Spheroid Formation of Bovine Endometrial Stromal Cells with Non-Adherent Culture Plate

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Abstract. A multicellular mass (spheroid) was developed from bovine endometrial stromal cells with non-adherent culture plates. Cells cultured in a spheroid-plate were spontaneously aggregated and formed a cell mass rough in appearance 24 h after the start of culture. After 3 days of culture, the cell mass became oval-shaped in the range 375 to 900 µm in diameter. The size of the spheroid depended on the number of cells plated in each well. Histological examination indicated that each spheroid was covered with a single layer of squamous cells, and cuboidal cells occupied the inside of the spheroid. The size of the spheroid decreased gradually and the inner cells of the spheroid became sparse and irregular in size after culture for more than 7 days. ProMMP-2 mRNA was found both in monolayer culture cells and the spheroid, but the active form of MMP-2 protein was found only in the condition medium of the spheroid. The present study demonstrated that the spheroid was easily generated from bovine endometrial stromal cells by using a non-adherent plate. Expression of the active form MMP-2 indicates that the cells in the spheroid changed their character not only morphologically but also functionally. These results indicate that the spheroid developed by the present method could provide a new tool for analyzing the functions of bovine endometrium.

Key words: Spheroid, Bovine, Endometrial stromal cells, Non-adherent culture plate


Spheroids, as a spherical mass composed of cells and extracellular matrices, have been extensively studied with cancer cells and embryonic cells [1–4]. Malignant cells in solid tumors, fetal cells in various organs, and liver cells in newborn rats aggregated and formed spheroids when they were cultured in dishes coated with nonadhesive substrate. Agarose, agar, and poly(2-hydroxyethyl methacrylate) have been used as materials for nonadhesive substrata in previous studies. Furthermore, several investigators have developed various types of spheroids from normal adult cells. Koide et al. [5, 6] established a spheroid from adult rat hepatocytes, with either on a positively charged surface or on a proteoglycan-coated surface plate. In their study, cells in the spheroid maintained greater ability to produce albumin than those cultured in monolayer cells. Therefore multicellular spheroid may be a very useful tool for the investigation of cell behaviour and interaction in vivo.

To investigate the functions of endometrial stromal cells is important in order to understand the basic information of implantation and pregnancy. Nevertheless, there is no report which analyzes the function of the endometrial stromal cells by means of three-dimensional culture, including spheroids. The aim of this study is to develop spheroids from bovine endometrial
stromal cells as an in vitro model for analyzing the functions of the bovine endometrium. A non-adherent cell culture plate was used for the spheroid formation of bovine endometrial stromal cells. The process of spheroid formation was examined histologically. And expression of matrix-metalloproteinases (MMPs), which is a one of the indexes of cells in the three-dimensional culture [7], was analyzed in the spheroids and compared with those of cultured monolayer cells.

**Materials and Methods**

**Preparation and culture of cells**

The stromal cells were separated and purified from bovine endometrium at day 140 of pregnancy. After washing in PBS three times, endometrial tissues were digested at 37°C for 1 h in DMEM/F12 medium containing 0.1% collagenase. Dissociated cells were cultured in DMEM/F12 containing 10% fetus bovine serum after being washed three times with the culture medium. In collecting stromal cells, the following procedures were done: two to three hours after the primary culture, the medium was changed to fresh medium, and the remaining cells were cultured as stromal cells. Stromal cells lifted more easily from dishes than epithelial cells in trypsinization. Using this characteristic, contaminating epithelial cells were eliminated by treatment with 0.25% trypsin solution in several passages. Finally, uniformity of the cell population was confirmed immunohistochemically. Stromal cells were a monolayer of flat, spindle- and fibroblast-like cells which stained vimentin positive and cytokeratin negative (Fig.1) [8]. After more than 20 passages, the cells were used for the experiment. All cell culture procedures were done at 37°C in humidified air with 5% CO₂.

