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Probucol, a Hypocholesterolemic Agent, Prevents the Development of Uterine Adenomyosis Induced by Pituitary Grafting in Mice

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Abstract. *This in vivo experimental study was designed to investigate the effects of probucol, a hypocholesterolemic agent, on uterine adenomyosis which is frequently induced by pituitary grafting in mice. SHN mice, which are known to develop uterine adenomyosis spontaneously, and much sooner after pituitary grafting, were used and histopathological study on the uteri in pituitary gland-implanted mice with or without probucol treatment was performed. Four out of 10 pituitary gland-implanted mice developed uterine adenomyosis with dilated blood vessels, but none of the probucol-treated mice. There were no differences between pituitary-grafted mice with or without probucol treatment in body weight and wet weights of uterus, ovaries, kidney and liver except spleen. Probucol markedly reduced the serum levels of total cholesterol, free cholesterol, free fatty acids, phospholipids and triglycerides and, thus, this agent inhibited the incidence of uterine adenomyosis induced by pituitary grafting in mice.*

The SHN strain of mice is known to develop uterine adenomyosis spontaneously and, furthermore, very soon after pituitary grafting (1). It was suggested that an early stage in the development of uterine adenomyosis was the marked invasion by stromal fibroblasts of the myometrium along the branches of blood vessels (2). As previously reported (3), we demonstrated, using von Willebrand factor (vWF), a broad-based endothelial cell marker, that pituitary grafting increased the number of dilated venules with alteration of microvessels in the mouse uterus, suggesting that vascular changes are an important etiological factor in the development of adenomyosis. Probucol, 4,4'-isopropylidene-dithiobis[2,6-di-*t*-butylphenol], is a hypocholesterolemic agent effective in animals and humans (4). This agent is transported and incorporated into endothelial cell membranes to act as a radical-trapping antioxidant, protecting the endothelial cells against oxidative stress (5), and lowering the incidence of ischemic heart disease (6).

In the present study, in an attempt to establish whether this agent can inhibit the development of uterine adenomyosis, we investigated the effects of probucol on serum levels of lipids and uteri in mice that received pituitary grafting.

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Key Words: Hypocholesterolemic agent, probucol, uterine adenomyosis, pituitary-grafted mice.

Materials and Methods

Animals and experimental procedures. Virgin female SHN mice, maintained at the Medical Research Institute, Tokyo Medical and Dental University, Japan, were used in the present study. They were housed in plastic cages with wood shavings under controlled conditions (24 ± 0.5˚C and 12 h of light from 0600 to 1800 h), in accordance with the principles outlined in the Guide for Animal Care and Use of the Committee of Tokyo Medical and Dental University. All mice had free access to a commercial diet (CE-7, CLEA Japan, Tokyo, Japan) and tap water in the animal room of the University. All experimental procedures conformed to the regulations described in the Guide to the Care and Use of Laboratory Animals of the U. S. National Institute of Health (NIH).

Twenty mice were implanted with a single pituitary gland each, under the capsule of the right kidney at 7 weeks of age and then were divided randomly into 2 groups of 10 animals each. One day after the pituitary grafting, 10 mice were subcutaneously injected with probucol (1.0 mg/ 30 g of body weight; a gift from Otsuka Pharmaceuticals, Tokyo, Japan) dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd, Osaka, Japan) twice a week for 6 weeks at a

