PMID- 28818454

Cytotherapy, 2017; 19: 1233–1245

International Society for Cellular Therapy ISCT

UROTHELIAL CELLS

The anti-tumor effect of intravesical administration of normal urothelial cells on bladder cancer

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Abstract

Background aims. Urothelial bladder cancer (UBC) is the second most common cancer of the genitourinary tract and for advanced forms of the disease it has a high mortality rate. There are no approved new molecularly targeted agents or chemotherapeutics for advanced UBC beyond cisplatin-based chemotherapy except the recently approved anti-programmed death ligand 1 (anti-PD-1/PD-L1) antibody. With complex genetic and epigenetic alterations in tumors, despite several druggable targets identified, to cure UBC is still a challenging unmet medical need. Like other cancers, UBC to the host body is considered as a wound, aging stem cell disease and immunosuppressive disorder. Therefore, we proposed a novel cellular approach to target the host body by intravesical instilling of normal urothelial cells that could repair the injury and reduce inflammation by activating body-reparative capacity and because non-self cells are transplanted, host body immune responses could be induced in the tumor microenvironment of UBC to restrain and even eliminate tumor cells. *Methods.* In this study, we isolated and expanded normal male murine urothelial cells and intravesically administered them into the bladders of female mice of two orthotopic bladder tumor models and one urothelial injury model. *Results.* We showed that the instillation of normal urothelial cells containing stem/progenitor cell population into bladders could have anti-tumor effect in orthotopic tumor models, possibly by activating immune responses and helping injured urothelium tissue recovery in a chemically induced urothelial injury model. *Conclusions.* Our findings could lead to an innovative and revolutionary cell therapy modality with normal urothelial cells as an effective and safe therapeutic option for UBC.

Key Words: *bladder cancer, immune, intravesical, rejection, repair, urothelial cell*

Introduction

There has been no major breakthrough in treating advanced urothelial bladder cancer (UBC) beyond chemotherapy and surgery in the past 30 years with no widely recognized second-line therapy and no approved molecularly targeted agents [1,2]. Molecular analysis has identified major changes in multiple signaling pathways with genetic and epigenetic alterations in muscle-invasive UBC [3,4]. Although the druggable targets could be discovered in these altered pathways and applied in combination or sequentially to battle UBC, the tumor heterogeneity and acquired resistance may cause therapy failure [3,4]. Recent trials assessed treatments with anti-programmed death ligand 1 (PD-L1) or PD-1 antibodies showed durable activity and good tolerability in patients with locally advanced or metastatic UBC that has progressed after platinum-based chemotherapy [5,6]. Despite the fact that these trial results hold great promise for immune checkpoint inhibitors in treating advanced UBC, the beneficial response rate is limited and, in some cases, there were severe immune adverse events. Therefore, it is critical to develop an innovative therapeutic approach to circumvent these obstacles and to contain the tumors at bay from eroding the vitality of patients.

In Virchow's hypothesis, he hypothesized that the origin of cancer was at sites of chronic inflammation because some classes of irritants, together with the tissue injury and ensuing inflammation they cause, enhance

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(Received 7 March 2017; accepted 25 June 2017)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2017.06.010