**Preparation of spheroids**

Spheroids were prepared in a 96-well spheroid-plate® (Celltight Spheroid Culture Plate, Sumitomo Bakelite, Tokyo, Japan), which has a non-adhesive surface and round bottom. The cells spontaneously aggregated in 24 h after plating and formed a single cell mass in each well. The density of the cells first plated on the spheroid plate was 1×10³, 5×10³, 1×10⁴, 5×10⁴ or 1×10⁵ per well. After 3 days of culture in the spheroid plate, aggregated cell masses were transferred to an agarose-coated dish. The diameters of the cell masses were measured under a phase-contrast microscope. The culture medium was changed every 3 days for continuous culture.

**Histology**

After 1, 3, 7 or 14-day culture, the spheroids were washed three times with PBS, embedded in TissueTek (Sakura Finetechnical Co., Tokyo, Japan) and stored for histological examination at −30°C. Cryostat sections (6 μm) were made by means of a cryostat (HM 500 OM, MICROM Laborgeräte GmbH, Walldorf, Germany with), mounted on Poly-L-Lysin-coated (Sigma, St.Louis, MO) slides and dried at room temperature. For histological

![Fig. 1. Immunofluorescent staining of purified bovine endometrial stromal cells. Stromal cells were stained with anti-vimentin antibody (A) and anti-cytokeratin antibody (B). Scale bar represents 100 μm.](image-url)
examinations, these sections were stained with hematoxylin and eosin (HE).

**Reverse transcription (RT) and amplification of cDNA**

Total RNA was isolated from monolayer cells and spheroids by using ISOGEN (Nippon Gene CC, Toyoma, Japan) according to the manufacturer’s instructions. After determination of RNA purity and concentration by means of a spectrophotometer, two µg of total RNA was used for reverse transcription in a 20 µl reaction volume. The total RNA was reverse transcribed with 200 units of Super Script™ II reverse transcriptase (Gibco BRL, Life Technologies, Inc., New York, USA) in the presence of 0.5 µg of oligo dT primer, 500 µM dNTP, 2.5 mM MgCl₂ and 10 mM DTT at 42°C for 50 minutes. After heat inactivation of reverse transcriptase at 70°C for 5 minutes, 2 units of ribonuclease H was added to the reaction mixture and incubated at 37°C for 20 minutes before polymerase chain reaction (PCR). The proMMP-2 primers designed from human proMMP-2 sequence [9] were 5’-CCACGTGACAAGCCCATGGGCCGCCC-3’ (sense: 1448–1472) and 5’-GCCAGCTCAGCAGCCTAGCCAGTCG-3’ (antisense: 1919–1943). PCR was done in a thermal cycler (MJ Research, Inc., Watertown, MA) and the reaction mixture consisted of cDNA template, 0.5 µM primers, 0.2 mM dNTP mixture and 0.1 units/µl AmpliTaq gold DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA). PCR conditions were as follows (in order of denature, annealing and extension): 93°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute. Ten µl of PCR product and 1 µl of 10 × loading buffer were then mixed and electrophoresised at 100V on a 2% agarose gel in TAE buffer (40 mM Tris-acetate, 10 mM EDTA, pH 7.8). PCR products were visualized by ethidium bromide staining.

**Zymography**

The culture medium was changed to DMEM/F12 medium without fetal bovine serum 24 h before collection of the conditioned medium for analysis of MMP activity. One µg of protein per sample was subjected to SDS-PAGE with a 10% (w:v) acrylamide slab gel containing 0.6 mg/ml gelatin under nonreducing conditions. Then SDS in the gel was removed by rinsing with extraction buffer [50 mM Tris-Cl, 5 mM CaCl₂, 1 µM ZnCl₂ and 0.02% (w:v) NaNO₃ (pH 7.5) containing 2.5% (w:v) Triton X-100]. The gel was then incubated at 37°C for 16 h in the incubation buffer which was the same as the extraction buffer without 2.5% Triton X-100. After incubation, the gel was stained with 0.1% (w:v) Coomassie Brilliant Blue R-250 in 50% (v:v) methanol/20% (v:v) acetic acid and destained in 45% (v:v) methanol/9.2% (v:v) acetic acid. In order to determine the type of proteinases observed on thezymograms, 10 mM ethylenediaminetetraacetic acid (EDTA) was added to the buffer during the incubation period. All procedures were performed as aforementioned (for a gel without EDTA in the incubation buffer).

