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Characterization of *in vivo* tumorigenicity tests using severe immunodeficient NOD/Shi-scid IL2Rγ^{null} mice for detection of tumorigenic cellular impurities in human cell-processed therapeutic products

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ABSTRACT

The contamination of human cell-processed therapeutic products (hCTPs) with tumorigenic cells is one of the major concerns in the manufacturing and quality control of hCTPs. However, no quantitative method for detecting the tumorigenic cellular impurities is currently standardized. NOD/Shi-scid IL2Rγ^{null} (NOG) mice have shown high xeno-engraftment potential compared with other well-known immunodeficient strains, e.g. nude mice. Hypothesizing that tumorigenicity test using NOG mice could be a sensitive and quantitative method to detect a small amount of tumorigenic cells in hCTPs, we examined tumor formation after subcutaneous transplantation of HeLa cells, as a model of tumorigenic cells, in NOG mice and nude mice. Sixteen weeks after inoculation, the 50% tumor-producing dose (TPD_{50}) values of HeLa cells were stable at 1.3×10^4 and 4.0×10^5 cells in NOG and nude mice, respectively, indicating a 30-fold higher sensitivity of NOG mice compared to that of nude mice. Transplanting HeLa cells embedded with Matrigel in NOG mice further decreased the TPD_{50} value to 7.9×10 cells, leading to a 5000-fold higher sensitivity, compared with that of nude mice. Additionally, when HeLa cells were mixed with 10^6 or 10^7 human mesenchymal stem cells as well as Matrigel, the TPD_{50} values in NOG mice were comparable to those of HeLa cells alone with Matrigel. These results suggest that the *in vivo* tumorigenicity test using NOG mice with Matrigel is a highly sensitive and quantitative method to detect a trace amount of tumorigenic cellular impurities in human somatic cells, which can be useful in the quality assessment of hCTPs.

1. Introduction

Cell-processed therapeutic products (CTPs) derived from human somatic/stem cells are eagerly expected to treat patients with severe diseases involving functional damage of organs and tissues. To transplant hCTPs into patients, however, tumorigenicity is raised as one of the issues of these products. Tumorigenicity is defined as the capacity of a cell population transplanted into an animal model to

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produce a tumor by proliferation at the site of transplantation and/or at a distant site by metastasis [1]. Assessment of tumorigenicity is quite important to manufacture products with consistent quality. Currently, the World Health Organization (WHO) Technical Report Series (TRS) No. 878 Annex 1 is the only international guideline that addresses tumorigenicity tests of animal cells for the production of biologicals. However, the tumorigenicity test described in WHO TRS 878, which involves the administration of 10^7 cells to ten nude mice, would not be sensitive enough to detect a trace amount of tumorigenic cellular impurities in hCTPs [2]. In addition, the *in vivo* tumorigenicity test proposed in WHO TRS 878 covers only viable animal cells used as cell substrates for manufacturing biological products but not cells used directly for therapy by transplantation into patients. Thus, to date, no suitable tumorigenicity test has been established for hCTPs.

To establish methods to detect a trace amount of tumorigenic cellular impurities in hCTPs, the usage of several new generations of highly immunodeficient animal models are proposed. Rag2-γC double-knockout mice [3], NOD/Shi-scid IL2Rγ^{null} (NOG) mice [4], and NOD/SCID/IL-2rγKO (NSG) mice [5] indicate multiple immunodeficiencies, including defects in T, B, and natural killer (NK) cells, and a reduction in the function of macrophages and dendritic cells. NOG mice exhibit extremely high engraftment rates of human HeLa S3 cells compared with T-cell-deficient nude mice and T and B-cell-deficient SCID mice [6]. NSG mice are reported to show efficient tumor formation by single human melanoma cells in combination with Matrigel, a basement membrane-like extracellular matrix extract [7]. However, for the use of these highly immunodeficient mouse strains to detect tumorigenic cellular impurities in hCTPs as a part of the quality assessment/control, the performance of the tumorigenicity tests using these strains shall be validated using well known tumor cell lines.

In the present study, we examined the tumor formation potential of HeLa cells transplanted in NOG mice with Matrigel and compared their tumorigenicity with that in nude mice. To determine the sensitivity for the detection of tumor cells contamination in non-tumorigenic human somatic cells, we mixed various dose of HeLa cells in human mesenchymal stem cells and conducted tumorigenicity tests using NOG mice and Matrigel. We also performed soft agar colony formation assay, which is commonly used to detect anchorage-independent cell growth *in vitro*, and compared tumor cell detection level by soft agar with the *in vivo* tumorigenicity test.