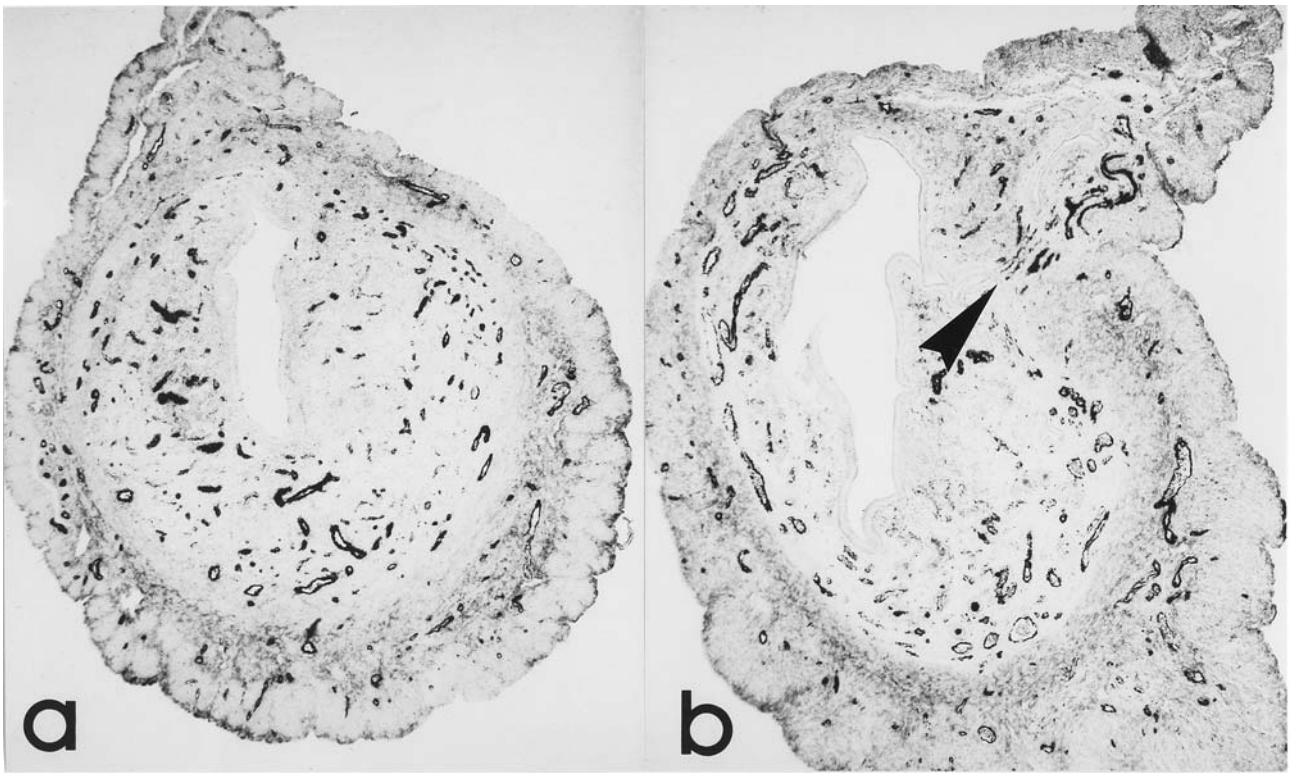

Figure 1. *Immunohistochemical staining using von Willebrand factor (vWF) in the uteri of SHN mice. Uterine sections from pituitary-grafted mice (b: PG-control group) demonstrate increased numbers of dilated venules in the endometrium and mild adenomyosis near the mesometrium (arrowhead indicates a focus of adenomyosis) compared with the untreated control mice (a: Normal-control group), i.e. numerous small microvessels in the endometrium. Uteri of probucol-treated mice (PG-probucol group) show no-sign of development of adenomyosis. (Original magnification x 72).*

different skin site every time (PG-probucol group). The remaining 10 mice were injected with the same dosage of DMSO (50 μl) as a control vehicle, according to the same procedure (PG-control group). Food intake and body growth were checked weekly throughout the experiment. All mice were killed at 13 weeks of age by decapitation under light ether anesthesia, after the estrous cycle had been checked by vaginal smear. Blood was collected for measuring the serum levels of lipids in each mouse, and the obtained sera were commercially measured afterwards (SRL, Inc., Tokyo, Japan).

At autopsy, removed uteri, ovaries, liver, spleen and kidneys were weighed. The obtained uteri were fixed in neutral buffered 10% formalin solution (pH 7.4), dehydrated and embedded in paraffin wax. The sections of uterus (5 μm in thickness) were prepared for immunohistochemical staining of von Willebrand factor (vWF), using the alkaline phosphatase avidin-biotin-complex (ABC-AP) method as reported (3).

Statistical analyses. All parameters were expressed as the mean±SEM. Statistical analysis was performed using the unpaired *t*-test for body and organ weights and uterine blood vessels, and Fisher's exact probability test for the incidence of uterine adenomyosis. A p value less than 5% was considered statistically significant.

Results

Immunohistochemical staining of vWF in the uteri of mice. Markedly dilated venules in the endometrium and mild adenomyosis near the mesometrium were frequently found in mice with pituitary grafting (PG-control group) (Figure 1b), compared with untreated mice (Normal-control group) (Figure 1a).

Body weight, organ weights, estrous cycle and incidence of uterine adenomyosis. The body weight of the mice and the weights of uterus, ovary and liver except spleen ($p<0.05$) in the probucol group differed little from those in the PG-control group (Table I). All mice showed diestrous vaginal smears just before autopsy. Four mice (40%) in the PG-control group, but none in the PG-probucol group, developed early signs of uterine adenomyosis ($p<0.01$) (Figure 1b) (Table I).

