RESEARCH ARTICLE

Metastatic failure of B16FO melanoma cells inoculated in different and non-typical organs of athymic male nude mice and female C57BL6 mice

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Abstract
Background: Cancer is the second leading cause of death in the world. Metastasis is the process by which tumor cells leave the primary site and form colonies in different locations. The risk of metastasis makes cancer difficult to treat and accounts for a 90 percent mortality rate. A major problem in studying metastasis has been a lack of suitable models that faithfully represent the metastatic process as it occurs in vivo. Purpose: In order to mimic the potential of tumor metastasis in humans, we used a highly aggressive B16FO murine melanoma cell line, and showed that B16FO melanoma can metastasize to various sites. In the current study we investigated whether B16FO cells inoculated in the non-typical organs in male nude mice and female C57BL6 mice have the potential to metastasize to distant organs. As such, we injected B16FO cells in prostate, seminal vesicles and the pancreas of male nude mice and in mammary pads of female C57BL6 mice. The animals were kept on recommended diet and sacrificed after three weeks. Results: We observed that while the animals did develop tumors at the sites of inoculation, the B16FO cells did not induce metastasis in any of the vital organs. It is possible that although B16FO are very aggressive cells, the three weeks were not enough for metastasis to develop. Otherwise it could be the tumor dormancy period. Conclusion: We conclude that out of the four inoculation sites we tested in this series of experiments such as the prostate, seminal vesicles, the pancreas and mammary pads of nude or C57BL6 mice are not the best route for future investigation of melanoma metastasis.

Key Words: Metastasis, B16FO, nude mice, C57BL6 mice, melanoma

1. Introduction:

Cancer remains the second leading cause of death in the world and despite advances in research on cancer prevention and treatment approaches the outcome for a cancer patient has not improved much. In 2019, 1,762,450 new cancer cases and 606,880 cancer deaths were projected to occur in the United States alone.[1] If this trend continues, cancer has the potential to become the number one leading cause of death. More advancement in cancer research is still needed with regards to prevention and specifically in the treatment of cancer metastasis. Metastasis is the primary cause of cancer related deaths.[2] Until recently, most research has focused on the biology of primary tumors versus metastatic tumors. While the advancement in treatment options highlight the progress in cancer fields, more than 90% of patients still succumb to the metastasis of cancer.

Metastatic tumors are very common in the last stages of cancer. Metastasis is the process by which tumor cells leave the primary site and form colonies in different locations. Metastasis of cancer occurs through the circulatory or lymphatic system or both. The most common sites are the lung, the liver, bones and the brain. Critical events in tumor metastasis include cell attachment, proteolytic degradation of the extracellular matrix and migration through the disrupted matrix.[3] The risk of metastasis makes cancer difficult to treat and accounts for a 90 percent mortality rate. A major problem in studying metastasis has been a lack of suitable models that faithfully represent the metastatic process as it occurs in vivo. While some human xenograft models can approximate primary tumor growth in mice, replication of tumor metastasis is more problematic.[4] Generally, human tumor cells metastasize poorly in mice and are associated with unexpected characteristics. In contrast, murine tumor cell models often metastasize more effectively and display metastatic characteristics similar to those observed in cancer patients.[5] In order to mimic the potential of tumor metastasis in humans, a highly invasive and metastatic murine B16FO melanoma cancer cell line was chosen.

Melanoma is a very serious and highly metastatic form of skin cancer, which causes the most skin cancer-related deaths. It is the seventh leading type of cancer in the US. In its advanced stages, melanoma is resistant to existing therapies. The incidence of melanoma is ten times higher in Caucasians than in African-Americans. Exposure to the sun is the principle cause of melanoma. Individuals with fair skin, blue eyes, blonde or red hair are at highest risk for melanoma. Prognosis varies depending on the stage of the malignancy at the time of diagnosis. For small lesions, surgical resection can be an effective cure. At the other extreme, Stage III metastatic malignant melanoma has a 5-year survival rate as low as 27%.[6, 7]

Breast cancer is the most common cancer diagnosed in women and is the second leading cause of death in women after lung cancer. When diagnosed at an early stage, breast cancer can be treated using surgery, radiation, chemotherapy or hormonal therapy. There is no complete cure and the 5-year survival rate of breast cancer patients is 88% when given the appropriate treatment.[6, 7]

