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A red fluorescent nude mouse model of human endometriosis: advantages of a non-invasive imaging method

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ABSTRACT

Objectives: To establish red fluorescent human endometriosis lesions in a nude mouse model and dynamically and non-invasively to compare intraperitoneal and subcutaneous injection models.
Study design: Primary cultures of endometrial stromal cells (ESCs) and epithelial cells (EECs) isolated from 24 patients with a normal uterine cavity were transfected with 2.5×10^8 (Group 1) and 1.25×10^8 (Group 2) plaque-forming units (PFU) of adenovirus encoding red fluorescent protein (Ad-RFP). Transfection efficiencies, fluorescence intensity and apoptosis rate of the two types of cells were compared in vitro. A mixture of 2.5×10^8 PFU Ad-RFP-infected approximately 400 EECs cell mass and 2×10^6 ESCs for 36 h was injected individually into 24 female nude mice subcutaneously (Group A) or intraperitoneally (Group B). From Day 5 after injection, an in vivo imaging system (IVIS) was used to non-invasively observe and compare the lesions of the two groups every week until Day 33. Specifically, the fluorescent intensity, positive rates, persistence time and lesion weight in the implanted human endometriosis lesions were compared. A parametric Student's *t*-test and two-way analysis of variance were used for statistical analysis.
Results: Compared with 1.25×10^8 PFU RFP, a titre of 2.5×10^8 PFU RFP ESCs and EECs incubated for 36 h exhibited higher transfection efficiencies and higher fluorescence intensities in vitro. In vivo imaging of the fluorescent human endometriosis lesions originating from an RFP titre of 2.5×10^8 PFU showed that the intensity and lesion weight in Group A were significantly higher than in Group B. However, the two groups had the same RFP-positive rates and fluorescence persistence. The structure of each lesion was evaluated by immunohistochemistry to confirm its human endometrial origin.
Conclusions: The red fluorescent human endometriosis model established by subcutaneously injecting 2.5×10^8 PFU RFP-transfected stromal cells and epithelial cells into nude mice had a higher fluorescent positive rate from Day 5, higher intensity and weight but the same persistence as the intraperitoneal injection model.

1. Introduction

Endometriosis is a mysterious disease, especially among women with infertility, and is defined by the presence of endometrial fragments outside of the uterine cavity. The prevalence rate approaches 10–15%, with partial cases regressing after the menopause or ovariectomy. The major symptoms are dysmenorrhea, infertility and pelvic mass formation. Although endometriosis is a benign disease, it has tumour-like characteristics, such as invasiveness and metastatic potential. To date, the main aetiological factors of endometriosis have been unclear, partly due to the lack of an appropriate model for this condition.

Recently, endometriosis animal models have been improved by the use of non-invasive fluorescent imaging approaches, including fluorescent dyes, gene transduction and transgenic animals [1–4]. In a previous study [5], we applied an isolation–transfection–incubation method using separated human endometrial stromal cells (ESCs) and endometrial epithelial cells (EECs), which were

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then individually co-cultured with green fluorescent protein (GFP). After an 18-h incubation, the cells were harvested and immediately subcutaneously injected together into nude mice. Although fluorescent lesions were detected up to 25 days after injection, most of the fluorescent signal disappeared after Day 15.

In the present study, we aimed to establish a more stable and effective model of human endometriosis with a high rate of positivity for red fluorescence. We also compared subcutaneous injection with intraperitoneal injection to identify the optimal conditions for the establishment of the red endometriosis model.

2. Materials and methods

2.1. Collection of endometrium

Endometrial samples were collected between January and April 2011 from 24 patients aged 20–45 years (mean age 31) diagnosed by hysteroscopy as having a normal uterine cavity, who had normal menstrual cycles and had not received any hormone treatments within the preceding 3 months. No abnormalities, malignancies or internal complications were present in these cases. All endometria obtained by dilation and curettage were immediately transferred into DMEM/F12 (Gibco, Carlsbad, CA, USA) supplemented with 1% penicillin and streptomycin (HyClone, Logan, UT, USA) within 1 h. This protocol was approved by the First Affiliated Hospital of Sun Yat-sen University Ethical Review Committee, and all recruited patients provided informed written consent. All samples were in the proliferative phase of the menstrual cycle, as confirmed by endometrial histology.

