Tumorigenicity Evaluation of Umbilical Cord Blood-derived Mesenchymal Stem Cells

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(Received November 24, 2015; Revised February 18, 2016; Accepted June 1, 2016)

Mesenchymal stem cells (MSCs) have been identified in multiple types of tissue and exhibit characteristic self-renewal and multi-lineage differentiation abilities. However, the possibility of oncogenic transformation after transplantation is concerning. In this study, we investigated the tumorigenic potential of umbilical cord blood-derived MSCs (hUCB-MSCs) relative to MRC-5 and HeLa cells (negative and positive controls, respectively) both in vitro and in vivo. To evaluate tumorigenicity in vitro, anchorage-independent growth was assessed using the soft agar colony formation assay. hUCB-MSCs and MRC-5 cells formed few colonies, while HeLa cells formed a greater number of larger colonies, indicating that hUCB-MSCs and MRC-5 cells do not have anchorage-independent proliferation potential. To detect tumorigenicity in vivo, hUCB-MSCs were implanted as a single subcutaneous injection into BALB/c-nu mice. No tumor formation was observed in mice transplanted with hUCB-MSCs or MRC-5 cells based on macro- and microscopic examinations; however, all mice transplanted with HeLa cells developed tumors that stained positive for a human gene according to immunohistochemical analysis. In conclusion, hUCB-MSCs do not exhibit tumorigenic potential based on in vitro and in vivo assays under our experimental conditions, providing further evidence of their safety for clinical applications.

Key words: Tumorigenicity, Mesenchymal stem cells, Immunohistochemistry, Soft agar assay

INTRODUCTION

Mesenchymal stem cells (MSCs) have been identified in multiple types of tissue including bone marrow, umbilical cord, and adipose (1). They came to prominence as a potential source of stem cells for cellular and genetic therapies due to their inherent abilities of self-renewal, proliferation, and functional multi-lineage differentiation (i.e., bone, cartilage, neurons, adipocytes, and cardiomyocytes). Clinical use of MSCs is emerging, providing valuable insight into regenerative medicine and tissue engineering (2-6).

However, therapeutic applications of MSCs are limited, owing to concerns that they could be prone to malignant transformation. It was reported that ex vivo expansion of mouse bone marrow MSCs induced transformation with subsequent generation of sarcomas after implantation in baboons (7). Furthermore, one study documented that bone marrow-derived cells, presumably MSCs, progressed from metaplasia and dysplasia to gastric epithelial cancer in mice with chronic mucosal injury (8).

Therefore, the tumorigenic potential of MSCs is a major concern in clinical applications, and regulatory guidelines recommend evaluating their tumorigenicity using immunocompromised animals to assess stem cell safety (9). To use MSCs safely in stem cell therapy in humans, it is important to determine whether human MSCs progress to malignant transformation. The purpose of this study was to investigate the potential malignant transformation of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) using in vitro and in vivo tumorigenicity assays.

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MATERIALS AND METHODS

Cell lines and experimental animals. hUCB-MSCs were provided by Kang Stem Biotech (Seoul, Korea) and maintained as previously reported (10). Cells at passage 6 were used for all experiments (11). To compare the in vitro and in vivo tumorigenic potential of hUCB-MSCs, HeLa (human adenocarcinoma) and MRC-5 (human lung fibroblast) cell lines were used as positive and negative controls, respectively (American Type Culture Collection, VA, USA). HeLa and MRC-5 cells were cultured in Eagle’s Minimum Essential Medium (EMEM, ATCC, VA, USA) supplemented with 10% fetal bovine serum (FBS, ATCC, VA, USA) in an incubator at 37°C with 5% CO₂.

To examine tumorigenicity in vivo, 4-week-old BALB/c-nu/nu female mice were purchased from Japan SLC Co. (Shizuoka, Japan). Mice were housed two per filter-top polycarbonate cage with aspen bedding at 23 ± 3°C with a relative humidity of 50 ± 10%, air ventilation of 10–20 times/hr, and light intensity of 150–300 Lux under a 12-hr light/dark cycle throughout the experimental period. The temperature and relative humidity of the room were maintained automatically, and ventilation frequency and light intensity were monitored periodically. Sterile pellet food (Labdiet #5053, PMI Nutrition International, St. Louis, USA) and water were available ad libitum.

To examine tumorigenicity in vitro, anchorage-independent growth was assessed with the soft agar colony formation assay using the 96-well plate. Briefly, the base agar layer was prepared from a 0.6% soft agar solution containing Dulbecco’s Modified Eagle’s Medium (DMEM) containing FBS in a 96-well plate. Then, 1 × 10⁵ cells were suspended in DMEM containing FBS and 0.4% agar solution and plated onto the base plate. Plates were incubated at 37°C with 5% CO₂ for 7 days, after which colony formation was observed under a microscope.

