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Renal Subcapsular Space of Balb/c Nude Mice as a Route for Evaluating Subpopulations of Human Bladder Carcinoma Cells

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Abstract. *Background/Aim: Subpopulations of bladder cancer (BC) cells have been found in tumors, with different abilities for malignancy and chemotherapy resistance. The BC cell line T24 has frequently been used to evaluate this phenomenon. Since technical limits exist in orthotopic procedures, we evaluated the renal subcapsular space as an alternative route for analyzing subpopulations of T24 BC cells in vivo. Materials and Methods: Balb/c nude mice underwent renal subcapsular inoculation with T24 cells, suspended in two different volumes of PBS. Four weeks post-inoculation, histology and immunohistochemistry were carried out. Results: In all the animals inoculated with a 10 μl volume of suspended cells, a pseudo-bladder structure in the renal subcapsular space was observed, with differential expression of mesenchymal and epithelial markers. T24 cells infiltrating the renal parenchyma towards the medulla and vessels were also observed. The volume used for inoculation was an important factor for the success of this technique. Conclusion: Renal subcapsular inoculation is an effective route for analyzing subpopulations and differentiation of T24 cells.*

Urinary bladder cancer (BC) is the most common malignancy of the urinary system (1). The most common form of BC is non-muscle invasive. However, almost half of such cases progress to the muscle-invasive form after repeated resection, leading to cancer-specific death and metastasis (2). Once metastatic disease is established, the only treatment option is chemotherapy, which may be limited if resistant cells are presented in the tumors.

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Emerging lines of evidence have suggested that for the progression of BC, the continuum phenotypic and genotypic changes leading to the emergence of more aggressive subpopulations is needed. In addition, cancer stem-like cells have been found in tumors, being considered precursors of neoplastic cells, as well as being involved in the maintenance and progression of tumors due their characteristics such as unlimited proliferative activity, self-renewal, and the ability to differentiate into other cell types (3). These abilities were clearly demonstrated in T24 BC cells, which represent major human epithelial BC with clinical relevance (4).

In one study, cell subpopulations were obtained from T24 BC using fluorescence-activated cell sorting technology (4). Using the limiting dilution method, Ware *et al*. were also able to isolate T24 subpopulations (5). Another study demonstrated that subpopulations of cancer stem-like cells from the T24 cell line contribute to cisplatin resistance (6), and have a different capacity for differentiation and migration (6).

Although T24 subpopulations may be acquired and evaluated *in vitro*, the tissue influence cannot be assessed in another way except though animal models. Orthotopic inoculation of human T24 cells in nude mice (immunodeficient mice) is an essential route for evaluating the behavior of these cells *in vivo*. However, there are technical limitations to this procedure. An alternative route is the subcutaneous space, but T24 cells are not tumorigenic when inoculated in nude mice by this route. Thus, we hypothesized a novel method for studying subpopulations of T24 cells in nude mice by which different phenotypes can be analyzed. Here, we evaluated the renal subcapsular inoculation of T24 cells as an alternative route for analyzing the differentiation of T24 cells *in vivo*.

Materials and Methods

Animals. Eleven male Balb/c nude mice (6-8 weeks old) were obtained from the Central Animal Facility of the Nove de Julho University. The mice were maintained under a controlled environment, allocated to mini-isolators with sterile water and food

Table I. *Frequency of events 28 days post-inoculation of T24 cells under the renal capsule. Evaluation of two volumes of inoculation.*

Inoculation with 1.0×10^6 T24 cells suspended in	Mice n	Subcapsular engraftment n (%)	Pseudo-bladder formation n (%)	Parenchymal infiltration n (%)
50 μl of PBS	3	0 (00.0)	0 (0.00)	0 (0.00)
10 μl of PBS	8	7 (87.5)	7 (87.5)	4 (50.0)

ad libitum. All procedures were approved by the Ethics Committee on Animal Research of the Nove de Julho University (number AN0027/2012).

