G72 (against 6-sulfo Lewis X, 6-sulfo *N*-acetyllactosamine, and 6-sulfo sialyl Lewis X, respectively) were prepared as described previously [13,14].

Preparation of soluble recombinant GlcNAc6ST-1, -2, -3, -4, and -5

The cDNAs encoding the lumenal domains of GlcNAc6ST-1 (amino acids 28-483), -2 (28-386), -3 (28-390), -4 (33-486), and -5 (27-395) [9] were prepared from the full length cDNAs generated previously [8] by polymerase chain reaction using gene-specific primers. The amplified cDNAs with the correct sequence were ligated into pQE-9-EK, which was generated by inserting the enterokinase cleavage site (DDDDK) into pQE-9 (QIAGEN) by using the QuikChange Site-Directed Mutagenesis Kit(STRATAGENE) and the oligonucleotide 5′-CCATCACCATCACGATGACGATGACAAAGGATCCG-TCGACC-3′ along with its complementary oligonucleotide. The *E. coli* M15 strain was then transformed with each of the resulting expression vectors. Recombinant soluble proteins were produced and purified using Ni-NTA agarose (QIAGEN) according to the manufacturer's instructions.

Immunohistochemical analysis

Immunohistochemistry was performed with the immunoperoxidase method using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Wako Pure Chemical, Osaka, Japan). The paraffin-embedded tissue sections were deparaffinized in xylene and then rehydrated in a graded ethanol series. Endogenous peroxidases in all tissues were quenched with 0.3% hydrogen peroxide in methanol for 30 min at room temperature, after which the tissue sections were washed in 10 mM Tris-buffered saline (TBS, pH 7.4). The tissue sections were then blocked in 1% bovine serum albumin (BSA) in TBS for 1 h, followed by incubation overnight at 4°C with antibodies or normal rabbit serum diluted in TBS containing 0.1% BSA (1:500 dilution for the anti-GlcNAc6ST-2 antibody; 1:8 dilution for the AG107, AG223, and G72 antibodies; 1:500 dilution for normal rabbit serum). The sections were washed in TBS three times, incubated with biotinylated anti-mouse or anti-rabbit immunoglobulins for 10 min, washed, and then incubated with HRP-conjugated streptavidin for 15 min (DAKO LSAB2 System, DAKO Cytomation, Denmark). Finally, the sections were incubated with 0.1% DAB and 0.01% hydrogen peroxide in TBS for 10–15 min. After being rinsed in distilled water, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted with Malinol (Muto Pure Chemical, Japan).

Detection of GlcNAc6ST-1, -2, - 3, -4 and -5 transcripts by RT-PCR

Total RNAs were isolated from ovarian tumor tissues by using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. cDNAs were synthesized from 1 μg of total RNA in a total volume of 20 μl of reaction mixture using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) primers. After cDNA synthesis, the reaction mixtures were diluted 5-fold with H_2O and 1–3 μl of each was used as a template for each PCR procedure. To normalize the mRNA quantities according to the β-actin mRNA levels, competitive PCR was performed using Gene Taq NT polymerase (Nippon Gene, Japan) in a total volume of 12 μl of reaction buffer containing 1 pg competitor plasmid DNA and 250 nM β-actin–specific primers. The competitor plasmid DNA was prepared previously [8]. PCR was performed under the following conditions: 25 cycles consisting of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, and elongation at 72°C for 1 min. After PCR, an 8-μl aliquot was electrophoresed in a 0.9% agarose gel, followed by staining with ethidium bromide. The intensities of the amplified fragments were quantified by using an FLA-2000 multi-imager (Fuji Photo Film, Japan) and NIH Image software. The template volumes that yielded equivalent β-actin transcript levels were determined by acquiring the band intensity ratio of target and competitor DNAs; these volumes were used in the following procedures. PCR was performed with the determined template volume and the respective GlcNAc6ST-1, -2, -3, -4 and -5–specific primers [8], under the same conditions as for β-actin except that 33 cycles were performed.

