STO Feeder Cells Are Useful for Propagation of Primarily Cultured Human Deciduous Dental Pulp Cells by Eliminating Contaminating Bacteria and Promoting Cellular Outgrowth

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STO feeder cells, a line established from mouse SIM embryonic fibroblasts, have been frequently used for establishing embryonic stem cells and maintaining them in an undifferentiated state. There are some reports demonstrating that fibroblastic cells have the ability to phagocytose Gram-positive bacterium (e.g., streptococci and staphylococci). In this study, we examined the possibility that STO cells could phagocytose *Streptococcus mutans* (a bacteria causing tooth decay), which always contaminates cultures of primarily isolated human deciduous dental pulp cells (HDDPCs). Simple cultivation of the primary HDDPCs in the absence of STO cells allowed *S. mutans* to massively propagate in the medium, thus leading to an opaque medium. In contrast, there was no bacterial contamination in the cultures containing mitomycin C (MMC)-inactivated STO cells. Furthermore, STO cells indicated bacterial phagocytic activity under fluorescent microscopy with the dye pHrodo. Besides removal of contaminating bacteria, STO feeder cells allowed the HDDPCs to spread out. These data suggest that MMC-treated STO cells can be useful for propagation of HDDPCs by eliminating contaminating bacteria and by promoting cellular outgrowth.

Key words: Feeder cells; STO cells; ES cells; Pluripotent stem cells; Phagocytosis; Primary tooth;
Dental pulp cells

INTRODUCTION

Oral tissues are useful cell resources for regenerative medicine (6) because they contain a rich source of stem cells from dental pulp, gingiva, tooth germ, and the periodontal ligament. In 2000, human dental pulp stem cells (DPSCs) were identified from human dental pulp cells, and these had phenotypic characteristics similar to those of bone marrow-derived mesenchymal stem cells (28). Stem cells were also isolated from human deciduous dental pulp cells (HDDPCs) from exfoliated deciduous teeth (SHED) (19). Both DPSCs and SHED had definitive stem cell properties, such as multidifferentiation and self-renewal (9,19,28).

For isolation of stem cells from dental pulp, primary culture of the dental pulp cells (DPCs) is a prerequisite. According to the protocol of Gronthos et al. (10),

enzymatically dissociated DPCs are subjected to simple cultivation on a plastic dish with normal medium with several supplements, such as fetal bovine serum (FBS), L-ascorbic acid-2-phosphate, D-glucose, and L-glutamine. During this culture period, actively proliferative fibroblastic cells generate, and almost all of these are stem cells. However, for successful cultivation, researchers must avoid possible contamination from tooth-derived bacteria normally present in the human oral cavity. To date, the presence of more than 700 bacteria has been reported in the human oral cavity (1,15). Therefore, highly concentrated multiantibiotic drugs are always included in the culture medium of primary oral tissue-derived cells to remove as much oral bacteria as possible. Unfortunately, despite extensive efforts, bacterial contamination often occurs in the primary culture

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system, leading to failure of propagation of the primary cultured cells.

Besides the above possible bacterial contamination, selection of the culture surface in the cultural dish is an important factor affecting propagation of the primary cultured cells. They often exhibit inability to grow out onto the plane surface of the dish. To increase affinity of cells to the surface of the culture dish, a dish coated with extracellular matrix, such as type I collagen and gelatin, has been often used (34). Seeding of feeder cells prior to cultivation of primary cells is also frequently used for various types of cells, including epidermal keratinocytes (8,25), endothelial cells (16,24), enamel organ epithelial cells (12), and salivary gland cells (11,13). For example, the use of growth-arrested mouse fibroblasts has been a standard cultural technique for massive propagation of human keratinocytes and corneal epithelial cells for skin grafts (8,21,23). Because feeder cells provide growth factors as well as anchorage, to which cells attach via cell surface adhesion molecules, they appear to be superior to dishes coated with an extracellular matrix. The possible usefulness of feeder cells in the fields of tissue engineering (13) and gene therapy (14) has been recently suggested.