cell proliferation, which has been supported in current studies [7,8]. Based on the studies on inflammation and cancer, wound healing and tumor stroma formation share many important properties and this similarity was phrased as "tumors: wounds that do not heal" [9]. Cancer is also an aging and stem cell disease except for a few pediatric cancers because most cancers occur in older people and the probability of being diagnosed with an invasive cancer is also higher in the elderly [10]. For bladder cancer, it is typically diagnosed in older individuals, with a median age at diagnosis of 69 years in men and 71 years in women [11]. Although not common, bladder cancer can be seen in children and young adults, where it usually presents as a low-grade, non-invasive disease [12]. Young patients with bladder cancer have more favorable pathological features and clinical outcomes than older patients [13]. These clinical observations strongly indicate that the regenerative and reparative ability of urothelial cells in the young may help the prevention and recovery of urothelial carcinoma. The urothelium, composed of specialized epithelium, lines the distal portion of the urinary tract, which comprises the renal pelvis, ureters, urinary bladder and upper urethra, and the urothelium has a remarkable regenerative capacity to repair tissue damage and restore urothelial integrity because urothelial cells can rapidly proliferate and differentiate under stimulus by pathological damage [14,15]. The initiation, progression and therapeutic failure of UBC are demonstrated to be linked to the transformation of a subpopulation of urothelial stem cells in the urothelium into cancer stem cells (CSC) and their presence [14,16]. These urothelial CSCs takeover normal urothelial stem cell signaling pathways such as sonic hedgehog (Shh), Stat3 and prostaglandin E2 (PGE2) for tumor formation, malignant transformation and chemoresistance [17,18]. Therefore, it is proposed that CSCs hijack stem cell signaling pathways to facilitate cancer progression and recurrence with renewal ability and to create a diverse heterogeneous population through multi-potential differentiation [19]. Similarly, the concept that carcinomas as caricatures of tissue renewal has been noted, in that they are composed of a mixture of malignant stem cells with high proliferation potential and a limited capacity for differentiation under normal homeostatic conditions [20]. Therefore, we believe that to prevent the rise of cancer, it is essential to regenerate and repair the injury sites, not letting the cancer cells dominate the sites, proliferate and eventually metastasize. During the tissue-healing process, normal cells could compete with cancer cells on stem cell signaling to repair the injury sites.

The human immune system is considered to be the combination of cells and proteins that fight infection, but it has also been proposed to be involved in cancer immune surveillance by the host body [21]. Because the immune system works essentially by discriminating self from non-self, the immune system is activated to detect and eliminate foreign subjects including mutated tumor cells [22]. However, tumors could develop immune resistance and tolerance by multiple mechanisms, such as dysregulating the expression and function of immune checkpoint proteins and inducing regulatory T cells [23,24] to induce an immunosuppressive milieu of the host body. In addition, higher cancer risk was observed in patients who are immunosuppressed, such as transplant recipients who were treated with immunosuppressive drugs [25]. Therefore, the immunotherapeutic strategies to boost host immune responses to treat cancers with immune checkpoint inhibitors have made great progress in several advanced cancers whose treatment has changed little in decades, such as melanoma and bladder cancer [5,6,26], indicating that enhancing host immune responses could be an effective strategy to fight cancer. Epithelial cells are able to respond to environmental changes by increasing the production of mediators to induce innate immunity to regulate inflammation, immunity and wound repair [27,28]. The immune responses could also be induced by the transplantation of non-self cells as shown in a skin epidermal cell graft [29]. These immune responses from epithelial cells and the rejection of transplanted non-self cells could boost anti-tumor immune responses to protect hosts from tumor cells.

Because we believe that cancer is a chronic wound, an aging stem cell disease and an immunosuppressive disorder for the host, we hypothesized that the transplantation of normal epithelial cells like urothelial cells could facilitate the urothelium to regenerate and repair injured stem cell–depleted/signaling hijacked and dysfunctional cancerous tissues with functional normal stem/progenitor cells and thus affect tumor progression. Furthermore, the non-self–transplanted cells from donors could induce immune responses and rejection reactions by the host, which could concurrently boost anti-tumor responses that could help the elimination of cancer cells. In the current study, we used non-self normal murine urothelial cells containing a stem/progenitor population and intravesically instilled cells into bladders to examine the anti-tumor and repair effect of normal urothelial cells in two orthotopic bladder tumor models and one urothelial injury model. This study demonstrates the potential of non-self normal urothelial cells to treat UBC with activated immune responses and increased repair capacity.