In vitro tumorigenicity assay. To evaluate tumorigenicity in vitro, 4-week-old BALB/c-nu/nu female mice were purchased from Japan SLC Co. (Shizuoka, Japan). Mice were housed two per filter-top polycarbonate cage with aspen bedding at 23 ± 3°C with a relative humidity of 50 ± 10%, air ventilation of 10–20 times/hr, and light intensity of 150–300 Lux under a 12-hr light/dark cycle throughout the experimental period. The temperature and relative humidity of the room were maintained automatically, and ventilation frequency and light intensity were monitored periodically. Sterile pellet food (Labdiet #5053, PMI Nutrition International, St. Louis, USA) and water were available ad libitum. This animal study was reviewed and assessed by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology (KIT).

In vivo tumorigenicity assay. Mice were divided randomly into four groups of six. Vehicle control (EMEM), hUCB-MSCs, MRC-5 cells, and HeLa cells (1 × 10⁵ cells/head) were inoculated subcutaneously once into the lateral trunk region. After inoculation, mice were observed for 13 weeks twice daily for clinical signs and twice weekly for the presence of tumors. Tumor dimension was assessed using a vernier caliper, and tumor size was estimated using the following equation: ½ × minor axis² × major axis. Body weight was measured once weekly throughout the study period.

Thirteen weeks after inoculation, animals were fasted overnight and necropsied under deep isoflurane inhalation anesthesia. Blood samples were collected from the posterior vena cava of animals for hematologic analysis. Complete gross examinations were conducted on all animals. Organs including the liver, brain, spleen, heart, kidney, adrenal gland, and lung were collected and weighed. In addition, relative organ weights were calculated as the ratio of organ weight-to-fasted body weight before necropsy.

Routine hematological examinations were conducted using blood with EDTA-2K, an anticoagulant, on a hematological auto-analyzer (ADVIA120 Hematology System, Bayer, USA) to assess: white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), differential leukocyte count (neutrophil (NEU), lymphocyte (LYM), monocyte (MON), eosinophil (EOS), basophil and large unstained cell (BAS) and reticulocyte count (RET).

The organs collected for histopathology including the kidney, liver, spleen, heart, brain, lung, mandibular lymph node, stomach, mesenteric lymph node, pancreas, thoracic cavity, skin, and injection site were fixed in 10% neutral-buffered formalin. Fixed organs from all animals were embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and examined under a microscope.

Human gene expression at the injection site. To determine whether inoculated hUCB-MSCs persisted at the injection site and whether tumor formation in HeLa cell-treated mice originated from inoculated cells, immunohistochemistry (IHC) was performed on injection site tissues or observed tumor tissues of animals from each group using an automated staining system (DISCOVERY XT, Ventana, Tucson, USA). Heat-induced epitope retrieval was applied using citrate-based buffer (pH 6.0). Sections were incubated with an anti-human mitochondria mouse monoclonal antibody (1 : 1000, ab92824, Abcam, Cambridge, UK) for 2 hr. For detection, diaminobenzidine chromogen was used and counterstained with hematoxylin.

Statistical analysis. The data were examined for variance homogeneity using Bartlett’s test. When Bartlett’s test indicated no significant deviations from variance homogeneity, a one-way analysis of variance (ANOVA) was performed at α = 0.05. When significance was noted, Dunnett’s test for multiple comparisons was conducted to determine which pairs were significantly different. When pairs of group homogeneity were observed, the non-parametric Kruskal-Wallis comparison test was conducted. When a significant difference was observed in the Kruskal-Wallis test, Dunn’s Rank Sum test was conducted to determine the specific pairs of group comparison. Statistical analyses were performed using the PATH/TOX System (Version 4.2.2,
In vitro tumorigenicity assay. To compare colony formation of hUCB-MSCs with HeLa and MRC-5 cells, we performed the soft agar colony assay using $1 \times 10^4$ cells/well in a 96-well plate. hUCB-MSCs showed a notably lower ability to form colonies than HeLa cells (positive control). The vehicle control and MRC-5 groups did not form colonies (Fig. 1).