The most widely used feeders are mouse embryonic fibroblasts (MEFs), Sandos inbred mouse (SIM)-derived 6-thioguanine- and ouabain-resistant (STO) cells, and several types of human cells. MEFs are primary cells derived from midgestational fetuses and can be maintained for only several passages before senescence. They have been adopted to provide a reprogramming microenvironment for generating induced pluripotent stem/embryonic stem (iPS/ES) cells (7,22,29,32). However, MEFs have some drawbacks. First, it is often difficult to obtain MEFs transfected with exogenous DNA (i.e., a drug-resistance gene expression vector) because their proliferation ability is generally limited compared with that of other immortalized cells. Second, isolation of MEFs is a laborious and time-consuming process requiring samples from many mice, and the quality of MEFs themselves may often differ depending on the cell isolation skill of individuals.

STO cells, a line established from mouse SIM embryonic fibroblasts (33), have also proven useful for establishing and maintaining ES cells (2,27,30). They can continue to proliferate in vitro, because of their immortalized nature, and are also known to cease proliferation after treatment with mitotic inhibitors such as mitomycin C (MMC) (18). These properties appear to make them superior to MEFs for use as a feeder for generation of genetically engineered ES cells. These cells can propagate without undergoing senescence, unlike MEFs. Several types of feeder cells of human origin have also been used for supporting the growth of human ES cells (3–5,17), but like MEFs, they cannot propagate themselves.

Given these backgrounds, we have explored the possibility of STO cells as useful feeder cells to support growth of primary HDDPCs. During this process, we noted that STO cells possess phagocytotic activity as well as promote outgrowth of HDDPCs. Some species of bacteria invade host nonphagocytic cells, such as epithelial cells, and are largely degraded by the endocytosis pathway (31). However, fibroblastic cells can exhibit autophagy against *Streptococcus pyogenes*, a bacterium that can potentially invade the host cells (26). Unfortunately, whether STO cells possess such phagocytotic activity remains unknown. Therefore, our research was focused on the phagocytotic property of STO cells as well as their outgrowth-promoting activity using primary HDDPCs.

MATERIALS AND METHODS

Mitotic Inactivation of STO Cells

STO cells [#1503; American Type Culture Collection (ATCC), Manassas, VA, USA] were maintained in Dulbecco's modified Eagle medium (DMEM; #11995-081; Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS (#SFMB30-2239; Equitech-Bio, Kerrville, TX, USA), 50 U/ml penicillin, and 50 mg/ml streptomycin (#15140-122; Invitrogen, Carlsbad, CA, USA) (hereafter referred to as DMEM-FBS) in a humidified atmosphere of 5% CO₂ in air at 37°C. Upon mitotic inactivation, these cells were treated with 10 µg/ml MMC (M4287; Sigma-Aldrich, St. Louis, MO, USA) for 3 h and then harvested by trypsinization (2.5% trypsin; #15090-046; Invitrogen). Approximately 2 × 10⁵ cells were seeded in 60-mm gelatin-coated dishes (Iwaki Glass Co. Ltd., Tokyo, Japan) containing DMEM-FBS prior to use for cocultivation with HDDPCs.

Primary Culture of HDDPCs

HDDPCs were obtained with the informed consent of the patients (or appropriate guardian), and the protocols of this study were approved by the Ethical Committee for Use and Experimentation of Kagoshima University Graduate School of Medical and Dental Sciences. Deciduous teeth were donated by five young patients (male and female, 9–12 years old; see Table 1). Isolation of HDDPCs was performed using the protocol of Gronthos et al. (10) with some modifications. First, the pulp mass was removed from the deciduous primary teeth with successional replacement or ectopic eruption. Second, the dissected pulp mass was then digested in a solution of 3 mg/ml collagenase type I (#17100-017; Invitrogen) and 4 mg/ml dispase (#410810077; Roche Applied Science, Upper Bavaria, Germany) for 60 min at 37°C. Finally, the mass was crushed by a pair of forceps, and the resulting single cells were cultivated on MMC-inactivated STO cells in a collagen-coated 35-mm dish