Serum levels of lipids. Serum levels of total cholesterol, free cholesterol, free fatty acids, phospholipids and triglycerides

Table I. *Body and organ weights in each group, and incidence of uterine adenomyosis between groups.*

	PG-control	PG-probucol
Final body weight (g)	27.7 ± 0.6	27.8 ± 0.5
Liver (mg/g B.Wt.)	46.3 ± 2.0	47.4 ± 1.2
Spleen (mg/g B.Wt.)	2.95 ± 0.43	4.16 ± 0.33*
Kidney (mg/g B.Wt.)	9.00 ± 0.33	9.61 ± 0.23
Ovaries (mg/g B.Wt.)	0.44 ± 0.02	0.45 ± 0.04
Uterus (mg/g B.Wt.)	2.31 ± 0.20	2.22 ± 0.19
Incidence of uterine adenomyosis	4/10	0/10**

PG: pituitary-grafted, probucol: probucol-treated (Refer to M & M).
B.Wt.: body weight.
Data are the mean ± SEM. $^{**}p<0.01$, $^{*}p<0.05$.

Table II. *Serum levels of lipids in each group.*

	PG-control	PG-probucol
Total cholesterol (mg/dl)	47.0 ± 2.6	33.5 ± 5.3**
Free cholesterol (mg/dl)	10.1 ± 0.7	7.8 ± 0.5*
Free fatty acids (mEQ/l)	0.65 ± 0.05	0.46 ± 0.05*
Phospholipids (mg/dl)	80.0 ± 4.0	62.2 ± 6.2*
Triglycerides (mg/dl)	18.5 ± 1.6	9.6 ± 0.8**

PG: pituitary-grafted, probucol: probucol-treated (Refer M & M).

Data are the mean ± SEM. $^{**}p<0.01$, $^{*}p<0.05$.

were markedly lowered in the PG-probucol group compared to those in the PG-control group ($p<0.01$ and 0.05) (Table II).

Discussion

Pregnancy, delivery, deep curettage of the uterus and cesarean section are the main risk factors for uterine adenomyosis (7). It is of note that marked vascularization and/or weakness of the myometrium always accompany these risk factors. It has been reported that, in patients with uterine adenomyosis, the degree of endometrial vascularization was markedly increased and dilated microvessels were frequently seen in adenomyosis (8). As the main blood vessels reach the uterine body through the mesometrium, the vascular network near the mesometrium is extensive (3). Furthermore, we found that the endometrial tissues penetrating the muscular layers were always accompanied by blood vessels. In cases when blood vessels could not be found, the ectopic endometrial tissues generally penetrated in the direction of the mesometrium (3). These findings indicate that uterine blood vessels provide favorable avenues for the development of adenomyosis in mice. Mori and Nagasawa (2) described the early signs of adenomyosis in detail, *i.e.* at first, the appearance of highly-developed and markedly-dilated blood vessels running straight across the inner myometrium, followed by the invasion of stromal fibroblasts into the myometrium along the branches of blood vessels. Our previous study using immunohistochemical vWF quantitatively confirmed that the uterine microvessels increased in diameter after pituitary grafting (3). Pituitary grafting results in hyperprolactinemia, associated with the early development of uterine adenomyosis in mice. However, in the absence of estrogen and progesterone, prolactin can not induce adenomyosis, since estrogen and progesterone are essential hormones for the growth and proliferation of uterine tissues (9). Subcutaneous implantation of progesterone enhanced the development of uterine adenomyosis in intact mice (10). Huseby *et al.* (11) stated that a hormonal imbalance involving high levels of circulating progesterone is a primary factor in the pathogenesis of adenomyosis. As previously reported (12), a potent antiprogestin, mifepristone (RU486), markedly reduced the incidence of uterine adenomyosis along with a decreased number of dilated blood vessels in mouse endometrium and myometrium. Thus, the dilated venules provide a convenient channel for the endometrial stromal cells, and the increase in cell death in the inner muscle layer facilitates this invasive process (13). Probucol is a potent hypocholesterolemic agent effective in animals and humans (4). This agent is transported and incorporated into endothelial cell membranes to act as a radical-trapping antioxidant, protecting the endothelial cells against oxidative stress (5). In the present study, treatment with probucol markedly lowered the serum levels of lipids resulting in the reduction of oxidative stress on the endothelial cells of blood vessels in the endometrium and myometrium, and then suppressed the incidence of uterine adenomyosis in pituitary-grafted mice compared with the control.

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