Materials and methods

Cell culture

Primary normal murine urothelial cells (NMUs) were isolated and *ex vivo* expanded as previously described

[30]. In brief, bladders were dissected, washed with phosphate-buffered saline (PBS) and everted through the neck of the bladder using dissection forceps. Everted bladders were placed in 5 mg/mL Collagenase type I (Sigma) in growth medium and incubated at 37°C for 1 h. Urothelial cells were collected by gently scraping with a scalpel blade, and the muscle and lamina propria layers were discarded. Urothelial cell sheets were further dissociated by pipetting up and down for 5 min and rinsed twice in PBS, centrifuged and washed with media containing 10% fetal bovine serum (FBS) before plating onto tissue culture dishes. Murine urothelial cells used had undergone between 5 and 15 passages. The MBT-2 murine urothelial carcinoma cell line was obtained from Dr. T.-F. Hsieh (Tzu Chi University/Hospital at Taichung Branch, Taiwan) in 2014 and, for MBT-2 lines (MBT-2-luc) stably expressing luciferase gene, the pGL4.51[luc2/CMV/Neo] vectors (Promega) encoding the luciferase reporter gene luc2 (*Photinus pyralis*) were stably transfected into MBT-2 cells with lipofectamine (Invitrogen), and 48 h after infection selected using 400 μg/mL G418 (Invitrogen) for 10 days and then verified with luciferase activity and bioluminescent imaging analysis. MBT-2 cells used in the experiments had undergone between 5 and 20 passages after receiving them. MBT-2-luc cells used in the experiments had undergone less than 10 passages. The cells were not tested for mycoplasma and authenticated due to their murine origin.

Flow cytometry

These cell suspensions were stained for flow cytometric analysis using a cocktail of anti-mouse CD44 allophycocyanin and anti-mouse CD49f fluorescein isothiocyanate antibodies from eBioscience. Stained samples were read on a BD LSR II cytometer and analyzed using BD FACSDiva software.

Immunofluorescent staining

Cells were grown the chamber slides at 50–70% confluence. To start immunofluorescent staining, cells on the slides were fixed with 4% paraformaldehyde, washed with PBS, permeabilized in 0.2% Triton/PBS and blocked for 1 h in PBS and 10% FBS. Primary antibody against CK5 or CK14 (Santa Cruz) was added overnight at room temperature. On the next day, cells were washed with PBS and incubated with Labeling&detection Alexa fluor 488 DAM (Thermo Fisher Scientific) for 1 h and then 4′6,-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Cells were washed with PBS, mounted and imaged with a microscope.

Western blot

Proteins were extracted by radioimmunoprecipitation assay buffer and separated on 4% to 12% stacking sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk and then incubated with the primary antibody overnight at 4°C. Following Tris Buffered Saline with Tween 20 (TBST) washing, membranes were incubated with peroxidase-conjugated secondary antibody for 1 h and exposed on film using the Enhanced Chemiluminescence (ECL) Detection System (Thermo Scientific). Antibodies used were as follows: anti-CK5, anti-CK14 and anti-β-actin antibodies (all from Santa Cruz).

Syngeneic orthotopic transplantable murine urothelial tumor model, murine N-Butyl-N-(4-hydroxybutyl)nitrosamine–induced urothelial tumor model and intravesical instillation of NMUs

For a syngeneic orthotopic transplantable murine urothelial tumor model, MBT-2-luc cells were harvested from 70–80% confluent cultures and washed once in serum-free Roswell Park Memorial Institute medium and then resuspended in PBS. Female C3H/He mice were anesthetized by isoflurane. A small lower abdominal incision was made and the bladder was exposed. MBT-2-luc cells were injected into the bladder wall muscle using a sterile syringe with a 30-gauge needle. The injection site was pressed with a cotton swab for 30 sec and the incision was closed with sutures. For N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-induced bladder carcinogenesis, a 0.05% concentration of BBN (Sigma) was dissolved in drinking water, and BBN-containing water in a dark bottle was provided to C57BL/6 mice *ad libitum* for 20–24 weeks until the hematuria score was >2+ (Arkray Aution Sticks urine strip). Bladders were collected and analyzed after 4 weeks of treatment. For intravesical instillation of normal murine urothelial cells, subconfluent NMUs (5–10 passages) were trypsinized, and >90% cell viability was confirmed using the trypan blue exclusion method. Tumor-bearing mice were anesthetized with isoflurane. Urine was void from the bladder by mild pressure on the abdomen. A 24-gauge catheter was introduced into the lumen of the bladder through the urethra. NMUs, 1×10^6 in a 100 μL suspension of normal saline, were then injected into the bladder. The control group received 100 μL normal saline instead. All mouse experiments were approved by the Institutional Animal Care and Use Committees (IACUC) review board at China Medical University.