In vivo tumorigenicity assay. No significant body weight changes were observed in any of the groups during the experimental period (Fig. 2). Upon clinical observation,
mice treated with hUCB-MSCs or MRC-5 cells had no clinical signs, but HeLa cell-treated mice exhibited swollen skin (6/6), a palpable mass (6/6), and hard skin (1/6) with an increased tumor volume during the experimental period (Fig. 3). Upon hematologic evaluation, there were no meaningful differences between control and hUCB-MSC-treated

### Table 1. Hematology after transplanting hUCB-MSCs, MRC-5 cells, and HeLa cells in BALB/c-nu mice

| Parameter       | Control | hUCB-MSC | MRC-5     | HeLa       |
|-----------------|---------|----------|-----------|------------|
| WBC (× 10^9/L)  | 3.45 ± 2.443 | 4.56 ± 1.722 | 3.36 ± 1.260 | 61.80 ± 166.819 |
| RBC (× 10^12/L) | 10.62 ± 0.584 | 9.90 ± 0.855* | 9.37 ± 2.030 | 9.91 ± 1.237 |
| HGB (g/dL)      | 1.59 ± 0.98 | 14.0 ± 1.46** | 14.1 ± 3.10 | 13.7 ± 1.78** |
| HCT (%)         | 51.5 ± 2.72 | 49.0 ± 4.55 | 46.5 ± 10.10 | 46.0 ± 5.33** |
| MCV (fL)        | 48.6 ± 1.68 | 49.5 ± 0.79 | 49.5 ± 1.08 | 46.6 ± 3.41 |
| MCH (pg)        | 14.9 ± 0.46 | 14.2 ± 0.93 | 15.1 ± 0.36 | 13.9 ± 1.27* |
| MCHC (g/dL)     | 30.7 ± 0.68 | 28.6 ± 2.23** | 30.4 ± 0.65 | 29.8 ± 0.71** |
| PLT (× 10^9/L)  | 1256 ± 205.0 | 1214 ± 519.4 | 1170 ± 208.4 | 1519 ± 426.1* |
| RET (%)         | 512.6 ± 384.77 | 477.7 ± 205.50 | 347.4 ± 99.68 | 561.8 ± 179.57 |
| NEU (%)         | 1.57 ± 0.835 | 2.29 ± 0.709 | 1.77 ± 0.560 | 56.81 ± 154.338* |
| LYM (%)         | 1.73 ± 1.641 | 2.01 ± 1.016 | 1.42 ± 0.786 | 1.26 ± 1.843 |
| MON (%)         | 0.04 ± 0.030 | 0.05 ± 0.028 | 0.04 ± 0.018 | 0.81 ± 2.413 |
| EOS (%)         | 0.06 ± 0.035 | 0.10 ± 0.072 | 0.09 ± 0.105 | 2.28 ± 7.024 |
| BAS (%)         | 0.01 ± 0.008 | 0.02 ± 0.015 | 0.01 ± 0.013 | 1.19 ± 3.664 |

Values are expressed as the mean ± SD.
* Significant difference compared to the control (p < 0.05).
** Significant difference compared to the control (p < 0.01).

### Table 2. Macroscopic findings with abnormal lesions after transplanting hUCB-MSCs, MRC-5 cells, and HeLa cells in BALB/c-nu mice

| Parameter          | Control | hUCB-MSC | MRC-5 | HeLa |
|--------------------|---------|----------|-------|------|
| No. of animals     | 6       | 6        | 6     | 6    |
| Mass               | 0       | 0        | 0     | 6    |
| Mass in injection site | 0     | 0        | 0     |      |
| Mass in thoracic cavity | 0   | 0        | 0     | 1    |
| Abnormal lesion    | 0       | 0        | 0     | 3    |
| Splenomegaly       | 0       | 0        | 0     |      |

### Table 3. Microscopic findings after transplanting hUCB-MSCs, MRC-5 cells, and HeLa cells in BALB/c-nu mice

| Parameter          | Control | hUCB-MSC | MRC-5 | HeLa |
|--------------------|---------|----------|-------|------|
| No. of animals     | 6       | 6        | 6     | 6    |
| Kidneys            | 0       | 0        | 3     | 0    |
| Liver              | 1       | 0        | 1     | 0    |
| Extramedullary hemopoiesis | 0 | 0        | 0     | 1    |
| Spleen             | 0       | 0        | 0     | 5    |
| Extramedullary hemopoiesis | 0 | 0        | 0     |      |
| Heart              | 1       | 1        | 0     | 0    |
| Cardiomyopathy     | 0       | 0        | 1     | 0    |
| Hemorrhage         | 0       | 0        | 0     | 2    |

mice treated with hUCB-MSCs or MRC-5 cells had no clinical signs, but HeLa cell-treated mice exhibited swollen skin (6/6), a palpable mass (6/6), and hard skin (1/6) with an increased tumor volume during the experimental period (Fig. 3). Upon hematologic evaluation, there were no meaningful differences between control and hUCB-MSC-treated...
Table 3. Continued

