Development of an experimental model of endometriosis using mice that ubiquitously express green fluorescent protein

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BACKGROUND: Aiming at improving an animal model of endometriosis, we developed a homologous mouse model using ‘green mice’ that ubiquitously express green fluorescent protein. METHODS: Endometrial fragment obtained from estradiol (E2)-supplemented ovariectomized ‘green mice’ was minced and injected into the peritoneal cavity of ovariectomized wild-type mice. The recipient wild mice were raised with or without E2 supplementation for 2 weeks, and then were euthanized. Endometriotic lesions that developed in the abdomen were examined both macroscopically and microscopically under fluorescence, and weight of the lesions was measured. RESULTS: The endometriotic lesions were more clearly detected under fluorescence imaging than by conventional macroscopic examination. Histologically, endometriotic lesions deriving from ‘green mice’ were sharply distinguished from surrounding host tissues under fluorescence microscopy. More lesions developed in E2-supplemented than control recipient mice. The measured fluorescence intensity of endometriotic lesions showed significant positive correlation with their weight ($R = 0.844$, $P < 0.0001$), and was significantly higher in E2-supplemented mice than in vehicle-supplemented mice ($P = 0.0062$). CONCLUSION: The present endometriosis model using ‘green mice’ would be useful for expeditious identification and quantitative evaluation of endometriotic lesions.

Key words: animal model/endometriosis/estrogen/green fluorescent protein/histology

Introduction

Endometriosis, an enigmatic disease, affects women of reproductive age, causing pelvic pain and infertility (Momoeda et al., 2002; Osuga et al., 2002). Implantation and growth of endometrial tissues in retrograde menstrual flux is a widely believed etiology of the disease. However, it remains to be elucidated why only a limited number of women develop the disease even though retrograde menstruation is seen in almost all women.

A good experimental animal model is needed for the research to elucidate the disease mechanism and to develop new therapeutic drugs. However, endometriosis spontaneously occurs only in primates, which is costly for experimental use. Hence, endometriosis models have been surgically created in small animals, such as rabbit, rat and mouse (Schenken and Asch, 1980; Zamah et al., 1984; Bergqvist et al., 1985; Vernon and Wilson, 1985; Bruner et al., 1997; Somigliana et al., 1999). These models are classified into two types, i.e. homologous models and heterologous models. In homologous models, the endometrium is obtained from the uterus of a congenic animal, and sutured or dispersed into the peritoneal cavity. In heterologous models, human endometrial fragments are injected into immunodeficient mice, such as nude mice and severe combined immunodeficient (SCID) mice, either i.p. or s.c.

In both types of model, endometriotic-like lesions, which are identified histologically, are formed in the animals. Nevertheless, the lesions are sometimes too unclear to distinguish from surrounding normal tissues. This ambiguity makes it difficult to determine number or weight of the lesions, which is an essential variant in the experiment.

Along with recent advances in in vivo bioluminescence imaging, animal models for various diseases have been improved with the use of green fluorescent protein (GFP) (Hoffman, 2002; Sata et al., 2002; Sutton et al., 2003). In a heterologous model of endometriosis, an adenovirus-mediated transient expression of GFP in endometriotic tissues has been reported recently (Fortin et al., 2003, 2004). In the present study, we developed a homologous mouse model of endometriosis with permanent expression of GFP in endometriotic tissues.
Materials and methods

Animals
All procedures involving experimental animals were performed in accordance with protocols approved by the Animal Care and Use Committee of the University of Tokyo. Transgenic mice (C57BL/6 background) that ubiquitously express enhanced GFP (gfp mice) were a generous gift from Dr M.Okabe (Osaka University) (Okabe et al., 1997). Female, 6–8 week old, wild-type C57/B6 mice (wt mice) were obtained from Tokyo Laboratory Animal Science (Tokyo, Japan). Mice were fed on mouse diet (MF; Oriental Yeast, Japan) and water and kept on a light/dark cycle of 12/12 h under controlled conditions. The diet contains phytoestrogens that correspond to 0.05 μg E2 per 1 g diet. Accordingly, only a small amount of estrogen is estimated to be taken from the diet. Prior to any invasive procedure, the mice were anaesthetized by 100 mg/kg ketamine hydrochloride (Sankyo, Japan) s.c. Every surgical technique was performed under sterile conditions.

Induction of endometriosis
Induction of endometriosis was performed according to the method reported by Somigliana et al. (1999). First, both donor (gfp mice) and recipient mice (wt mice), 6–8 weeks old, were subjected to ovariectomy.