Results

Immunohistochemical analysis of GlcNAc6ST-2 expression in ovarian mucinous adenocarcinoma tissues

We previously demonstrated that GlcNAc6ST-2 gene transcripts are ectopically expressed in colonic mucinous adenocarcinomas. To elucidate whether GlcNAc6ST-2 is also ectopically expressed in the mucin-producing carcinomas of various other organs, we raised a polyclonal antibody that recognizes the catalytic domain of GlcNAc6ST-2. To assess whether the polyclonal antibody specifically recognizes GlcNAc6ST-2 protein or not, we performed the Western blot analysis (Fig. 1). In SDS-PAGE analysis, recombinant GlcNAc6STs were detected at positions of the respective molecular sizes by SYPRO Orange staining. The polyclonal antibody recognized only GlcNAc6ST-2 protein, indicating the specificity of the antibody for GlcNAc6ST-2.

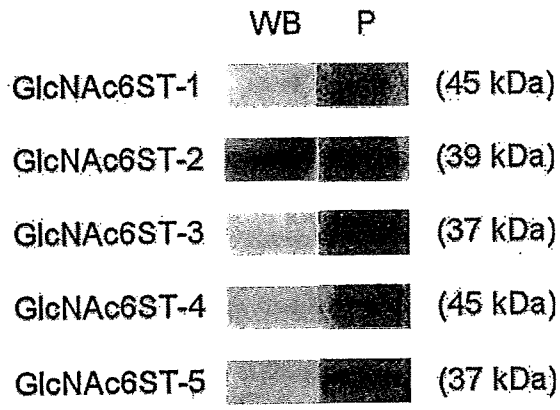

Fig. 1 Confirmation of specificity of anti-GlcNAc6ST-2 polyclonal antibody raised in this study by Western blot analysis using recombinant GlcNAc6STs. WB, Western blot analysis detected by anti-GlcNAc6ST-2 polyclonal antibody; P, Ponceau 3R stain of correspondent bands for five GlcNAc6ST isozymes.

To further confirm this result, we performed immunostaining of normal colonic mucosa and adenocarcinomas (Fig. 2). Our previous results [8,15] showed that GlcNAc6ST-2 is ectopically expressed in colonic mucinous adenocarcinomas, but not in normal coloninc mucosa and non-mucinous adenocarcinomas, using enzymological and RT-PCR methods. As shown in Fig. 2, normal colonic mucosa and non-mucinous adenocarcinomas were not immunostained by the polyclonal antibody, while mucinous adenocarcinomas were strongly immunostained, in good agreement with our previous results. Considering previous reports showing that GlcNAc6STs-1 and -3 are expressed in normal colonic mucosa [16,17], it is supported that the polyclonal antibody does not recognize at least GlcNAc6ST-1 and -3 in immunohistochemical staining. These results also support that the polyclonal anti-GlcNAc6ST-2 antibody is specific for the GlcNAc6ST-2 protein and can be applicable to immunohistochemical analyses.

We then used the anti-GlcNAc6ST-2 antibody to determine whether ovarian mucinous adenocarcinomas also express GlcNAc6ST-2. For this purpose, we tested 10 surgically obtained primary mucinous adenocarcinoma tissues. As shown in Fig. 3A and Table 1, all of the malignant ovarian mucinous adenocarcinoma tissues strongly expressed GlcNAc6ST-2, while none of the five benign mucinous adenoma cases we tested did so (Fig. 3B). GlcNAc6ST-2 was expressed in the mucin-producing cell layers in mucinous adenocarcinoma tissues, but not in interstitial cells.

RT-PCR analysis of GlcNAc6ST-2 transcripts in ovarian mucinous adenocarcinoma tissues

To confirm that GlcNAc6ST-2 is expressed in malignant ovarian mucinous adenocarcinomas but not in benign adenomas, we performed RT-PCR using RNA samples extracted from the surgically obtained tissues (three mucinous adenocarcinomas and two benign adenomas). To normalize the amounts of each cDNA, competitive PCR with β-actin cDNA was performed prior to PCR for detecting GlcNAc6ST-2 transcripts. The GlcNAc6ST-2 transcript was strongly or moderately expressed in the mucinous adenocarcinomas tested but not expressed at all in the mucinous adenomas (Fig. 4). In contrast, all mucinous adenoma and adenocarcinoma cases expressed GlcNAc6ST-1 transcripts while none expressed GlcNAc6ST-3, -4 or -5 transcripts (data not shown).