| Parameter                        | Group          | Control | hUCB-MSC | MRC-5 | HeLa |
|----------------------------------|----------------|---------|----------|-------|------|
| Adrenal glands                   |                |         |          |       |      |
| Subcapsular cell hyperplasia     |                | 5       | 4        | 4     | 4    |
| Corticomedullary vacuolation     |                | 5       | 4        | 4     | 2    |
| Mandibular lymph node            |                |         |          |       |      |
| Extramedullary hemopoiesis       |                | 3       | 3        | 2     | 3    |
| Stomach                          |                | 0       | 1        | 1     | 2    |
| Mesenteric lymph node            |                |         |          |       |      |
| Extramedullary hemopoiesis       |                | 0       | 0        | 0     | 2    |
| Inflammatory cell infiltration, granulocytes |          | 0       | 1        | 0     | 0    |
| Giant cell infiltration          |                | 0       | 1        | 0     | 0    |
| Injection site                   |                |         |          |       |      |
| Tumor mass/HeLa cells, subcutaneous |            | 0       | 0        | 0     | 6    |
| Thoracic cavity                  |                |         |          |       |      |
| Tumor mass                       |                | 0       | 0        | 0     | 1    |
| Skin                             |                |         |          |       |      |
| Keratin cyst                     |                | 0       | 0        | 0     | 1    |

Fig. 4. Histopathology of injection sites and formation of subcutaneous and metastatic cell masses. No cell mass formation was observed in the (a) control, (b) hUCB-MSCs, or (c) MRC-5 cell injection sites. However, the (d) HeLa cell injection site showed a subcutaneous mass (sm). Mitotic figures (asterisks) were observed in the dotted square. These were confirmed in the magnified picture (e), which also showed rounded or pleomorphic cells with hyperchromatic nuclei. (f) A metastatic cell mass (mm) in the thoracic cavity had the same cellular components as the subcutaneous mass. Esophagus (eso). Bar = 200 µm.
groups (Table 1). There were no treatment-related findings according to macroscopic examination in the vehicle control, MRC-5, or hUCB-MSC-treated mice (Table 2). Microscopic examination revealed no tumors in the vehicle control, MRC-5, or hUCB-MSC-treated groups. However, all mice inoculated with HeLa cells developed tumors at the injection site, with one case presenting metastasis to the thoracic cavity (Table 2, Table 3). The tumor lesions presented mitotic figures, necrosis, and rounded or pleomorphic cells with hyperchromatic nuclei (Fig. 4). In addition, extramedullary hemopoiesis with splenomegaly was observed in HeLa cell-treated mice (Table 3).

**Human gene detection at the injection site.** Tumor formation of inoculated human cells can be detected by immunostaining the injection site tissue sections of animals with a human-specific antibody (12). No human mitochondrial antigen expression was observed at the injection site in the vehicle control, hUCB-MSCs, or MRC-5 cell-treated groups, while the HeLa cell-treated group showed high human mitochondrial antigen expression in tumors at the injection site (Fig. 5).

**DISCUSSION**

Mesenchymal stem cells are a promising tool for cell therapies because they are easily sourced from various tissues and have properties of tissue revascularization and repair. Previous non-clinical and clinical studies have reported on the therapeutic value of MSCs (13-16). However, some studies have reported adverse effects of MSCs, including tumor growth and mutation into tumor cells (17-20). Moreover, one patient with the neurodegenerative hereditary disorder ataxia telangiectasia treated with human fetal neural stem cells by intracerebellar and intrathecal injection developed a multifocal brain tumor of non-host origin. This suggested that the tumor was derived from the transplanted neural stem cells (21). These reports indicate the importance of...
investigating the tumorigenicity of MSCs for their use in clinical applications.

In this study, we evaluated the tumorigenicity of hUCB-MSCs in vitro with the soft agar colony formation assay, which is used to measure cell anchorage-independent proliferation potential. Anchorage-independent growth is a hallmark of transformation and the most accurate in vitro indication of tumorigenicity (22). In this study, hUCB-MSCs formed few colonies, while HeLa cells formed a greater number of larger colonies, indicating that hUCB-MSCs do not possess anchorage-independent proliferation potential. The less tumorigenic effect of hUCB-MSCs in vitro than HeLa cells corresponds with recent results of other MSCs (23,24).

With increasing passages of stem cell lines, there is increased potential for chromosomal aberrations, a hallmark of tumor formation (9). The hUCB-MSCs in this study were used at passage 6 and had no evidence of tumorigenicity, suggesting that hUCB-MSCs may be chromosomally stable at least up to passage 6 under our culture conditions, as they were not prone to malignant transformation. In agreement with our results, Ra et al. (25) reported that chromosomal abnormalities were not observed in human adipose tissue-derived MSCs (hAdMSCs) from passages 4–12.

In our 13-week tumorigenicity study in which hUCB-MSCs were transplanted into female BALB/c-nu/nu mice, there were no tumor formation-related findings in body weight, clinical signs, gross findings, organ weight, hematology, or macro- and microscopic examination. However, all HeLa cell-treated mice developed tumors. These findings correlate with the clinical observations and macro- and microscopic examination. However, further studies are required to address the long-term tumorigenicity of human MSCs in animals.

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