Prostate cancer is the most common cancer in men and the second deadliest cancer in the US, primarily affecting males 55 years and older. Prostate cancer is more common in African-American males than Caucasion males and occurs more frequently in developed countries. Developed countries account for 75% of the incidence rate; with the highest found in Europe, North America and Australia. Current

diagnostic methods like PSA testing and digital rectal examinations have helped in early stage detection. Standard treatments consist of surgery (prostatectomy), hormonal therapy and radiotherapy.[6, 8]

Primary cancer of the seminal vesicle cancer is rarely encountered in clinical settings, with adenocarcinoma being the most common type of primary malignancy in the seminal vesicles. However, metastasis to the seminal vesicles is common in cancers of the prostate, urinary bladder and rectum. Immunohistochemistry for PSA and prostate specific phosphate are negative.[9]

Cancer of the pancreas is the seventh most leading cause of cancer related deaths in the world. Although rare, pancreatic cancer is the fourth leading cause of cancer-related deaths in both men and women and is almost always fatal.[10]

In our previous studies using different cancer cell lines, we inoculated fifty different human cancer cell lines subcutaneously.[11, 12] Although we were able to induce tumor growth, we failed to demonstrate metastasis to vital organs. In order to mimic the potential of tumor metastasis in humans, highly aggressive B16FO murine melanoma cells were used and showed that B16FO melanoma can metastasize to various sites. In one of our studies, when we injected B16FO cells into the tail vein and testicles, and noted metastasis to the lungs.[13, 14] When B16FO cells were injected into the spleen, we noted metastasis to the liver[15,] and when the cells were injected into the kidney, metastasis was found in the lungs and spleen.[16]

The aim of the current study is to investigate whether B16FO cells inoculated in the prostate, seminal vesicles and pancreas of male nude mice; and into the mammary pads of female C57BL6 and nude mice had the potential to metastasize to distant organs.

2. Materials and methods:

2.1 Cancer Cell Line and Culture:

Murine B16FO melanoma cells obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The media and sera used were obtained from ATCC, while the antibiotics (penicillin and streptomycin) were purchased from Gibco-BRL (Long Island, NY, USA).

2.2 Animals:

Male and female athymic nude mice ~~of~~ 8-10 weeks of age, and C57BL6 female mice of approximately the same age, were obtained from Simonsen Laboratories (Gilroy, CA, USA). The mice were kept in micro-isolator cages under pathogen-free conditions on a 12-hour light/dark schedule for 1 week. The animals were cared for in accordance with institutional guidelines for the care and use of experimental animals.

2.3 Experimental Design:

2.1.1) B16FO melanoma cells in nude mice:

After housing for one week the mice were anesthetized by inhalation utilizing isofluorane USP (Abbott Laboratories, Chicago, IL, USA). The 50,000 B16FO melanoma cells in 50 µl of PBS were inoculated in the prostate, seminal vesicles and the pancreas of male nude mice using a 26-gauge needle. Two-three mice were used per group. The needle was carefully removed. The injection area was blotted; the skin was sutured and clamped. Three weeks later the animals were sacrificed. The abdominal cavity was opened and the tumor growth was observed at the injection sites of the prostate, seminal vesicles and the pancreas. The tumors were excised and processed for histopathology. The abdominal cavity of each

animal was examined for any visible metastasis to the vital organs.

2.1.2) B16FO melanoma cells in female nude mice and and female C57BL6 mice: In another set of experiments, the one million B16FO melanoma cells in 100 µl of PBS were inoculated in the mammary pad of four C57BL6 female mice and 3 nude female mice. The mice were returned to their cages and put on a normal diet. Three weeks later, all the female mice were sacrificed and the tumors were excised and weighed. The tumors were further processed for histopathology. The abdominal cavity was opened and examined for any metastasis to the vital organs.

All procedures were performed according to humane and customary care and use of experimental animals and conducted under protocols approved by the Internal Animal Care and Use Committee (IACUC).

2.4 Histopathology: The tumor samples excised from the prostate, seminal vesicles and the pancreas from male nude mice and the female C57BL6 mice were processed for histopathology. They were fixed in 10% buffered formalin, embedded in paraffin and cut into 4- to 5-µm sections. The sections were deparaffinized through xylene and graduated alcohol series to water and stained with hematoxylin and eosin for microscopic evaluation by IDEXX Reference Laboratories.