Immunohistochemical analysis of GlcNAc6ST-2 expression in other types of ovarian adenocarcinomas

To elucidate whether GlcNAc6ST-2 is expressed by other histological types of ovarian adenocarcinomas, we subjected surgically obtained primary serous, endometrioid, and clear cell adenocarcinoma samples to immunohistochemical analysis with the anti-GlcNAc6ST-2 polyclonal antibody. As shown in Table 1, 10 of 14 (71%) clear cell carcinomas and all six (100%) papillary serous carcinomas expressed GlcNAc6ST-2 at high frequency (Fig. 3C and D, respectively). Clear pools observed in clear cell adenocarcinomas were not stained while confined cytoplasms of the tumor cells were strongly stained. With respect to papillary serous

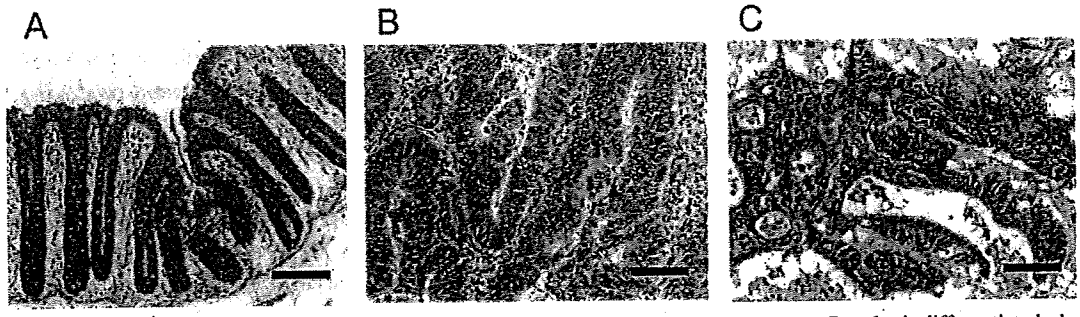

Fig. 2 Immunohistochemical staining for GlcNAc6ST-2 in colon tissue sections. A, normal colon mucosa; B, colonic differentiated adenocarcinoma; C, colonic mucinous adenocarcinoma. (Scale bars: A, 500 μm; B and C, 100 μm).

Table 1 Summary of immunohistochemical staining of GlcNAc6ST-2 in ovarian tumor tissue sections.

Histological classification	No. of cases with positive GlcNAc6ST-2 staining/total cases	Rate of GlcNAc6ST-2-positive staining
Mucinous adenoma	0/5	0%
Mucinous adenocarcinoma	10/10	100%
Clear cell adenocarcinoma	10/14	71%
Serous adenocarcinoma		
Papillary growth	6/6	100%
Solid growth	1/14	7%
Endometrioid adenocarcinoma	4/17	24%