2. Materials and methods

2.1. Cells

Human cervical cancer HeLa cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). The cells were maintained in Eagle's minimum essential medium (Sigma), supplemented with 10% fetal bovine serum (FBS; Sigma), 0.1 mM non-essential amino acids (Life Technologies), 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). Human mesenchymal stem cells (hMSCs) were purchased from Lonza and cultured in MSCGM™ medium (Lonza). Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, and were passaged upon reaching 80% confluence. hMSCs were used at passage 6 and passages 6–8 for *in vivo* tumorigenicity tests and soft agar colony formation assay, respectively.

2.2. Preparation of cell suspensions for transplantation

Upon reaching approximately 80% confluence, cells were washed twice with phosphate buffered saline (PBS) and treated with 0.25% trypsin-EDTA solution (Life Technologies) for detachment from culture dishes. HeLa cells and/or hMSCs were counted and prepared in 100 μl of ice-cold HeLa cell culture medium or a 1:1 (v/v) mixture of HeLa cell culture medium and Matrigel (product #354234, BD Biosciences, San Jose, CA) for transplantation.

2.3. Tumorigenicity test with immunodeficient mice

Male BALB/cA nu/nu mice (nude; CLEA Japan, Inc., Tokyo) and male NOG mice maintained in the Central Institute for Experimental Animals (CIEA, Kanagawa, Japan) were used for *in vivo* tumorigenicity studies. Prepared cell suspensions were injected using 1 ml syringes with a 25 G needle (Terumo) into 8-week-old mice (n = 6 or 10). The mice were palpated weekly for 16 weeks to observe nodule formation at the injection site. Tumor size was assessed by external measurement of the length and width of the tumors in two dimensions using a caliper as soon as tumors reached measurable size. The tumor volume (TV) was calculated using the formula volume = 1/2 × length (mm) × (width [mm])^2. The successive engraftment was determined according to progressive nodule growth at the injection site. Mice were euthanized and necropsied when tumors reached approximately 20 mm in any dimension or when a sign of deconditioning was noted. The tumorigenicity of HeLa cells was evaluated by measuring tumor-forming capacity, which indicates the tumorigenic phenotype [8,9]. Tumor-forming capacity is defined as 50% tumor-producing dose (TPD_{50}), which represents the threshold dose of cells forming tumors in 50% of the animals. TPD_{50} values were calculated using the Spearman-Kärber method [10–12] at each time point. Not all animals transplanted with the highest dose formed tumors, in which case it was assumed that the tumor incidence of animals at 10 or 100 times the uppermost dose step (a dummy set of data) would have been 100% for the Spearman-Kärber method to be applicable [12].

The protocol of the present study was reviewed beforehand and approved by the Animal Ethics Committees of CIEA (Permit Number: 13041A) and the National Institute of Health Sciences (NIHS, Tokyo) (Permit Number: 359, 359-1, 359-2, 359-3). All animal experiments were performed according to the Ethical Guidelines for Animal Experimentation from the CIEA and the NIHS. All animals were sacrificed under isoflurane inhalation anaesthesia, and all efforts were made to minimize suffering.

2.4. Histology and immunohistochemistry

The engrafted tumors were isolated and fixed with 10% neutral buffered formalin (Wako). The paraffin-embedded sections were investigated by hematoxylin and eosin (H&E) stain and immunohistochemical studies using Bond-max stainers (Leica Biosystems). Some sections were incubated at 100 °C for 10 min in a target retrieval solution consisting of 10 mM citrate buffer (ER1; Leica Microsystems), and then placed at room temperature for 20 min. Mouse anti-human HLA class I-A, B, C monoclonal antibody (EMR8-5; Hokudo, Sapporo, Japan), and rabbit anti-vimentin monoclonal antibody (SP20; Nichirei Bioscience, Tokyo) were used as the primary antibodies. The antibodies for mouse immunoglobulin were visualized using Bond polymer refine detection kits (Leica Microsystems). Sections were counterstained with hematoxylin.

2.5. Soft agar colony formation assay

A soft agar colony formation assay was performed using a CytoSelect™ 96-well Cell Transformation Assay kit (CellBio labs, San Diego, CA) as previously described [13]. Prewarmed 25 μl of 2 × DMEM/20% FBS and 25 μl of 1.2% agar solution were mixed and