Urothelial injury murine model

Chemical injury of the urothelium of the bladder was induced by intraperitoneal injection of a cyclophosphamide (CPP; Sigma) solution in PBS (250 mg kg^{-1}). Bladders were collected at the indicated time points after administering CPP. Bladder tissues were fixed in 4% paraformaldehyde at 4°C for 2 h, thoroughly washed in PBS, placed in 30% sucrose overnight and frozen in optimal cutting temperature (OCT) compound (Tissue Tek, Sakura). Frozen 7-μm sections were obtained using a cryostat and stained with DAPI for fluorescent microscopy.

Immunohistochemistry

Slides were deparaffinized, dewaxed in xylene and gradually rehydrated with ethanol. Immunohistochemical staining was performed on the slides using automated Leica Bond III-autostainer. Slides were baked at 60°C for 30 min and then the temperature was increased to 72°C. Slides were then rinsed three times with Bond Dewax followed by absolute alcohol and Bond Wash. The temperature was then increased to 100°C, and Bond ER Solution 1 was applied and incubated for 30 min. Slides were rinsed with Bond Wash and the temperature was allowed to come down to room temperature. Anti CD4 and CD8 antibodies (Genetex) were applied at 1:50 dilution and incubated for 1 h at room temperature followed by rinsing with Bond Wash. Bond Polymer was applied and incubated for 10 min followed by rinsing with Bond Wash and distilled H_2O. Diaminobenzidine was applied and incubated to visualize the signals of the antibody staining. Hematoxylin was used as counterstain. The slides were then dehydrated and mounted for photos under a microscope.

In vivo imaging systems (IVIS) analysis

Mice were anesthetized under 2.5% isoflurane and then D-luciferin solution (150 mg/kg) was injected intraperitoneally. After 7 min, mice were imaged using Xenogen IVIS system with a 3-min exposure time. Bioluminescent signals were quantified using Living Image 3.1 (Caliper Life Sciences). Total photon flux of tumors was analyzed.

Statistical analysis

All numerical data are presented as mean ± standard deviation (SD). *P* values were acquired with the unpaired two-tailed Student *t* test. Survival plots were generated using the Kaplan–Meier method. The log-rank test was used to compare survival distributions between groups. $P < 0.05$ was considered statistically significant.

Results

The NMUs repress the growth and migration of murine urothelial carcinoma cells

Previously, we have successfully isolated and expanded NMUs from mouse urothelium [30] and we used them to test their interaction with murine urothelial carcinoma cells (MBT-2), derived from a carcinogen-induced bladder tumor in female C3H mice [31]. As shown in Figure 1A–1C, the isolated and expanded NMUs have the characteristic of urothelial stem cells. The NMUs express both CD44 and CD49f urothelial stem cell surface makers [16] as demonstrated by flow cytometry analysis (Figure 1A). NMUs also express both CK5 and CK14 urothelial stem cell cytokeratin makers [15] as demonstrated using Western blotting and immunofluorescent staining (Figure 1B and 1C). To determine the interaction between normal urothelial cells and urothelial carcinoma cells, we first generated MBT-2-luc reporter cells by stably transfecting luciferase genes into cells. Next we co-cultured MBT-2-luc and NMUs for 2 days and cells then were harvested for the measurement of firefly luciferase activity to determine the cell growth. After a 2-day incubation, the presence of NMUs inhibited the growth of the MBT-2-luc cells compared with the cell growth alone without NMUs (Figure 1D). To determine whether normal urothelial cells also affect the migration of urothelial carcinoma cells, we also co-cultured MBT-2-luc and NMUs in the upper well of a transwell chamber and measure the migrated cells. The MBT-2-luc cell migration was inhibited by the addition of NMU cells (Figure 1E and 1F). These results indicate that the normal urothelial cells may pose the capacity to suppress cancer cells from growing and spreading.