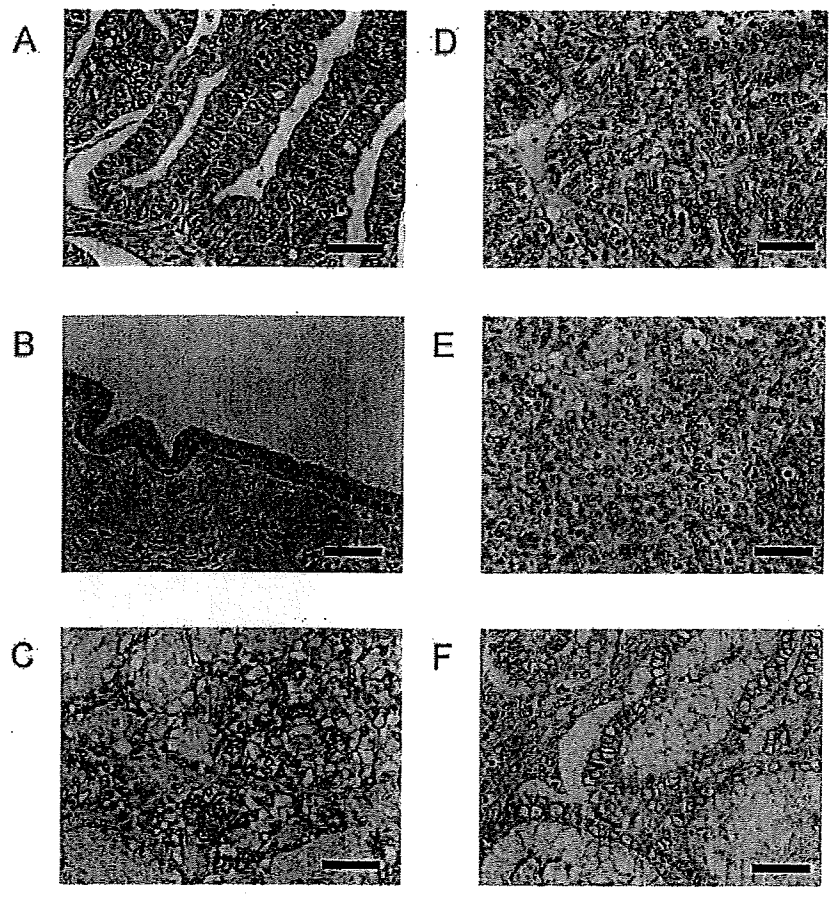

Fig. 3 Immunohistochemical staining for GlcNAc6ST-2 in ovarian tissue sections. A, mucinous adenocarcinoma; B, mucinous adenoma; C, clear cell adenocarcinoma; D, papillary serous adenocarcinoma; E, solid serous adenocarcinoma; F, endometrioid adenocarcinoma. (Scale bars, 50 μm).

carcinomas, most of the tumor cells expressed GlcNAc6ST-2. In contrast, only one of 14 (7%) solid serous carcinomas and four of 17 (24%) endometrioid carcinomas expressed GlcNAc6ST-2 (Fig. 3E and F, respectively). This indicates that the GlcNAc6ST-2 protein is expressed at quite different frequencies by ovarian cancer cells of diverse histological classifications. Thus, most or all of the ovarian mucinous, clear cell, and papillary serous adenocarcinomas ectopically expressed GlcNAc6ST-2.

It should be noted that chemotherapy-resistant types of ovarian adenocarcinomas exhibit frequent expression of GlcNAc6ST-2, while chemotherapy-sensitive types exhibit quite lower frequency of GlcNAc6ST-2 expression. It remains unclear whether or not there is molecular relationship between chemotherapy sensitivity and GlcNAc6ST-2 expression, but GlcNAc6ST-2 could be a tumor marker for chemotherapy-resistant types of ovarian adenocarcinomas as discussed later.

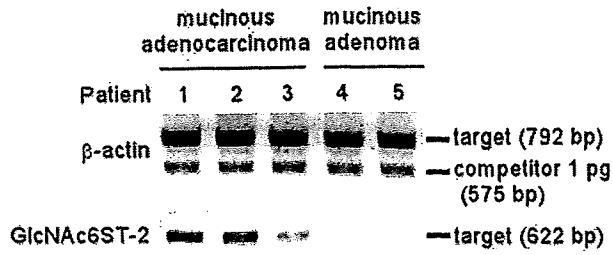

Fig. 4 Analysis of GlcNAc6ST-2 transcripts in mucinous adenocarcinomas and mucinous adenomas. Total RNAs were isolated from tissue blocks obtained from surgical operations and cDNAs were obtained by reverse transcription. The relative amount of cDNA of each sample was estimated by competitive PCR with 1 pg of β-actin–specific competitor DNA.