All donor mice and a half of the recipient mice (E2-supplemented mice) were injected s.c. with 100 mg/kg estradiol valerate (Teikoku Zouki, Japan) in corn oil every week from the time of ovariectomy. Estrogen dependency of the growth of endometriotic lesions is a well-known phenomenon (Dizerega et al., 1982). To ascertain the quality of the model, we studied the effect of estrogen in this model. For this purpose, the other half of the recipient mice (control mice) were injected s.c. with only corn oil every week from the time of ovariectomy. Two weeks after ovariectomy, donor mice were killed by cervical dislocation. Uterine horns were removed and put into a dish containing phosphate-buffered saline (PBS). Endometrial fragments, obtained by peeling off the serosa and myometrium gently, were minced using a razor blade. The fragments suspended in 0.6 ml PBS were injected with an 18-gauge needle through the abdominal wall just below the umbilicus into the peritoneal cavity of recipient mice with a ratio of one donor to two recipients. In total, 12 control mice and 14 E2-supplemented mice were provided for further analysis.

Fluorescence imaging of GFP-expressing tissues
Two weeks after the injection of the endometrial fragments, mice were euthanized with an overdose of ketamine hydrochloride and perfused via the left ventricle with 0.9% sodium chloride solution, followed by perfusion fixation with 4% paraformaldehyde in PBS. Laparotomies were performed by a vertical midline incision. Peritoneal cavity of mice was illuminated with a GFP-lighting system (VB-L12; Keyence, Japan) and observed using cooled CCD camera (VB-6010; Keyence). Images were analysed with use of image analysis software (VH Analyzer; Keyence). After evaluating the fluorescence intensity of each endometriotic lesion, we measured its weight by an electronic scale. The fluid contents of cystic lesions were excluded by gentle aspiration with a 26-gauge needle before measuring the weight. We chose weight for assessment of the lesions because it is a convenient and precise measurement.

Assessment of GFP expressing lesions ex vivo
Each endometriotic lesion detected under fluorescence imaging was carefully excised to exclude the surrounding tissue. Removed endometriotic lesions were illuminated with a GFP-lighting system and observed using cooled CCD camera. Images were analysed with use of image analysis software (VH Analyzer). After evaluating the fluorescence intensity of each endometriotic lesion, we measured its weight by an electronic scale. The fluid contents of cystic lesions were excluded by gentle aspiration with a 26-gauge needle before measuring the weight. We chose weight for assessment of the lesions because it is a convenient and precise measurement.

Statistical analysis
Data are expressed as mean ± SEM. Comparison of total fluorescence intensity in control mice and in E2-supplemented mice was performed by a non-parametric Mann–Whitney test. The correlation between the fluorescence intensity and the weight of the lesion was calculated by linear regression analysis.

Results
Macroscopic appearance
Of a total of 26 mice which received endometrial fragments, all mice developed endometriotic lesions. The endometriotic lesions were formed on the peritoneum, the omentum, the perivesical fat tissue, and the intestinal and uterine surface. On gross examination, these lesions consisted of a white, pink or tan multicystic structure. As shown in Figure 1, all lesions that could be detected under gross examination were GFP-positive under fluorescence imaging. Furthermore, under fluorescence imaging, we could detect additional lesions that were unclear on gross examination.

Microscopic appearance
Histologically, all GFP-expressing tissues showed typical endometriotic appearance with endometrial glandular epithelium accompanied by endometrial stroma (Figure 2A, B). Under fluorescence microscopy, glandular epithelium and stroma of the endometriotic lesions expressed GFP regardless of E2 supplementation (Figure 2C, D). In contrast, the surrounding tissues (e.g. peritoneal epithelium, adipose tissue, etc.) did not express GFP. The boundary between stroma of endometriotic lesions derived from donor mice and stromal tissues of recipient mice was clear in merged image (Figure 2D), whereas it was ambiguous in haematoxylin and eosin staining (Figure 2A).

Correlation between fluorescence intensity and weight of endometriotic lesions
All endometriotic lesions were evaluated from all mice. Figure 3 shows correlation between fluorescence intensity and weight of lesions in five control mice and six E2-supplemented mice. The number of the lesions per mouse was significantly lower in the control mice (2.2 ± 0.4,
mean \pm SEM) compared with the E2-supplemented mice (5.0 \pm 0.5). There is a strong positive correlation ($R^2 = 0.844, P < 0.0001$) between measured fluorescence intensity and weight of the lesions. The weight per lesion was 1.68 ± 1.44 mg (mean ± SD) in the control mice and 7.80 ± 5.70 mg in the E2-supplemented mice. The relative fluorescence intensity per lesion was 407 ± 450 (mean ± SD) in the control mice and 1370 ± 857 in the E2-supplemented mice.