The cell-based experiment showed that normal urothelial cells could have a suppressive effect on the growth and migration of urothelial carcinoma cells. Therefore, we then applied MBT-2-luc in an *in vivo* orthotopic graft mouse model in syngeneic mice to form bladder tumors to identify the possible effect of normal cells on carcinoma cells in the animals with a competent host immune system. In the orthotopic model, the carcinoma cells are directly injected into the bladder wall to generate an invasive bladder cancer in an immune intact environment. At first, we implanted MBT-2-luc (C3H/He strain) cell and MBT-2-luc plus NMUs into the intramural portion of the bladder dome of female C3H/He mice to test whether normal urothelial cells could repress urothelial carcinoma cells in tumor formation and development as demonstrated in *in vitro* data. The tumor progression was monitor by IVIS imaging every 5 days by detecting the chemiluminescence radiated by tumor cells. The result showed that MBT-2-luc plus NMU

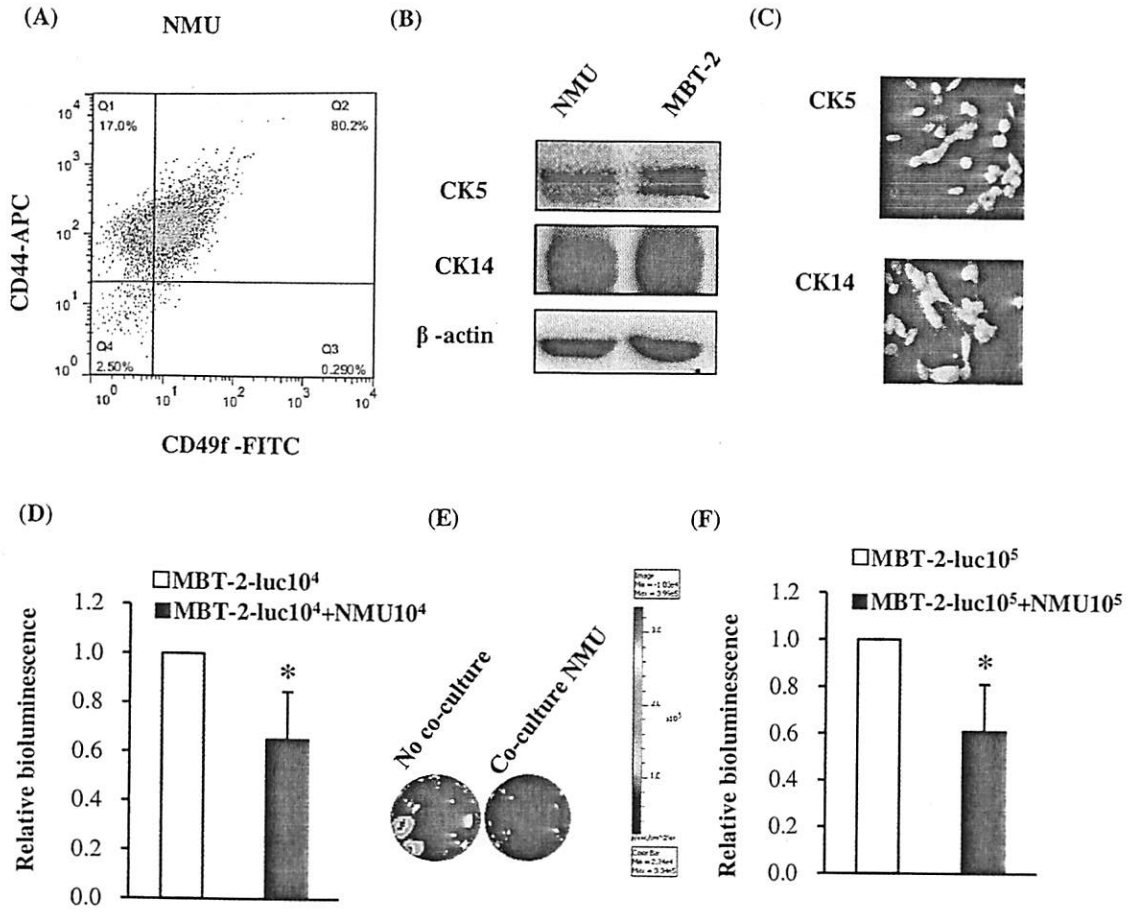

Figure 1. NMUs repress the growth and migration of murine urothelial carcinoma cells. (A) NMUs were labelled with anti-CD44 and anti-CD49f antibodies and analyzed with a flow cytometer. (B) CK5 and CK14 expression with Western blotting on NMUs and MBT-2 cells. Lysates from NMUs and MBT-2 cells were harvested and protein lysates were subjected to Western blotting analyses with the indicated antibodies. (C) NMUs were fixed and performed with immunofluorescent staining for cytokeratin CK5 and CK14 (green). DAPI was used for nuclear staining (blue). (D) *In vitro* effects of NMUs on MBT-2 cells on cell growth effect between NMUs and MBT-2-luc cells. The MBT-2-luc cell growth with or without NMUs. MBT-2-luc cells (1×10^5 cells) were co-cultured with NMUs (1×10^5 cells) for 48 h. The cell lysate was collected for luciferase activity. The luciferase activity of MBT-2-luc cells without co-culture was defined as 1. (E) MBT-2-luc cells were cultured in the presence or the absence of NMUs on the upper well of the transwell for 48 h before being visualized by bioluminescent imagining. (F) Quantitation results of bioluminescence imaging photon emission count for MBT-2-luc cells migrating through filter to the bottom well with or without NMU co-culture.