Detection of sulfated glycans in GlcNAc6ST-2-expressing ovarian adenocarcinomas by immunohistochemical analysis

To assess whether sulfated carbohydrate epitopes, which are the enzymatic products of GlcNAc6ST-2, are present in GlcNAc6ST-2-expressing ovarian adenocarcinoma tissues, we subjected one sample each of mucinous adenocarcinoma, mucinous adenoma, clear cell adenocarcinoma, papillary serous adenocarcinoma, solid serous adenocarcinoma, and endometrioid adenocarcinoma to immunohistochemical analyses using monoclonal antibodies specific for either 6-sulfo LacNAc (AG107), 6-sulfo Lewis X (AG223), or 6-sulfo sialyl Lewis X (G72). The three L-selectin ligand-related sulfated glycans examined are synthesized as follows. GlcNAc6ST-2 catalyzes the 6-*O*-sulfation of non-reducing terminal GlcNAc residues, followed by β1,4-galactosylation and α1,3-fucosylation of 6-sulfo GlcNAc, which generate 6-sulfo LacNAc and 6-sulfo Lewis X. The 6-sulfo sialyl Lewis X, an L-selectin ligand, is subsequently formed by the α2,3-sialylation and α1,3-fucosylation of 6-sulfo LacNAc. Thus, the 6-*O*-sulfation of GlcNAc by GlcNAc6ST-2 is the first step in the biosynthesis of all three sulfated glycans.

As shown in Fig. 5A and C, mucinous adenocarcinoma tissues expressed the 6-sulfo LacNAc and 6-sulfo Lewis X epitopes, as detected by mAb AG107 and mAb AG223, respectively. In contrast, the mucinous adenoma tissues did not express the carbohydrate epitopes recognized by mAb AG107 (Fig. 5B) or mAb AG223 (data not shown). The 6-sulfo sialyl Lewis X epitope detected by mAb G72 was also present in mucinous adenocarcinoma tissues (data not

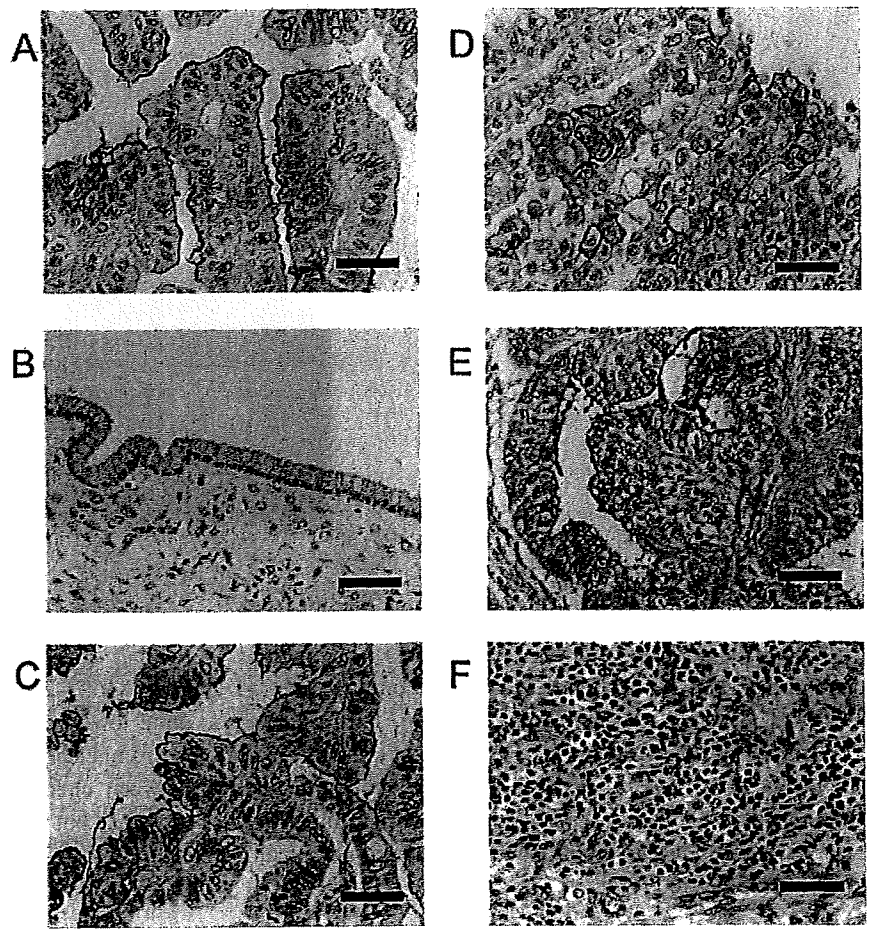

Fig. 5 Immunohistochemical staining with antibodies specific for 6-sulfo-LacNAc (AG107; A, B, D-F) or 6-sulfo-Lewis X (AG223; C). A and C, mucinous adenocarcinoma; B, mucinous adenoma; D, clear cell adenocarcinoma; E, papillary serous adenocarcinoma; F, solid serous adenocarcinoma. (Scale bars, 50 μm).