3. Results:

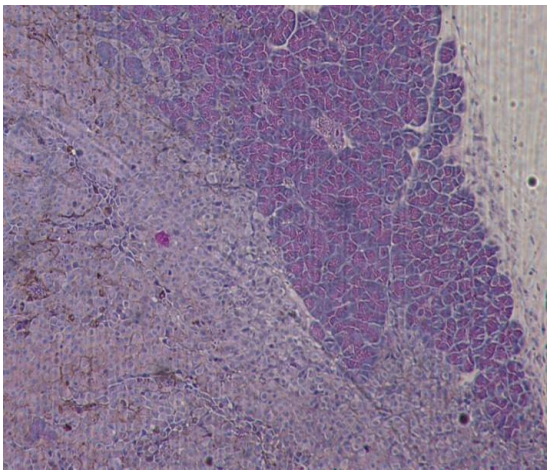
The mice were sacrificed three weeks after the inoculation. The abdominal cavity was opened and the tumors were excised. The rest of the abdominal cavity was examined for metastases to the vital organs.

3.1 B16FO melanoma cells inoculation in the prostate, seminal vesicles and the pancreas of male nude mice:

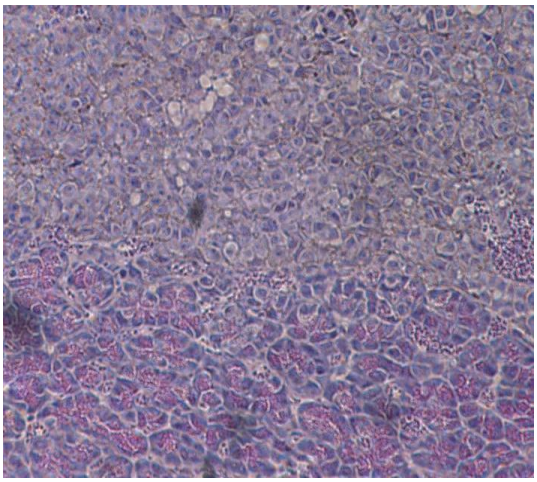
3.1.1 Gross examination of the tumors:

Figure 1: Shows the tumors that were excised from the prostate, seminal vesicle and pancreas. The excised tumors from prostate (leftmost above), seminal vesicle (middle above) and pancreas (rightmost above) showed extensive growth of melanoma cells at the site of inoculation.

3.1.2 Histopathological examination of the tumors:

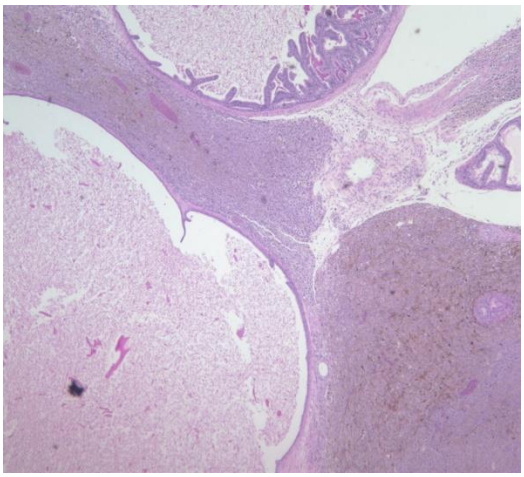


2A: Melanoma growth in prostate (10X)

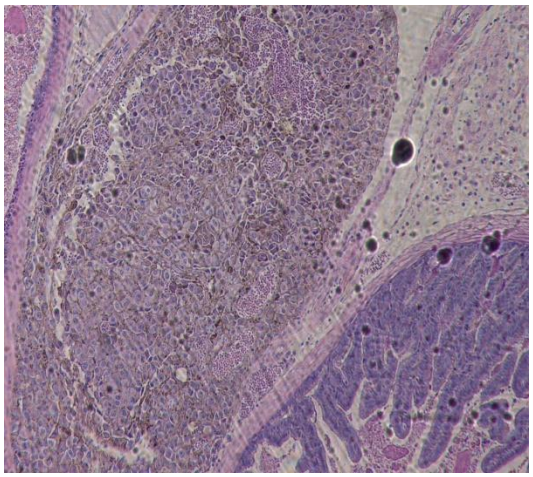


2B: Melanoma growth in prostate (20X)

Figure 2: Shows low and high magnification of B16FO tumor invasion of the prostate 10X and 20X respectively showing a border between normal prostate tissue and the melanoma invasion by large irregular large round mass of sheet with granular pigmentation with malignant melanoma.