Table 1. HDDPCs of Five Patients Were Tested for Cell Growth and Cell Types

HDDPCs* (Gender:Age)	STO Feeder	Cell Growth	Cell Type	Bacterial Contamination
A (M:9y0m)	(+)	Good growth	Fibroblast	(-)
	(-)	Moderate growth	Fibroblast	(-)
B (F:10y6m)	(+)	Good growth	Fibroblast	(-)
	(-)	Good growth	Fibroblast	(-)
C (F:10y5m)	(+)	Good growth	Fibroblast	(-)
	(-)	Poor growth	-	(+)
D (F:12y0m)	(+)	Good growth	Fibroblast	(-)
	(+)	Moderate growth	Fibroblast	(-)
E (F:11y10m)	(+)	Moderate growth	Fibroblast	(-)
	(+)	Poor growth	-	(+)

HDDPCs, human deciduous dental pulp cells; STO, Sandos inbred mouse (SIM)-derived 6-thioguanine- and ouabain-resistant cells.

*Established cell line by patients A to E.

(#4000-010; Iwaki Glass Co. Ltd.) containing α -modified minimum essential medium (MEM α ; #135-15175; Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 20% FBS, 100 μ M L-ascorbic acid-2-phosphate (#323-44822; Wako Pure Chemical Industries, Ltd.), 50 U/ml penicillin, and 50 mg/ml streptomycin (hereafter referred to as MEM α -FBS). These enzyme-digested cells were seeded onto STO feeder cells [hereafter referred to as “STO(+)”] or a feeder-free plate [hereafter referred to as “STO(-)”] as a reference. The growth of dissociated pulp-mass-derived cells was monitored periodically and photographed. Cells exhibiting confluency by 2 weeks after seeding were judged as “good growth.” Similarly, cells exhibiting confluency by 1 month after seeding were judged as “moderate growth.” Cells that failed to grow were judged as “poor growth.”

Culture of Bacterial Cells

Streptococcus mutans MT8148 (serotype c; obtained from Osaka University Graduate School of Dentistry, Osaka, Japan) was selected on Mitis Salivarius (MS) agar (Difco Laboratories, Detroit, MI, USA) and then cultured in Brain Heart Infusion (BHI) broth (Difco Laboratories) at 37°C in air, according to previously described methods (14).

Giemsa Staining

Giemsa stain was used to visualize the nucleus, cytoplasm, and bacteria. Bacterial concentration (optical density, OD) of *S. mutans* cultured in BHI broth was first determined by a spectrophotometer (BioSpec-mini; Shimadzu Corporation, Kyoto, Japan). The bacterial solution (100 μ l; OD=660) was seeded onto the STO feeder cells grown in a 60-mm dish (#4010-010; Iwaki Glass Co. Ltd.), and the dish was left at 37°C for 2 days. After the dish was gently washed with phosphate-buffered

saline, pH 7.2 (PBS; #12729018S; Invitrogen), cells were fixed with methyl alcohol (4987481259898; Wako Pure Chemical Industries, Ltd.) for 5 min and then exposed to Giemsa stain solution (079-04391; Wako Pure Chemical Industries, Ltd.) for 30 min at room temperature. The STO cells without the addition of *S. mutans* were also subjected to Giemsa staining as a control. After rinsing of the dish with distilled water, the dish was subjected to microscopic observation under a phase-contrast microscope (OLYMPUS CKX41, Tokyo, Japan).

