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Original article

Ex vivo culture of tumor cells from N-methyl-N-nitrosourea–induced bladder cancer in rats: Development of organoids and an immortalized cell line

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Abstract

Objective: We ex vivo cultured primary tumor cells from N-methyl-N-nitrosourea (MNU)-induced bladder tumors in rats and established an immortalized cell line from them.

Materials and methods: Bladder tumors in rats were induced by instillation of MNU into the murine bladder. Primary tumor cells were prepared by the cancer-tissue originated spheroid method. An immortalized cell line was established by co-culture with fibroblasts. The cultured tumor cells were molecularly and functionally characterized by quantitative real-time polymerase chain reaction, Western blot, growth assay, and transwell migration assay.

Results: Primary tumor cells were successfully prepared as multicellular spheroids from MNU-induced bladder tumors. The differentiation marker expression patterns observed in the original tumors were largely retained in the spheroids. We succeeded in establishing a cell line from the spheroids and named it T-MNU-1. Although basal markers (CK14 and CK5) were enriched in T-MNU-1 compared to the spheroids, T-MNU-1 expressed both luminal and basal markers. T-MNU-1 was able to migrate through a transwell.

Conclusions: Tumor cells in MNU-induced bladder tumors were successfully cultured ex vivo as organoids, and an immortalized cell line was also established from them. The ex vivo models offer a platform that enables analysis of intrinsic characteristics of tumor cells excluding influence of microenvironment in MNU-induced bladder tumors.

Keywords: Bladder cancer; N-methyl-N-nitrosourea; Primary culture; Spheroid; Organoid; Cell line

1. Introduction

Experimental animal models that recapitulate the human condition are essential tools for study of malignant neoplasms. Such animal models include engraftment models, genetically engineered models, and carcinogen-induced models [1,2]. Among them, carcinogen-induced models have autochthonous tumors generated by the exposure to carcinogens such as radiation, viruses, and chemicals. Carcinogen models are typically immunocompetent and have higher mutational burdens than do genetically engineered models and are therefore more appropriate for defining mechanisms involved in the effects of immunotherapy [2].

As for the development of bladder tumors in preclinical model systems, N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), which is a chemical delivered in drinking water, is the most widely used carcinogen to induce bladder tumors in rodents [3]. BBN-treated mice exhibit step-wise progression of bladder cancer phenotypes over time, including hyperplasia, dysplasia, carcinoma in situ (CIS), papillary non–muscle-invasive cancer,

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and muscle-invasive cancer [4–6]. Another carcinogen used for induction of bladder tumors in rodents is N-methyl-N-nitrosourea (MNU). In contrast to BBN, which is systemically delivered, MNU is instilled locally into the bladder via intravesical instillation and is more prone to development of papillary tumors with progression from dysplasia, CIS, as well as development of non–muscle-invasive tumors then muscle-invasive disease [7–9]. Difference between BBN- and MNU-induced bladder tumors at molecular levels is still unclear. Those carcinogen-induced rodent models demonstrate heterogeneous tumor development including CIS and noninvasive papillary disease in an immunocompetent host which enables investigation of systemic and local immunotherapies [7] and evaluation of newly formulated drugs [8] in a preclinical setting that is closely related to human bladder cancer.

Tumor cells in carcinogen-induced spontaneous models of bladder cancer interact with the host microenvironment including stromal cells, immune cells, and extracelluar matrices, providing a distinct advantage over xenograft and orthotopic models of bladder cancer. They allow investigation of the complex interactions that involve the host immune system and local paracrine/autocrine signaling. However, the ability to manipulate tumor cells ex vivo can maximize their usefulness in many ways as an experimental tool [10]. For example, analyzing intrinsic characteristics of tumor cells such as drug sensitivity or response to extracellular matrices is achievable by ex vivo culture of tumor cells prepared from the original tumors.

In this study, we isolated and cultured primary bladder tumor cells from MNU-induced bladder tumors in rats as multicellular spheroids ex vivo by applying the cancer-tissue originated spheroid (CTOS) method [11–13]. We molecularly characterized these spheroids to determine whether they would retain characteristics of the original tumors. We also succeeded in establishing an immortalized cell line, and molecularly and functionally characterized it.

2. Materials and methods

2.1. Bladder tumor induction in rats

All protocols involving animals followed US National Institutes of Health guidelines and were approved by the animal and care use committee of the Johns Hopkins Medical Institutions. MNU instillations were given every other week for a total of 4 instillations as previously described [7,8]. Briefly, Fischer 344 female rats aged 7 weeks (Harlan, avg. weight 160 g) were anesthetized with 3% isoflurane. After complete anesthesia, a 20 G angiocatheter was placed into the rat's urethra. MNU (1.5 mg/kg) (Spectrum, New Brunswick, NJ) dissolved in 0.9.% sodium chloride was then instilled and the catheter removed, with continued sedation lasting for 60 minutes to prevent spontaneous micturition and allow absorption. Rats' bladders were monitored by ultrasound every 4 weeks after 28 weeks from the initial instillation of MNU. Rats were sacrificed and the bladder was harvested when a tumor grew more than 5 mm under ultrasound as we have previously described [7,8].

2.2. Preparation of primary cell culture and growth assay

Primary cultures of bladder cancer cells were prepared according to the CTOS method [11–13]. Briefly, a piece of the tumor was minced, and mechanically and enzymatically digested. Clusters of cells were collected and cultured in in StemPro human embryonic stem cell culture medium (Invitrogen, Carlsbad, CA). For growth assays, spheroids were placed in suspension or embedded in Matrigel Growth Factor Reduced (Corning, Corning, NY). Images of spheroids were taken at day 1, 3, and 5 by EVOS Cell Imaging Systems (Thermo Fischer Sicentific). Relative growth of a spheroid was calculated by dividing the area of the indicated day by that of day 1.

2.3. Cell culture

Human bladder cancer cell lines RT4 and 5637 were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in RPMI1640 (Thermo Fischer Sicentific) containing 10% FBS and penicillin/streptomycin. 3T3J2 mouse fibroblast cells were purchased from Kerafast (Boston, MA) and maintained in DMEM (Thermo Fischer Sicentific) containing 10% FBS, glutamine, and penicillin/streptomycin. Cells were cultured at 37°C under 5% CO2.

2.4. Establishment of an immortalized cell line

An immortalized cell line was established using a co-culture method with fibroblast cells [14]. 3T3J2 cells were irradiated at 30 Gy and used as feeder cells. One million of irradiated 3T3J2 cells were plated in a T25 culture flask 24 hours before co-culturing with tumor cells. Primary tumor cell spheroids were collected and dissociated into single cells with TrypLE Express (Thermo Fischer Sicentific). Half million of tumor cells were plated in the flask with irradiated 3T3J2 cells in the co-culture media (Table). When cancer cells reached 80% confluency, cells were passaged by differential trypsinaization [14] and cultured with newly plated irradiated 3T3J2 cells. After 3 passages with irradiated 3T3J2 cells, cancer cells were cultured alone and challenged for proliferation and passage. Passageable cells were expanded and stored in the co-culture media containing 5% DMSO at −80°C accordingly, and used for subsequent experiments.

2.5. Histological analysis

Bladders were formalin fixed and paraffin embedded. Spheroids were embedded in iPGell (Diagnocine, Hackensack,