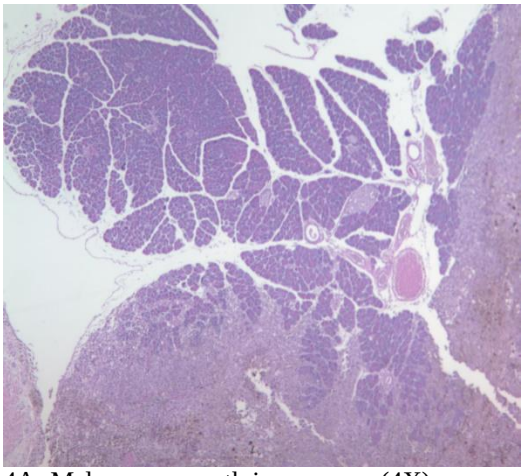


3A: Melanoma growth in seminal vesicle(4X)

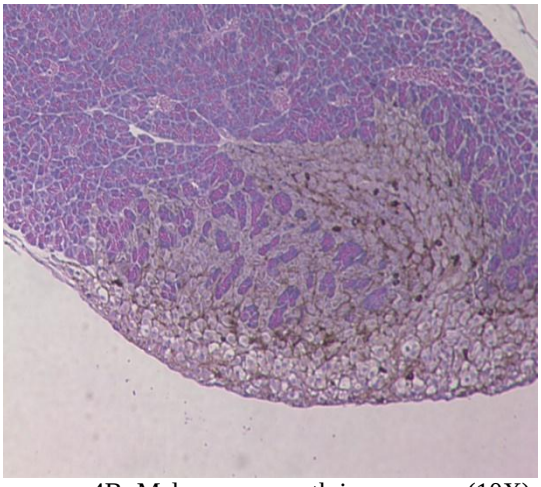


3B:Tumor growth in seminal vesicle (10X)

Figure 3: (A, B) Show the H&E staining of the seminal vesicles. A similar tumor is invading the interstitium of the seminal vesicle by large irregularly round mass composed of sheaths of large and irregularly round tumor cells showing a clear line of demarcation between normal tissue and melanoma invasion.



4A: Melanoma growth in pancreas (4X)



4B: Melanoma growth in pancreas (10X)

Figure 4: (A, B) Show the sections of pancreas where a similar melanoma tumor is seen invading the pancreatic tissue.

3.1.3 *Metastasis in vital organs:*

The abdominal cavity of all mice was opened and the metastasis to other vital organs such as the liver, spleen and the lungs were visually examined. We failed to see metastatic foci to any other vital organs of the male nude mice after inoculation of B16FO melanoma cells.

3.2 B16FO melanoma cells inoculation in the mammary pad of female C57BL6 and nude mice:

3.2.1 Gross examination of the tumors: Gross anatomy of representative C57BL/6 mice injected with 106 B16FO melanoma cells in the mammary pads.

Figure 5: Shows that the large tumors that were excised from the mammary pads of the C57BL6 mice after inoculation of B16FO melanoma cells.

3.2.2 Mean initial and final weights of the animals:

The body weight of both types of mice did not differ significantly. The average initial weight of C57BL6 mice was 23.8 ± 3.42 gms, while the average weight at the time of sacrifice of the mice was 25.2 ± 4.5 gms. The average initial weight of the female nude mice was 26.97 ± 2.0 gms while the average weight at the time of their sacrifice was 33.8 ± 4.2 gms.

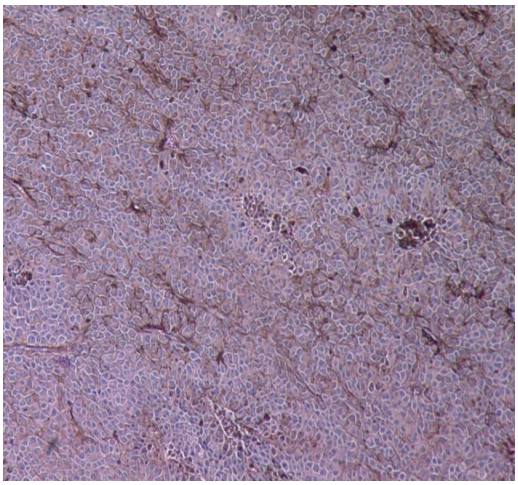
3.2.3 Tumor morphology:

The tumors were excised after 3 weeks and the average weight of the tumors developed in the mammary pads of C57BL6 mice was 4.63 ± 1.7 gms. The average weight of the excised tumors in the nude mice was 3.0 ± 0.2 gms.

