12 mice with DU 145 cell suspension. The tumor take rate was 100% for both cell lines. Palpable tumors were detected 3 weeks after implantation. The tumors were harvested within 6–10 weeks after implantation. Two mice died during the sixth and seventh week (one PC-3 and one DU 145 animal). Histological analysis was performed systematically and all harvested tumors were prostatic carcinoma. Tumor weight was 0.25–0.6 g at the sixth week. There was no macroscopic metastasis identified at autopsy.

Orthotopic model

Twenty Nod Scid mice have been orthotopically implanted: 10 mice with PC-3 cell suspension and 10 mice with DU 145 cell suspension. Two mice died spontaneously during the first 48 h after implantation (one PC-3 and one DU 145 animal). Animals were killed within 5–11 weeks after implantation. Two mice implanted with PC-3 suspension have had a generalized lymphoma. Macroscopic metastases were first observed between 8 and 9 weeks after implantation for both cell lines.

Histological analysis was performed in all orthotopic and metastatic tumors. There was no difference in terms of histological tumor pattern.

Table 1 Metastatic distribution after orthotopic implantation according to cell lines (PC-3, DU 145)

	Retroperitoneal node	Liver	Hepatic vessels	Pancreas	Spleen	Adrenal	Kidney	Lung	Wound lumbar	Diaphragm
PC-3 seven mice	3	1	0	2	1	3	1	1	0	2
DU 145 five mice	4	0	1	0	0	0	2	0	1	4

The tumor take rate was 83%. Fifteen orthotopic tumors out of 17 were prostatic carcinoma and two were lymphomas. One mouse did not show metastatic or prostatic tumor at the time of autopsy. Prostatic tumor weight at 6 weeks was between 0.15 and 0.55 g. In 12 mice (7 PC-3 and 5 DU 145), 26 macroscopic metastases were observed. The lymph node was the most frequent metastatic location in both cell lines. Parietal metastases were frequent in DU 145 animals. Results are summarised in Table 1.

Metastatic sublines

Seven out of 26 different metastatic foci were cultured *in vitro* and implanted orthotopically in new animals

Table 2 Metastatic location after orthotopic implantation of different sublines

Sublines	Mice implanted	Mice metastatic/mice autopsied	Metastases: number and location
M1 PC-3 Spleen	4	3/3	Retroperitoneal node: 2 Mediastinal node: 1 Kidney: 2 Pancreas: 2
M2 PC-3 Liver	4	3/3	Retroperitoneal node: 2 Splenic vessels: 1 Kidney: 3 Pancreas: 1
M3 PC-3 Adrenal	4	2/2	Retroperitoneal node: 1 Kidney: 2 Pancreas: 2
M4 PC-3 Lung	4	1/1	Retroperitoneal node: 1 Kidney: 1 Pancreas: 1 Liver: 1
M5 DU 145 Diaphragm	4	2/3	Retroperitoneal node: 1 Kidney: 2
M6 DU 145 Retroperitoneal node	4	2/4	Retroperitoneal node: 2 Kidney: 1
M7 DU 145 Kidney	4	3/4	Retroperitoneal node: 2 Hile splenic: 1 Pancreas: 1

M1: subline coming from splenic metastasis (PC-3). M2: subline coming from hepatic metastasis (PC-3). M3: subline coming from adrenal metastasis (PC-3). M4: subline coming from lung metastasis (PC-3). M5: subline coming from diaphragmatic metastasis (DU 145). M6: subline coming from retroperitoneal node metastasis (DU 145). M7: subline coming from renal metastasis (DU 145).

when cell cultures were viable. The aim of this experiment was to analyse the metastatic behavior of metastatic cell sublines in terms of aggressiveness and organ selectivity. For each subline, four mice have been implanted orthotopically as described earlier. All surviving mice were killed 8 weeks after implantation. At autopsy, organs were systematically harvested for histological analysis (prostate, lungs, liver, spleen, pancreas, kidneys, retroperitoneal lymph nodes).

All surviving mice implanted with PC-3 and DU 145 metastatic sublines developed one or more visceral metastases. Retroperitoneal lymph node metastases were more frequent in PC3 metastatic sublines compared with DU 145 ones. There was no metastatic organ specificity found for each subline. Thirty-three metastases have been harvested in 16 mice and flash-frozen for further investigations. Results are summarized in Table 2.