2.2. Experimental animals

All animal work was performed in the animal facility at the First Affiliated Hospital of Sun Yat-sen University in accordance with hospital guidelines. Twenty-four female nude mice (BALB/c), aged 4–5 weeks and weighing 14–15 g, were provided by the Sun Yat-sen University Laboratory Animal Resources and were housed under specific pathogen-free conditions at a humidity of 45–70% and a temperature of 22–24 °C. The mice were kept under a light/dark cycle of 12/12 h and fed with a standard diet. Anaesthesia with 0.5 ml/min isoflurane was applied for all surgical procedures and non-invasive observations.

2.3. Main reagents and instruments

DMEM/F12 culture medium and type I collagenase were purchased from Gibco (USA), and 0.25% trypsin, penicillin, streptomycin and 10% fetal calf serum (FCS) were purchased from HyClone (USA). Adenovirus particles encoding enhanced red fluorescent protein (Ad-RFP) were provided by Sunbio (Shanghai, China). Annexin V-FITC and 7-AAD were supplied by Bestbio (China). Mouse anti-human vimentin (Clone V9, ready for use) and mouse anti-human cytokeratin (Clone AE1/3, ready for use) primary antibodies were purchased from Dako (Tokyo, Japan). An inverted phase-contrast microscope (Ix71) and a CCD camera (DP71) were supplied by Olympus (Tokyo, Japan). Flow cytometry equipment was provided by Becton Dickinson, and the IVIS kinetic system was purchased from CS Biotech. Stainless-steel cell filters were purchased at the Shen Yue instrument store (Haizhu District, Guangzhou). Plates and centrifuge tubes were provided by Costar (Corning Incorporated, Corning, NY, USA).

2.4. Isolation, purification and culture of ESCs and EECs

Endometrial cells were prepared for 24 h before transfection as previously described by our group [5,6].

2.5. Adenovirus transfection of ESCs and EECs

The isolation–transfection–incubation procedure was performed according to our preliminary work [5]. Two different titres of red fluorescent protein (RFP) were tested: in each well, Group 1 received 2.5×10^8 plaque-forming units (PFU) adenovirus particles, and Group 2 received 1.25×10^8 PFU. Fluorescence expression was observed at 12, 36 and 60 h for three time points after transfection. After digestion with 0.25% trypsin, the ESCs and EECs were examined by flow cytometry to determine the transfection efficiency, fluorescence intensity and apoptosis rate.

2.6. Establishment of a red fluorescent human endometriosis nude mouse model

After 36 h of incubation with the adenovirus particles, the EECs were collected and washed three times with phosphate buffered solution (PBS). The ESCs were collected by 0.25% trypsin digestion and washed three times with PBS. Approximately 400 EECs cell mass and 2×10^6 ESCs were mixed. DMEM/F12 supplemented with 10% FCS was added to each tube after centrifugation. Twenty-four nude mice were then randomly divided into two groups of twelve each. Each mouse in each group received only one type of implantation in a single injection (Group A: subcutaneously injected mixed cells; Group B: intraperitoneally injected mixed cells).

2.7. Non-invasive observation of the red fluorescence human endometriosis nude mouse model

The two groups of mice were observed in vivo using the IVIS kinetic imaging system weekly, beginning at Day 5 after injection. The mice were anaesthetised and placed horizontally on a black box. The software was operated at the maximum table setting, with the minimum and maximum RFP absorption wavelengths at an excitation wavelength of 488 nm and an acceptance wavelength of 600 nm, respectively. Within this wavelength range, the red fluorescent area was identified as the model lesion area displaying photographable fluorescence. Analytical software was used to collect the fluorescence intensity and fluorescence area data. The fluorescence intensity, positive rate, and persistence time were recorded and compared. All mice were then sacrificed by cervical dislocation on Day 33 after implantation. Lesions were collected for weight and size analysis. Haematoxylin and eosin (HE) staining, along with immunohistochemistry for cytokeratin (glandular epithelial tissue) and vimentin (stromal tissue), was used to identify the lesions.

2.8. Statistical analysis

All data were normally distributed and reported as means ± standard deviation (SD). A parametric Student's *t*-test and two-way analysis of variance were used to determine the significance of differences between groups. All data analysis was performed using SPSS version 13.0 (SPSS, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Ad-RFP transfection of ESCs and EECs in vitro

RFP expression in both ESCs and EECs in each group began at 12 h and steadily increased for up to 36 h (Table 1). Greater transfection efficiency and higher fluorescence intensity were observed in Group 1 than in Group 2 ($P < 0.05$). Within these groups, the EECs exhibited higher transfection efficiencies and