**Results**

**Spheroid formation of bovine endometrial stromal cells**

The cells spontaneously aggregated and formed a single cell-mass with a rough appearance within 24 h after plating in a spheroid-plate®. Each cell-mass became oval-shaped after being transferred to the agarose-coated dish (Fig. 2). As shown in Fig. 3, the size of the cell mass in 3 days of culture depended on the number of cells plated. The cell-mass formed a spherical mass that averaged 375 to 900 µm in diameter. When 1 × 10⁵ cells were seeded in the well, the diameter of the spheroid was about 1250 µm at day 1 of the culture. After being transferred to the agarose-coated dish, the diameter gradually decreased to approximately 350 µm at day 21 of culture, and some cells were dissociated even at day 21.

**Histological characterization**

After 24 h in the spheroid plate (day 1), doughnut- or cone-shaped cell-masses were formed with a cavity inside (Fig. 4A). The cavity was filled with cells in 3 days (Fig. 4B). A single layer of squamous cells covered the spheroid in 24 h. This squamous cell layer covering the outer surface of the spheroid became thinner during the two week culture period (Fig. 4E-H). Cuboidal cells were found inside the spheroid on the first day of culture, and the cells became sparse and irregular in size by day 7 (Fig. 4G). Some pyknotic cells were found after 3 days of culture (Fig. 4F-H).
Expression of MMP in spheroids

ProMMP-2 mRNA was found both in monolayers and spheroids throughout the culture periods (Fig. 5A). Although monolayer cells faintly expressed an active form of MMP-2, strong expression of active MMP-2 was found in spheroids, especially at day 7 of culture. Several additional lower bands were detected by zymography in the culture medium of the spheroid (Fig. 5B). All gelatinase activities were inhibited by EDTA, which confirm that the bands on the zymogram (Fig. 5B) represent metalloproteinases (Fig. 5C).

Discussion

The spheroid is a three-dimensional cell mass and has been thought to be a suitable model for analyzing tissue and organ functions [6, 10]. The present study aimed to develop spheroids composed of bovine endometrial cells to analyze functions of bovine endometrium. Spheroids were prepared by using non-adherent cell culture plates. Bovine endometrial stromal cells were cultured on the plates, and aggregated spontaneously, resulting
Dishes coated with non-adhesive substrata were usually used for generating spheroids; agarose, agar or poly(2-hydroxyethyl methacrylate) has been used [1–4]. Because the generation of spheroids in non-adherent dishes mostly depends on occasional aggregation of the cells, it was unstable and difficult to control. In the present study we used a non-adherent plastic dish that has a positive charge on the surface. Bovine endometrial stromal cells
instantly aggregated and formed a single cell mass in each well, so that it would be a convenient tool for the formation of three-dimensional tissue-like spheroids and examination of the endometrial functions.

The cells on the outer layer of spheroids have a squamous appearance, which is a typical epithelial cell feature. Takezawa et al. [11] found that human dermal fibroblasts on the outer layer of the spheroid take on a squamous appearance. They also described how these fibroblasts were capable of making gap and tight junctions on the outer layer of spheroids, which are usually formed between epithelial cells [12]. Histological observations showed that bovine endometrial stromal cells expressed epithelial cell-like characteristic when they moved to the outer surface of the spheroid.

A doughnut-shaped cell mass was found histological examination in the spheroid at 24 h after seeding cells. This cavity in the spheroid may be caused by the losing cells during preparation of the cryosection because of loose assembly in cell to cell contact. When the spheroid was cultured for more than 7 days, cells inside it became irregular in size and some cells had pyknotic features. These results indicate that the spheroids generated by means of this non-adherent spheroid plate maintain their structure for only several days. A less intact appearance was reported in spheroids generated from cancer and normal adult cells, when the culture period was extended for more than 1 week [11, 13, 14]. Further studies are required to develop a suitable method for culturing spheroids that can maintain their structure and function in a long-term culture to analyze in vivo conditions.

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References