Quantification of fluorescence intensity of lesions in mice

The fluorescence intensity of endometriotic lesions in mice was quantified with the use of image analysis software. As shown in Figure 4, the total fluorescence intensity, measured 2 weeks after inoculation of endometrium, was significantly higher ($P = 0.0062$) in E2-supplemented mice than that in control mice. Three-dimensional histograms (Figure 1D, H) show GFP expressing area in $xy$-plane and fluorescence intensity in $z$-direction.

Figure 1. Macroscopic assessment of green fluorescent protein (GFP)-expressing lesions in recipient mice. Upper panels (A–D) show the peritoneal cavity of control mouse, and lower panels (E–H) show that of estradiol (E2)-supplemented mouse. Gross findings of ectopic endometrial implants in control mouse (A) or E2-supplemented mouse (E). Corresponding images under a GFP-lighting system and a cooled CCD camera are shown in B and F. Merged images are shown in C and G. Three-dimensional histograms (D and H) show GFP expressing area in $xy$-plane and fluorescence intensity in $z$-direction.

Figure 2. Histological aspects of a green fluorescent protein (GFP)-expressing lesion on the abdominal wall of recipient mice. (A) Haematoxylin and eosin staining of the lesion. (B) Hoechst 33258 staining (blue), demonstrating cellular nucleus. (C) GFP-positive cells (green) detected under fluorescence microscopy. (D) Merged image of B and C. Original magnification: \( \times 40 \).
In the present study, we presented a new mouse model of endometriosis using GFP technology. This model has several advantages as compared to traditional homologous models. First, endometriotic lesions can be detected in a more sensitive manner. Small endometriotic lesions are sometimes difficult to be identified by macroscopic examination. As presented in this study, GFP-emitted light, which can be detected easily in dark field, indicates the location of the lesions.

Second, the level of fluorescence of endometriotic lesions enables semi-quantification of their size. According to the finding that the weight of endometriotic lesions was positively correlated with the measured fluorescence intensity with high coefficient, the measured fluorescence intensity could be an alternative to the weight in quantification of the lesions. This computer-based measurement system would be useful for speedy evaluation in a drug screening-type experiment. Indeed, in accordance with estrogen-dependency of endometriosis, the present study showed that the fluorescence was significantly higher in the E2-supplemented mice as compared to the control mice.

Third, a remarkable advantage of this model is that endometriotic cells originating from donor mice are clearly identified in histological sections. In contrast to haematoxylin and eosin staining, in which it seemed difficult to discriminate endometriotic stromal cells from surrounding stromal cells of the host animal, GFP fluorescence clearly visualized endometriotic cells. Precise discrimination of endometriotic cells from other cells may have significant implications for the analysis of endometriotic tissues in animal models. As immunoinflammatory reactions and angiogenesis are suggested to play essential roles in the development of endometriotic lesions (Osga et al., 1999; Kyama et al., 2003; Yoshino et al., 2003), interactions among immune cells, endothelial cells, and endometriotic cells are major topics of endometriosis research. Further studies using the present model are expected to identify the origins of various cells in endometriotic lesions.

Recently, a heterologous mouse model was reported, in which human endometrial fragment transduced with GFP complementary DNA was implanted in nude mouse (Fortin et al., 2003, 2004). These studies, like ours, argue for various advantages of the use of GFP technology. However, as the gene transduction into human endometrial fragment was transient, the fluorescence of endometrial tissue faded out in a few weeks. In addition, due to the limited and variable efficiency of transduction, the model seems not to be suitable for the fluorescence-dependent identification in histological settings. Accordingly, our model may have some benefits in that GFP expression is permanent in cells derived from GFP mouse.

Finally, we admit the limitations of our mouse model because of its innate characteristics as a homologous model, mainly the use of non-human endometrial tissue. In addition, when test drugs for endometriosis are evaluated using fluorescent intensity as a marker in this model, an as-yet-unknown interaction between GFP and the drugs might interfere with the study. Nevertheless, in view of numerous contributions of homologous models to the study of endometriosis, we believe that our mouse model extends the possibilities of the animal model of endometriosis.

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References


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