Phagocytosis Assay

The phagocytic activity of STO cells was determined by using proprietary pH-sensitive pHrodo dyes (#A10010; Invitrogen) that served as specific sensors of phagocytosis and endocytosis. The dyes are almost non-fluorescent at neutral pH and fluoresce brightly in acidic environments. The increase in fluorescence signal at low pH makes it ideal for studying phagocytosis and endocytosis and its regulation by drugs or environmental factors. SYBR Green-stained (Takara Bio Inc., Shiga, Japan) live *S. mutans* organisms were infected onto STO cells at an infection ratio of bacteria to cells of 100:1 in a 60-mm dish (#4010-020; Iwaki Glass Co. Ltd.). As a control, the SYBR Green-stained *S. mutans* were simultaneously seeded onto a feeder-free dish. Streptococcal organisms were washed with PBS and suspended within PBS containing 50 μ g/ml of SYBR Green (Life Technologies). After incubation for 30 min at room temperature, the bacterial cells were washed with PBS. These dishes were stained with 750 μ g/ml of pHrodo for 2 h at 37°C in an atmosphere of 5% CO₂ in air. Increased lysosomal activity is correlated with the enhancement of pHrodo-derived red fluorescent density on the microscopic images, and therefore the red stained cells indicate active phagocytosis in those cells, whereas the green images demonstrated

that the cells incorporated only SYBR Green-stained *S. mutans* in their cytoplasm. Furthermore, cells expressing both red and green fluorescence are assumed to have incorporated *S. mutans* via phagocytosis. Fluorescence was observed and recorded under a Keyence BZ-9000 fluorescent microscopic system (Keyence Corporation, Osaka, Japan).

RESULTS

Difference in Outgrowth Activity of Primary HDDPCs Cultivated in the Presence or Absence of STO Feeder Cells

The property of primary HDDPCs isolated from five patients after cultivation (within 1 month after primary cultivation) in the presence or absence of an STO feeder layer was assessed. When enzymatically dissociated pulp-mass-derived cells are placed under a cultural environment, they attach to the surface of the dish and exhibit outgrowth away from the cell colony. The cells exhibiting cellular outgrowth appear to be actively proliferative, and in almost all cases, such cells are fibroblastic cells (Fig. 1). When primary HDDPCs were cultured in the presence of feeder cells (arrowheads in Fig. 1C, D), almost all grew well and exhibited outgrowth within 2 days after seeding (arrows in Fig. 1C). They finally reached confluency by 2 weeks after seeding (Table 1). In contrast, cultivation in the absence of feeder cells resulted in poor growth of primary HDDPCs (arrows in Fig. 1A, B), which only reached confluence over 1 month after seeding (Table 1). In each case,

cells exhibiting outgrowth appeared morphologically to be fibroblastic cells (Table 1) (Fig. 1D). Notably, bacterial contamination in primary HDDPCs isolated from patients C and E were evident when they were cultivated in a feeder-free condition, whereas such contamination was much reduced after coculture with STO cells (Table 1).

Phagocytosis of S. mutans by STO cells

To examine whether STO cells could clear contaminated bacteria by phagocytotic activity, *S. mutans* was infected onto the feeder cells (Fig. 2). Microscopic observation revealed that MMC-treated STO cells cultured in the presence of *S. mutans* had numerous Giemsa-positive granules in their cytoplasm (arrows in Fig. 2D), whereas those cultured in the absence of *S. mutans* had no such positive granules in their cytoplasm (Fig. 2B).

To indirectly confirm the above results, a phagocytosis assay using proprietary pH-sensitive pHrodo dyes was performed. The SYBR Green-stained *S. mutans* was infected onto the MMC-treated STO feeder cells. When labeled *S. mutans* were added to the dish containing STO cells, there were several cells with both red and green fluorescence (arrows in Fig. 3C, E). These results indicate that STO cells have the ability to phagocytose bacteria like *S. mutans*.