Culture of T24 cells. Human bladder cancer T24 cells (HTB-4; American Type Culture Collection, Manassas, VA, USA) were acquired from the cell bank of the Federal University of Rio de Janeiro. T24 cells were cultured in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Sigma-Aldrich), and maintained at 37˚C with 5% CO_2. For renal subcapsular inoculation, cells at 70% confluence were trypsinized, washed twice to remove medium and supplements, and suspended in phosphate buffered saline (PBS).

Inoculation of T24 cells under the renal capsule. For the renal subcapsular inoculation procedure, mice were anesthetized by intraperitoneal injection of xylazine and ketamine (100 mg/kg and 10 mg/kg, respectively). The left kidney of all animals was externalized and 3 of them received 1.0×10^6 T24 cells suspended in 50 μL of PBS, while 8 animals received 1.0×10^6 T24 cells suspended in 10 μL of PBS. The injections were performed using a Hamilton syringe with a 23G needle attached. The opening in the capsule was then cauterized to prevent reflux of the cells. After orthotopic repositioning, the muscle and skin incisions were sutured with nylon 6.0 (Ethicon Inc., Somerville, NJ, USA). After 3 inoculations using a volume of 50 μL, we noted that this amount was harder to be kept away from reflow when compared to a volume of 10 μL, thus concluding that this smaller volume was methodologically better and more precise. Tramadol hydrochloride was administered subcutaneously (5 mg/kg) immediately after surgery as an analgesic strategy. After 28 days, euthanasia was performed using overdose of xylazine and ketamine (400 mg/kg and 40 mg/kg, respectively). The kidneys were removed and a 5 mm fragment covering the region of the graft was sliced and fixed in 10% buffered formalin. The other organs were thoroughly analyzed to detect possible metastases.

Histology and immunohistochemistry. Sections of 3 μm-thick were stained with hematoxylin and eosin (H&E) and then used for immunohistochemistry. We analyzed a median of 25 H&E serial sections per case. For immunohistochemistry, renal tissues were cut at 4 μm thickness. The samples were then deparaffinized and an antigen recovery step was performed (citrate buffer, pH 6.0 in a microwave). Blocking for endogenous peroxidase and avidin/biotin was carried-out. To improve the blocking of other nonspecific antigens, the samples were exposed to a solution containing 6% milk diluted with PBS for 15 minutes. After removal of the excess milk solution, primary antibodies monoclonal mouse anti-human cytokeratin 19 (Code IS615; DAKO, Carpinteria, CA, USA) and anti-α-smooth muscle actin (αSMA, code IS611; DAKO) were used. Anti-cytokeratin 19 was diluted 1:100 in PBS, while anti-αSMA was used directly (supplied already to use). The sections were kept at 4-8˚C overnight (12 to 14 hours), inside a humidified chamber. For the negative controls, the primary antibodies were omitted. To complete the procedure, sections were incubated with LSAB+ System-HRP reagents (K0690; Dako Co., Glostrup, Denmark). Finally, 3,3'-Diaminobenzidine (DAB) substrate-chromogen was used to complete the reaction (K346811; Dako Co.). The tissue was then counterstained with hematoxylin (CS70030; Dako, Glostrup, Denmark) and coverslips were fixated with the use of Aquatex (1085620050; Merck, Darmstadt, Germany).

Results

The subcapsular inoculations of 1×10^6 T24 cells suspended in a volume of 50 μl failed to induce cellular engraftment (Table I), probably because a significant reflux of cells occurred during the inoculation. In contrast, no reflux was observed during the subcapsular inoculation of 1×10^6 T24 cells suspended in 10 μl. Of the eight mice that received 10 μl, only one failed to engraft the cells under the renal capsule (Table I). For the seven successful cases, all exhibited the formation of a subcapsular vesicle containing blood in its luminal space (Figure 1A). Surrounding the blood, an inner layer was found, formed by approximately five rows of cells presenting epithelial phenotype (Figure 1B), which were positive for cytokeratin 19, an epithelial marker (Figure 1B). Finally, over the vesicle wall, a second cellular layer was found in which the cells presented a mesenchymal-like phenotype, especially due to their elongated form. These cells lost positivity for cytokeratin 19, becoming αSMA-positive cells (Figure 1B). The external side of the vesicle was associated with a thickened renal capsule, in which vessels were found.

