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Short paper

Characterization of the cell growth analysis for detection of immortal cellular impurities in human mesenchymal stem cells

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ABSTRACT

The analysis of *in vitro* cell senescence/growth after serial passaging can be one of ways to show the absence of immortalized cells, which are frequently tumorigenic, in human cell-processed therapeutic products (hCTPs). However, the performance of the cell growth analysis for detection of the immortalized cellular impurities has never been evaluated. In the present study, we examined the growth rates of human mesenchymal stem cells (hMSCs, passage 5 ($P = 5$)) contaminated with various doses of HeLa cells, and compared with that of hMSCs alone. The growth rates of the contaminated hMSCs were comparable to that of hMSCs alone at $P = 5$, but significantly increased at $P = 6$ (0.1% and 0.01% HeLa) or $P = 7$ (0.001% HeLa) within 30 days. These findings suggest that the cell growth analysis is a simple and sensitive method to detect immortalized cellular impurities in hCTPs derived from human somatic cells.

1. Introduction

Human cell-processed therapeutic products (hCTPs) are expected to provide novel breakthrough therapies for currently life-threatening or incurable diseases. In the clinical applications of hCTPs to patients, however, one of the major concerns is the tumorigenic cellular impurities in the products. Since pluripotent stem cells (PSCs), such as embryonic stem cells and induced pluripotent stem cells, are tumorigenic [1–3], there is a risk of tumor formation if the products contain the residual undifferentiated PSCs [4]. On the other hand, somatic cells are considered to have little tumorigenic potential even after substantial manipulations like *in vitro* expansion, because they consistently pass into senescence [5]. Malignant transformation of the cells is believed to occur through multiple processes involving the accumulation of mutations in key regulatory genes that promote cell survival and proliferation [6,7]. Although a few individual groups reported the spontaneous transformation of human mesenchymal stem cells (hMSCs) during *in vitro* culture [8–11], two of them retracted their papers because the results appeared to be attributable to contamination with tumorigenic cells (fibrosarcoma, osteosarcoma, or glioma cell lines) [12,13]. The rest of the groups found the immortalization of the cells, which is closely associated with tumorigenicity, during *in vitro* culture, indicating that the good practices to avoid contamination with tumorigenic cells and the monitoring of cell growth are critical for the quality control of hCTPs derived from human somatic cells.

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Abbreviations

hCTP	human cell-processed therapeutic product
PSC	pluripotent stem cell
hMSC	human mesenchymal stem cell
STR	short tandem repeat
$P = n$	passage n
PBS	phosphate buffered saline
RT	room temperature
HPV	human papillomavirus

Cross contamination of cells with unidentified cells is usually evaluated by the short tandem repeat (STR) analysis [14]. However, the cell growth analysis which simply monitors the cell proliferation for a limited period may be adequately sensitive for the detection of the contamination of somatic cells with immortalized/tumorigenic cells, because somatic cells usually show slower growth, compared with that of immortalized/tumorigenic cells, as well as the attenuation of the growth after serial passaging [15–17]. In fact, the European Medicines Agency has considered that the evaluation of *in vitro* cell senescence after serial passaging is sufficient to prove the absence of immortalized/tumorigenic cells in a somatic cell-based product [18]. However, the performance of the cell growth analysis for detection of the immortalized/tumorigenic cellular impurities in somatic cells has never been studied. In the present study, we examined the growth of hMSCs contaminated with various doses of HeLa cells, a well-known cancer cell line, to determine the sensitivity of the cell growth analysis for the detection of the immortalized/tumorigenic cells in human somatic cells.

2. Materials and methods

2.1. Cells

hMSCs (Lonza, Walkersville, MD) at passage 2 ($P = 2$) were cultured in MSCGM BulletKit, a mesenchymal stem cell basal medium with mesenchymal cell growth supplement, L-glutamine, and gentamycin/amphotericin-B (Lonza). HeLa cells (the Health Science Research Resources Bank, Osaka, Japan) 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). Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, and were passaged upon reaching 90% confluence.