DISCUSSION

In this study, we demonstrated that STO cells are beneficial as feeder cells for supporting the outgrowth of primary HDDPC-derived fibroblastic cells. However, to

Figure 1. Cultivation of HDDPCs derived from patient A in the absence or presence of MMC-treated STO feeder cells. Note that cellular outgrowth from the dissociated dental mass is not extensive when the human deciduous dental pulp cells (HDDPCs; arrows) were cultured under feeder-free conditions for 48 h (A) and 72 h (B). In contrast, the HDDPCs cultured on mitomycin C (MMC)-treated Sandos inbred mouse (SIM)-derived 6-thioguanine- and ouabain-resistant (STO) cells (arrowheads) exhibited extensive outgrowth (arrows) 48 h (C) and 72 h (D) after cultivation. Scale bar: 100 μ m.

Figure 2. Cultivation of MMC-treated STO cells in the presence (C, D) or absence (A, B) of *S. mutans*. Giemsa staining revealed incorporation of contaminating bacteria (arrowheads in C), which could be identified as Giemsa-positive granules (arrows in D) in the cytoplasm of the MMC-treated STO cells. In contrast, the STO cells cultured in the absence of *S. mutans* had no obvious Giemsa-positive granules in their cytoplasm (B). The outline of a cell is shown as dotted lines. Scale bar: 100 μm.

our knowledge, the previous reports concerning cultivation of primary DPCs have not used such feeder cells along with collagen-coated dishes for propagation of the primary DPCs. When primary HDDPCs were cultured in the absence or presence of MMC-treated STO

cells in our study, outgrowth of fibroblastic cells in the former was poorer than that in the latter (see Table 1). Table 1 also shows the considerable variability in the cell growth response among the five subjects. This may depend on differences between the subjects. We also need

Figure 3. Phagocytosis assay. SYBR Green-labeled *S. mutans* was added to the culture medium in a dish containing STO cells (A), incubated for 2 h at room temperature, and then stained with proprietary pH-sensitive pHrodo dyes. The green fluorescence indicates the presence of *S. mutans* (B), and red fluorescence indicates active phagocytosis by STO cells (D). In (C), which is merged from (B) and (D), there were cells (arrows in C) having both red and green fluorescence, indicating that STO cells can, in fact, phagocytose *S. mutans*. Both red and green fluorescence observed in the cytoplasm of the MMC-treated STO cells (arrow in E) is shown in the two quadrants located at the bottom and right side. Scale bar: 100 μm.

to consider the kind of deciduous tooth donated and their age. This will be explored in future studies.

As mentioned previously, feeder cells secrete many types of growth factors and provide anchorage through which cells can normally proliferate, all of which are beneficial for the growth of primary HDDPCs. Feeder cells themselves can proliferate in the absence of a mitotic inhibitor such as MMC. Therefore, it is not recommended to use intact feeder cells for propagating primary DPCs because the feeder cells grow with the primary DPCs. Generally, the MMC-treated feeder cells cannot proliferate, survive for at best 1 week, and finally die. This property is beneficial for propagating a pure population of dental pulp-derived cells. When the dental pulp-derived cells are used for regenerative medicine, the use of human (not mouse) feeder cells is desirable. In this context, it would be interesting to test whether human deciduous dental pulp-derived cells could possess the ability to support the growth of primary DPCs.

Another merit in using MMC-treated STO cells for cultivation of primarily isolated DPCs is their ability to clear contaminating bacteria from the medium. In the presence of the MMC-treated STO cells, which phagocytose bacteria present in the medium, the primary DPCs can proliferate normally (see Table 1). Nakagawa et al. showed that autophagic machinery could effectively eliminate pathogenic group A *Streptococcus* (GAS) within non-phagocytic cells such as HeLa cells. After escaping from endosomes into the cytoplasm, GAS become enveloped by autophagosome-like compartments and are killed upon fusion of these compartments with lysosomes (20).

In conclusion, we demonstrated that MMC-treated STO cells are useful for propagation of primarily isolated HDDPCs because they support the outgrowth of human deciduous dental pulp-derived fibroblastic cells and clear contaminating bacteria from the medium.

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