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Maintaining viability and characteristics of cholangiocarcinoma tissue by vitrification-based cryopreservation

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ABSTRACT

Tumor tissue has great clinical and scientific value which relies highly on the proper preservation of primary materials. Conventional tumor tissue cryopreservation using slow-freezing method has yielded limited success, leading to significant cell loss and morphological damage. Here we report a standardized vitrification-based cryopreservation method, by which we have successfully vitrified and warmed 35 intrahepatic cholangiocarcinoma (ICC) tissues with up to 80% viability of the fresh tumor tissues. Cryopreserved ICC tissue could generate patient-derived xenografts (PDXs) with take rates of 68.2% compared to 72.7% using fresh tumor tissues. Histological and genetic analyses showed that no significant alterations in morphology and gene expression were introduced by this cryopreservation method. Our procedure may facilitate collection, long-time storage and propagation of cholangiocarcinoma or other tumor specimens for (pre)clinical studies of novel therapies or for basic research.

1. Introduction

Tumor tissue-based analysis plays an important role in clinical cancer diagnosis, treatment and basic research. The conventional preservation of tumor tissue for biomedical research either as formalin-fixed paraffin embedded (FFPE) samples or flash frozen in liquid nitrogen kills the cells, which can just provide morphological and genetic information. It may be expected that the “living” tumor tissues, besides being subject to traditional histopathological examination, will be used for various molecular analyses and functional tests.

Although PDX models is being fully appreciated for such applications [5,8,10,18,24], xenografting as a routine is not always feasible because it requires a highly efficient cooperation among clinical hospitals, research laboratories and animal facilities which are difficult to be brought together in space and in time [14]. Furthermore, it is important to use low passage PDX (<6) to model the original patient's tumor by retaining tumor heterogeneity, gene expression and similar response to treatment [2]. Therefore, a reliable and efficient cryopreservation method for living tumor tissue is indispensable and would ideally serve to preserve primary patient specimen as well as PDX samples for re-implantion when required.

The slow-freezing technique using fetal bovine serum (FBS) (90–95%) combined with DMSO (5–10%) has been used for tumor tissue preservation and xenograft implantation with limited success [2]. However, the percentage of viable tumor cells is severely compromised. This may be due to ice crystal formation, which can have a deleterious effect on cellular interactions and cell membranes. Compared with the conventional slow-freezing method, vitrification, which is defined as the conversion of a system from a fluid to a solid by increasing solution viscosity, has several advantages; avoiding ice crystallization by which the cells are damaged, and reducing the time and cost of the freezing. However, vitrification of tumor tissue and its subsequent application have not been thoroughly tested.

Here we have preserved 35 cholangiocarcinoma tissues by using

Abbreviations: ICC, Intrahepatic cholangiocarcinoma; PDXs, patient-derived xenografts; FFPE, formalin-fixed paraffin embedded.

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a practical and standardized vitrification procedure. We have carefully analyzed both fresh and rewarmed cholangiocarcinoma tissue in terms of viability and histomorphological characteristics using CCK-8 assays and immunohistochemistry, respectively. Primary tumor cells were isolated after cryopreservation and subjected to gene expression analysis. Moreover, fresh and rewarmed cholangiocarcinoma tissues were used to establish subcutaneous PDXs. We also showed that cryopreservation of established xenograft tumors for re-xenografting is also feasible.

2. Materials and methods

2.1. Human tissue specimen

All cholangiocarcinoma specimens were obtained from surgical resections of patients with intrahepatic cholangiocarcinoma (ICC) at the Renji Hospital (Shanghai, China) and Eastern Hepatobiliary Surgery Hospital (Shanghai, China). The procedure for human sample collection was approved by the Ethics Committee of Renji Hospital and Eastern Hepatobiliary Surgery Hospital. The application of samples, patient characteristics and overall results of xenografting experiments are summarized in Supplementary Table 1.

2.2. Animal protocol

Male NOD-SCID (6–8 weeks old) mice were purchased from Shanghai Experimental Center of Chinese Academy of Science, maintained under pathogen-free conditions and received sterilized food and water ad libitum. All animal experiments were approved by the Ethical Committee of the Second Military Medical University.