combination has a high take rate to form a tumor (Figure 2A). Once a tumor was formed at 1 week, the mice had palpable pubic mass-like bumps that were imaged using IVIS and displayed hematuria detected using urine strips at 2–3 weeks post–tumor implantation, the tumors progressed as visualized using IVIS imaging and most mice died at 3–4 weeks after forming notable tumor mass (Figure 2B). The result did not fit our expectation that normal urothelial cells could suppress urothelial carcinoma cells, but instead normal urothelial cells could facilitate tumor development when normal and carcinoma cells mixed together and implanted. The conflicting results of *in vitro* and *in vivo* experiments could be due to the complicate biological environments within a living organism. Because this graft tumor model is made by injecting tumor cells into the bladder wall intramurally, the co-implantation of tumor cells and normal cells could facilitate the formation of a more tumor-favorable microenvironment than implantation of tumor cells only at the implanting site. This phenomenon was demonstrated in other tumor graft models with co-implantation of tumor cells with normal cells such as fibroblasts and mesenchymal stromal cells [32,33].

The intravesical administration of normal urothelial cells has in vivo efficacy in a MBT-2 syngeneic orthotopic murine urothelial tumor model

We used these tumor-bearing mice to examine whether the non-self male NMUs could have anti-tumor effects when these cells were intravesically administered into bladders with developed tumors. Because of the unique bag-like structure of bladders, intravesical instillation

may participate in the repair and regeneration of tumor sites and induce immune responses that target remaining cancer cells.

In summary, this study proved our therapeutic concept that cancer as a wound, aging stem cell disease, and immunosuppressive disorder could be treated by enhancing host body–healing capacity with transplanted non-self cells. We believe that this therapeutic approach can provide a cell therapy modality that is not only for urothelial carcinoma but all cancers, treated with their own tissue-specific cells.

Acknowledgments

This work was supported by the Ministry of Science and Technology grant (MOST 103-2633-B-039-003-), China Medical University grant (CMU105-S-51), China Medical University Hospital grant (DMR-CELL-17020) and in part by the Taiwan Ministry of Health and Welfare Clinical Trial Center (MOHW105-TDU-B-212-133019).

Disclosure of interests: The authors disclose no potential conflicts of interest.

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