Additionally to the subcapsular vesicle, infiltrating T24 cells were found invading the renal parenchyma (Figure 1C), surrounding glomeruli and tubules. Half of the mice inoculated with a volume of 10 μl presented cellular infiltration (Table I), including the mouse that did not present subcapsular engraftment. Of the eight mice inoculated with 10 μl, only two presented T24 cells invading renal vessels, as demonstrated in Figure 1D. Even with blood vessel invasion, no sign of metastasis was found.

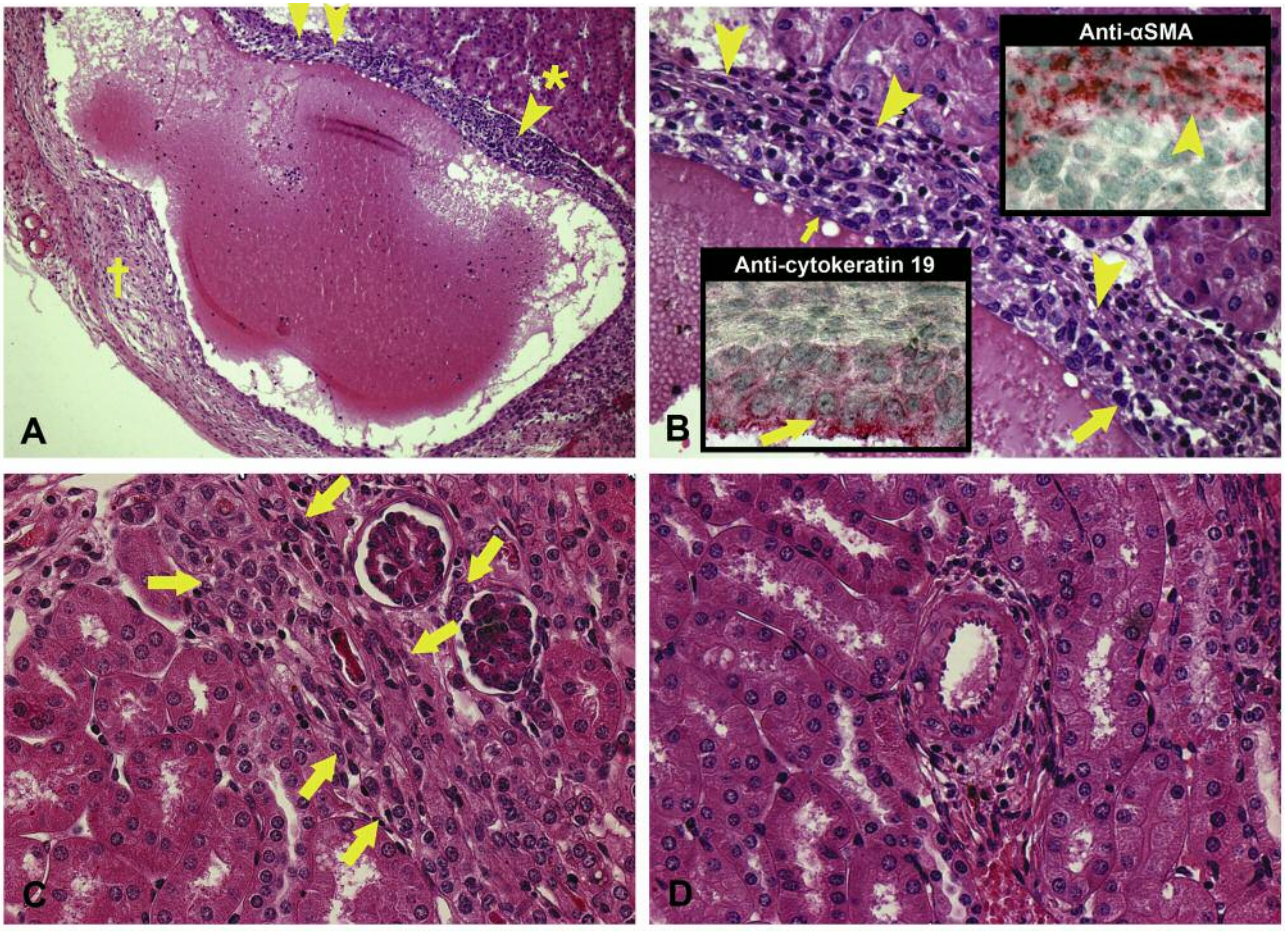

Figure 1. *A: Bladder-like vesicle created under the renal capsule 28 days post-inoculation of T24 cells. Blood was found in the luminal vesicle. A thickening of the renal capsule can be seen, with abundant collagen fibers (cross). T24 cells organized to form the vesicle wall (arrowheads), close to the renal parenchyma (asterisk). Bar=200 μm. B: T24 cells presented different phenotypes in order to form the bladder-like vesicle layers. There are epithelial-like cells facing the luminal vesicle (arrows), which were positive for cytokeratin 19, and mesenchymal-like cells in deeper layers close the renal parenchyma [α-smooth muscle actin (αSMA)-expressing cells] (arrowhead). Bar=40 μm. C: A subpopulation of T24 cells infiltrated the renal parenchyma (arrows) towards the renal medulla. Bar=20 μm. D: T24 cells surrounding a renal arteriole. Bar=20 μm*

Discussion

The renal subcapsular space has been explored by researchers into cancer since the 1980s, when cells and biopsy samples began to be deployed for this space. Using nude mice, Murahashi *et al*. inoculated cancer cell lines of kidney, pelvis, urethra, bladder, testis and prostate in the subcutaneous and renal subcapsular spaces, and demonstrated that both subcutaneous and under the renal capsule were interesting for the evaluation of tumor growth in response to Tegafur chemotherapy treatment (8).

In this study, we demonstrated that the renal subcapsular space is an attractive site for inoculation of T24 carcinoma cells in order to analyze different T24 subpopulations and differentiation. We tested two volumes for inoculation of 1.0×10^6 T24 cells. The results demonstrated that the inoculation of 50 μl was not adequate for inducing engraftment of the T24 cells under the renal capsule. The failure in this case may be related to the reflux of the cells, and also to the greater dilution. For this reason, we tested inoculation using a lower volume. The volume of 10 μl was efficient at engrafting T24 cells under the renal capsule. Only one mouse inoculated with 10 μl failed to present T24 cells under the renal capsule 28 days post-inoculation, however.