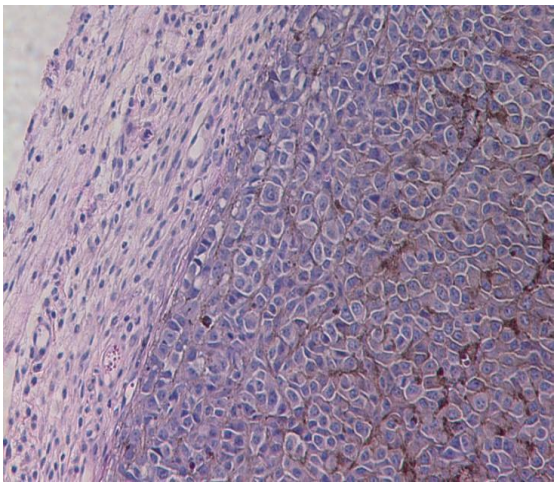
3.2.4 Metastasis in vital organs:

The abdominal cavity of all mice was opened and the metastasis to other vital organs such as the liver, spleen and the lungs were visually examined. We did not see any metastasis to any of the vital organs such as the lungs, liver, and the kidney.

3.2.5 Histopathological examination of the tumors:



6A: Melanoma growth in mammary pad (10X)



6B: Melanoma growth in mammary pad (20X)

Figure 6: Histopathological examination of the tumors excised from female C57BL6 mice showed sections of large subcutaneous masses composed of sheaths and nests of large irregularly round cells. Some of the cells associated with granular dark pigment consistent with malignant melanomas.

4. Discussion

Our objective in this study was to examine if aggressive B16FO melanoma cells would metastasize to vital organs when inoculated at four different sites atypical for this cell line in male and female mice. We inoculated the cells in the prostate, the seminal vesicles, and the pancreas of male nude mice and into the mammary pads of the C57BL6 of female nude mice. Our results showed that while the cells developed large tumors at the site of inoculation, they failed to metastasize to other organs, especially vital organs. Our previous experiments have shown that B16FO melanoma cells were able to successfully metastasize when injected via the mouse tail vein, intratesticular, kidney and intraperitoneal or intrasplenic route.[13-16]

However in this series of experiments we did not see metastasis of B16FO cells. This is not surprising since microenvironments and tumor-

host interactions play important roles in tumor cell behavior. It could be due to multiple reasons.

Cancer metastasis is a complex process and involves multiple steps. Simplistically, the cascade of metastasis involves detachment of cancer cells from the original tumor, its entry into the circulatory or lymphatic system, successfully avoiding the attack by immune cells, exiting the blood or lymph vessel, proliferates and establish in the distant organ.[17, 18] Although, the metastatic cascade is driven by a sequence of multiple factors, a favorable microenvironment and appropriate biomechanics are necessary for the eventual formation of a secondary tumor.[19] A transition from an epithelial cell to a mesenchymal cell is critical and is considered one of the major characteristics of cancer metastasis. The "seed and soil" theory of metastasis proposed by the English surgeon Stephen Paget more than 100 years ago is still widely accepted theory of metastasis.[20] He postulated that when a plant goes to seed, its seeds are carried in all directions but can only live and grow if they fall on congenial soil.

In our current study, one possible explanation of metastasis failure of B16FO melanoma cells could be due to a shorter span of experiments. We sacrificed the mice after three weeks versus four weeks in our previous studies where metastasis did occur. If we leave it a little longer, the animals would die. We did not think waiting longer to sacrifice the animals and see if the metastasis occurred later, met the ethical standards of the animal experiments.

Many cancer patients remain in post treatment remission for months or years before metastatic disease appears due to a phenomenon known as tumor dormancy. It is the lag time between dissemination and metastatic outgrowth during which the cancer cells remain in non-proliferative state for long periods.[21] Suzuki M reported that cancer cells retrieved from metastasis-free distant sites still retain their metastatic ability.[22] Our previous studies have shown that intraperitoneal injection of B16FO melanoma cells into C57BL6 mice demonstrated intraperitoneal growth and ascites, but did not result in metastasis to other organs.[14] In our previous experiments, we had observed metastasis after 2-3 weeks. In this case we sacrificed the animals after three weeks. Had we left them alive longer, there is a risk of animal death.

The purpose of the present study was to analyze which inoculation sites have the highest potential for melanoma cancer spread. We have used the B16FO murine melanoma cells; because we found that the human melanoma cells were not as aggressive as B16FO cells to form a tumor or their metastasis. Since this is an ongoing project, we wanted to explore which other non-typical sites are possible for metastasis. Out of the four inoculation sites we tested in this series of experiments, we conclude that the prostate, the seminal vesicles, the pancreas and the mammary pads of nude or C57BL6 mice are not the best route for melanoma metastasis.

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