Discussion

The Nod Scid mouse is a valid model to study human prostate carcinoma xenografts. In animals injected with PC-3 and DU 145 cell suspensions, the tumor take rate was 100% for subcutaneous implantation and 83% for orthotopic implantation.

Tumor growth was fast (5–6 weeks) and reproducible. The growth rates are close to those observed in other mouse models.^{6,16} Differences observed in tumor weight between different animals during the same implantation, are possibly due to cell leakage during injection.

In the literature, subcutaneous implantation can provide few distant metastases and mainly lymph node and lung metastases when observed.^{6,16–18} In the subcutaneous implanted mice, there were no macroscopic metastasis observed at autopsy performed between the sixth and tenth week after implantation.

Orthotopic implantation is very favorable to metastatic spread. In this study, macroscopic metastases were found in 80% of orthotopically implanted mice from 8 weeks after implantation. This percentage is close to those reported in the literature.^{6,17} Table 3 summarises the results reported in different studies. The most frequent metastatic site was retroperitoneal lymph nodes, as in other models.^{17,19} During the first five autopsies only, liver, spleen, lungs, kidneys were systematically harvested besides macroscopic metastases. However, no microscopic metastasis was found in macroscopically normal organs and only visible metastases have been harvested in the next animals, leading to a possible underscore of microscopic metastases.

All reported works on orthotopic mice models with cell suspension (PC-3 and DU 145) did not report bone metastasis.^{6,16,17} In our model, metastatic sites have been lymph nodes, pulmonary, adrenal, diaphragmatic,

Table 3 Incidence of tumorigenicity and metastatic frequency after orthotopic implantation with cell lines suspensions reported in different studies

Authors	Tumor cell line	Cell inoculum	Mouse type	Incidence of local tumor	Incidence of metastases	Metastatic sites
Stephenson <i>et al</i> ⁶	PC-3M	1.10 ⁶	Nude	100% (9/9)	66% (6/9)	Lymph node
Waters <i>et al</i> ¹⁷	PC-3	5.10 ⁵	Nude	91% (10/11)	100% (10/10)	Lymph node Lung Kidney
Rembrink <i>et al</i> ¹⁶	PC-3	1.10 ⁶	Nude	100% (5/5)	100% (5/5)	Lymph node Lung
This study	PC-3 DU 145	1.10 ⁶	Nod Scid	83% (15/18)	80% (12/15)	Lymph node Lung Liver Pancreas Spleen Kidney Adrenal Diaphragm

pancreatic, and hepatic sites. Most authors who have used the same technique of implantation have reported only pulmonary and lymph node metastases.^{6,16,17} Two recent studies using another technique of implantation (orthotopic implantation of tumor tissue fragments removed from subcutaneous xenograft established by injection of cell suspension) reported a metastatic mouse model with lymph node, pulmonary, pleural, hepatic, renal, adrenal, bone, cerebral sites.^{5,20} However, in these reported studies, several metastases were micrometastases. In two other studies using the same orthotopic implantation technique with tumor fragments reported metastatic sites were lymph node and lung only.^{21,22}

Diaphragmatic metastases observed in this study are rarely described in other orthotopic model and may be due to peritoneal seeding during implantation after cell leakage.²³ The unusual pattern of metastatic spread (kidney, pancreas, etc.) in the mouse model may be due to artificial orthotopic injection of cancer cells with a possible role of vascular spread during the procedure and/or to vascular and lymphatic prostate drainage differences between rodent and human.

The use of PC-3 and DU 145 metastatic sublines for orthotopic implantation has allowed us to obtain prostatic tumors and various metastases. We have observed a potential increase in metastatic spread with no metastatic propensity to a specific site. Further molecular biology studies are ongoing using DNA chip technology to study gene expression in primary, metastatic and cultured metastatic tumors. These studies will try to find differences in gene expression between primary and metastatic tumors.

Conclusions

The Nod Scid mouse is a relevant preclinical animal model to study human prostate cancer. It is efficient to develop orthotopic tumors and visceral metastases. Furthermore, metastatic sites seem more numerous in comparison to other metastatic mouse models described.

The Nod Scid model, characterised by a major immunodeficiency, is a very attractive animal model for gene

therapy studies. We validated this new model to begin a gene therapy program for prostate cancer using proapoptotic genes (ongoing work).

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