2.3. Cryopreservation procedure

All cryopreservation solutions (LT2601, LiveTissue™) and warming solutions (LT2602, LiveTissue™) are provided by Celliver Biotechnology Inc. (Shanghai, China). The cryopreservation of cholangiocarcinoma samples were performed according to the manufacturer's instruction. Briefly, 10 ml vitrification solution 1 (V1; DMEM 80%, DMSO 10%, EG 10%, BSA 3%, sucrose 1%, methylcellulose 0.05%, hydroxyethyl starch 0.25%) and 10 ml vitrification solution 2 (V2; DMEM 70%, DMSO 18%, EG 12%, BSA 3%, sucrose 20%, methylcellulose 0.05%, PVP 0.25%) in sterile 15 ml centrifugal tubes were pre-warmed in a 26 °C water bath. Primary cholangiocarcinomas were cut into slices of 1 mm thickness using sterile stainless slicer in a laminar flow cabinet. Cholangiocarcinoma tissue slices were first rinsed in sterile saline at room temperature. Subsequently, slices were transferred to V1 and then to V2 with an incubation time of 25 and 15 min at 26 °C, respectively. Afterwards, slices were placed in a minimum volume of solution onto a thin metal strip, and submerged directly into sterile liquid nitrogen for at least 5 min. Finally, the strip was inserted into a sterile cryotube and stored into a liquid nitrogen storage tank.

2.4. Rewarming procedure

For tumor tissue rewarming, the cryotube was removed from liquid nitrogen and the metal strip was immersed promptly into 30 ml of 37 °C warming solution 1 (T1) for 3 min. Then, the cholangiocarcinoma tissues were subsequently transferred into 10 ml of warming solution 2 (T2), 10 ml of warming solution 3 (T3) and 10 ml of warming solution 4 (T4) for 5, 10 and 10 min at 26 °C, respectively. Finally, slices were washed twice in sterile saline and kept in Williams E solution (BasalMedia) on ice until application.

2.5. Tissue viability assay

CCK-8 (Cell Counting Kit-8), being nonradioactive, allows sensitive colorimetric assays for the determination of the number of viable cells by utilizing highly water-soluble tetrazolium salt (WST-8). WST-8 is reduced by dehydrogenases in cells to give an orange colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. CCK-8 has been widely used in cell and tissue viability analyses [1]. For tissue viability assay, the round tissue specimen of same size were further prepared using a sterile hole puncher in a laminar flow cabinet. Fresh or rewarmed tissue wafers were cultured in 100 μl tissue culture medium in 96-well plate and incubated in a humidified incubator (37 °C, 5% CO2) for 2 h 10 μl of the Cell counting kit-8 (CCK-8, Dojindo Molecular Technologies) solution was added into each well of the plate. The plate was further incubated for 1 h and the OD values were measured at 450 nm using a microplate reader.

2.6. Primary cell isolation

Primary cell isolation was performed using Tumor Dissociation Kit (Miltenyi). Fresh or rewarmed cholangiocarcinoma slices of same weight were minced, transferred to Tube type C (Miltenyi) and mechanically dispersed with Gentle MACS™ Dissociator (Miltenyi) using installed software program “h_tumor_01” [20]. Cell suspension was centrifuged at 300 × g for 7 min. Pellet was re-suspended and cultured in DMEM/F12 supplemented with 10% FBS.

2.7. Real-time polymerase chain reaction (PCR) analysis

Quantitative PCR was performed using SYBR Green PCR Kit (Applied Biosystems, Foster City, CA). The messenger RNA (mRNA) level of specific genes was normalized against β-actin.

2.8. Establishment of patient-derived xenografts

Surgery was performed under sterile conditions in a laminar flow cabinet using sterilized surgical instruments. Two single cuts were made in the neck of the NOD-SCID mice; fresh or rewarmed cholangiocarcinoma slices were mixed with Matrigel and DMEM at a ratio of 1:30 and respectively subcutaneously transferred to either side of the flank [2].

2.9. H&E, immunohistochemistry and TUNEL staining

Formaldehyde-fixed, paraffin-embedded sections of fresh or rewarmed cholangiocarcinoma, and of patient-derived xenografts derived from fresh or rewarmed cholangiocarcinoma tissues were subjected to hematoxylin and eosin (H&E) and immunohistochemistry staining following routine protocols. Immunohistochemistry staining of paraffin-embedded sections were incubated with rabbit anti-Ki67 (Abcam) and rabbit anti-CK19 (Proteintech Group). TUNEL staining was done using a commercially available kit following manufacturer's instructions (Calbiochem), counterstaining was performed with DAPI. Representative images of H&E and immunohistochemistry staining were captured with an Olympus IX70, besides, representative images of TUNEL staining were captured with a Leica TCS SP8. The Ki67 and TUNEL index were calculated based on the eye-counting method within 10 independent microscopic fields.