2.2. Cell growth analysis

At $P = 5$ of hMSCs, 1×10^6 of hMSCs were mixed with 1000, 100, or 10 of HeLa cells and seeded into T175 flasks (Corning). The cells were maintained in 40 ml of Dulbecco's Modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Upon reaching approximately 90% confluence, the cells were washed with phosphate buffered saline (PBS) and treated with 0.05% trypsin-EDTA solution (Gibco) for detachment from the flasks. The cells were centrifuged at $450 \times g$ for 5 min and suspended with the fresh culture medium. Aliquots of the suspended cells were stained with Trypan Blue solution and counted by Countess Automated Cell Counter (Invitrogen) according to the manufacture's protocol. One million cells in the suspension were re-seeded into T175 flasks and cultured until the next passage. This process was repeated by $P = 10$. The growth rate (R_n) at $P = n$ was calculated by the following equation:

$$R_n = [\log_2(N_{n+1} - N_n)]/(D_{n+1} - D_n)$$

where N_k and D_k are the number of accumulated cells and the date at $P = k$, respectively.

2.3. Immunofluorescence microscopy

hMSCs contaminated with HeLa cells were fixed with 4% paraformaldehyde in PBS (Nacalai Tesque) for 10 min at room temperature (RT) and blocked in Blocking One (Nacalai Tesque) for 30 min at RT. The cells, then, were incubated with anti-HPV18 E7 antibody (8E2) (abcam) diluted at 1:500 in the blocking solution (PBS containing 5% Blocking One) for 1 h at RT for primary staining, and secondarily stained with goat anti-mouse IgG Alexa Fluor 488 (1:1000; Invitrogen) in the blocking solution for 45 min at RT. The cells were mounted with VECTASGIELD mounting medium with DAPI (VECTOR) and observed with a fluorescence microscope (IX71, Olympus).

3. Results and discussion

In the present study, we added 1000, 100, or 10 of HeLa cells to 1×10^6 of hMSCs of passage 5 ($P = 5$) and compared their growth with that of hMSCs alone (HeLa 0) until $P = 10$. The growth curves of three lots of hMSCs and the contaminated hMSCs are shown in Fig. 1. The cell numbers of HeLa 0 and the contaminated hMSCs were comparable at $P = 5$. The growth of HeLa 0 was constant during the early culture and getting slower with time (Fig. 1), while the growth of the contaminated cells was accelerated.

To confirm that the increases in the growth were attributable to the contamination with HeLa cells, we observed the cells with phase contrast microscopy. In the images of the contaminated hMSCs, we found small cells clearly different from hMSCs (Fig. 2A), and their relative abundance increased every passage. Because HeLa cells are infected with human papillomaviruses (HPV), we performed immunofluorescence analysis using HPV18 E7 antibody and confirmed that the cells were HeLa cells not transformed hMSCs (Fig. 2B). At $P = 10$, hMSCs were hardly identified in images of HeLa 1000 (Fig. 2C), because almost all of hMSCs were exchanged for HeLa cells at the five passages.

Next, we examined the growth rates of the contaminated cells (Fig. 3A). They were comparable to that of HeLa 0 at $P = 5$, and got significantly increased at $P = 6$ (HeLa 1000 and HeLa 100) or $P = 7$ (HeLa 10). These results indicated that the gross proliferation rate was not influenced by the spiked cells at $P = 5$ and then the population of HeLa cells in hMSCs increased in dose- and time-dependent manner. Eventually, the growth rate (doubling/day) of the contaminated hMSCs increased, and then reached plateau. The average growth rate of the contaminated cells at $P = 9$ and 10 was 0.73, suggesting that the growth rate of HeLa cells was approximately 0.7 in this culture condition. The average growth rates of the three lots are plotted along the passage number in Fig. 3B. The growth rates of HeLa 1000 and HeLa 100 at $P = 7$ and HeLa 10 at $P = 8$ were significantly increased compared with the growth rates at $P = 5$ ($^*P < 0.05$, two-way repeated measures ANOVA and Student-Newman–Keulis test). These results indicate that the cell cultures longer than $P = 7$ (about 20 days) and $P = 8$ (about 30 days) detect cross-contaminations of 100 (0.01%) and 10 (0.001%) HeLa cells, respectively, assuming that 10^6 hMSCs were contaminated at $P = 5$.

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