Interestingly, the T24 cells formed a vesicle in the renal subcapsular space. The presence of epithelial-like cells in the inner of the vesicle (cytokeratin 19-positive cells), and mesenchymal-like cells (αSMA-positive cells) in the

external layer suggest that the epithelial to mesenchymal transition phenomenon occurred, leading to the formation of a 'pseudo-urinary bladder'. This is possible since stem-like cells were found as a subpopulation of the T24 cell line (4). These stem-like cells are believed to be a source of neoplastic cells. However, it is plausible that they may influence differentiation in other cell types, as such as normal bladder cells. Cancer cells may produce growth factors required for cellular differentiation. Co-culture of cancer cells with stem cells has been used to regenerate other organs, for example lung (9), breast (10), and colorectal cells (11). Chung and Koh (12) demonstrated that differentiation to urothelial cells may be induced using co-culture of human amniotic fluid stem cells and immortalized bladder cancer lines, and that fibroblast growth factor 10 has an important role in this process (12). The mechanisms by which T24 cells are stimulated to form the pseudo-bladder when inoculated under the renal capsule remain unknown. Our model should be explored to clarify the molecular and cellular mechanisms involved in this process.

Histological analysis also demonstrated that a subpopulation of T24 cells infiltrated the renal parenchyma towards the medulla. In addition, T24 cells were found surrounding some renal vessels. These events probably occurred due to the presence of a more aggressive subpopulation of T24 cells. Thus, this model can be explored to investigate the molecular and cellular mechanisms involved in the metastatic phenotype of T24 cells.

Conclusion

Renal subcapsular inoculation is an effective route for analyzing subpopulations and cellular differentiation of T24 cells *in vivo*.

Disclosure

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Conflict of Interest

The Authors have declared that no conflict of interests in regard to this study.

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