shown). In addition, the clear cell adenocarcinoma and papillary serous adenocarcinoma tissues expressed the carbohydrate epitopes recognized by mAb AG107 (Fig. 5D and E, respectively), while the solid serous and endometrioid adenocarcinomas did not (Fig. 5 and data not shown, respectively). These results suggest that the ectopic expression of GlcNAc6ST-2 by ovarian adenocarcinoma tissues correlates with their expression of 6-sulfo GlcNAc–containing epitopes on their cell surface membranes.

Discussion

We found in our previous study that GlcNAc6ST-2 transcripts and its enzymatic activities are present in colonic mucinous adenocarcinomas but not in non-mucinous adenocarcinomas or normal colonic mucosa [8]. Here, we examined the expression of GlcNAc6ST-2 by ovarian carcinomas as a first step to investigate whether GlcNAc6ST-2 is universally expressed by mucinous adenocarcinomas regardless of the organ of origin. We clearly demonstrated in this paper that ovarian mucinous adenocarcinoma tissues ectopically express GlcNAc6ST-2 transcripts, proteins and its carbohydrate products while mucinous benign adenomas do not. Furthermore, we showed that ovarian clear cell and papillary serous adenocarcinomas have a high incidence of GlcNAc6ST-2 expression, while solid serous adenocarcinomas and endometrioid adenocarcinomas both have a low incidence. Mucinous adenocarcinomas, clear cell adenocarcinomas and papillary serous adenocarcinomas account for over 50% of the total incidence of ovarian epithelial adenocarcinomas and are resistant to anticancer reagents like *cis*-platinum, resulting in a poor prognosis [1–3]. It remains unclear why these ovarian adenocarcinomas are resistant to these reagents, but one possibility is that the large quantity of sulfated mucins in mucinous adenocarcinomas and the accumulation of viscous materials in clear cell adenocarcinomas either might prevent the reagents from accessing their target molecules or otherwise interfere with their action. It would therefore be of considerable interest to determine whether the ectopic expression of GlcNAc6ST-2 in ovarian adenocarcinoma tissues is involved in their resistance to *cis*-platinum–based chemotherapy.

We also demonstrated that sulfated glycans, including L-selectin ligands, are expressed by ovarian mucinous adenocarcinomas. To our knowledge, this is the first report showing that ovarian mucinous adenocarcinomas express 6-sulfo-lactosamine-related antigens. Notably, Federici *et al.* have shown that ovarian mucinous adenocarcinomas express sialyl-Tn (sTn), Lewis a, and sialyl Lewis a antigens more frequently than serous and endometrioid adenocarcinomas [18]. In addition, Tamada and coworkers showed that cell lines derived from ovarian mucinous and clear cell adenocarcinomas express sulfated Lewis a, sialyl Lewis X and sialylated MUC1 antigen more strongly than those derived from serous adenocarcinomas [19]. Thus, it is thought that most mucinous adenocarcinomas have a tendency to express mucin-related carbohydrate antigens, including 6-sulfo-sialyl Lewis X antigen. Since it has been reported that glycans containing Lewis X–related structures are involved in the metastasis of cancer cells [20–23], we are thus interested in the potential role that the GlcNAc6ST-2-synthesized L-selectin ligands or related glycans play in ovarian adenocarcinoma metastasis or dissemination.

It remains unclear how GlcNAc6ST-2 transcripts are ectopically expressed in ovarian mucinous, clear cell and papillary serous adenocarcinomas. The ectopic expression suggests aberrant deregulation of GlcNAc6ST-2 gene transcription. The K-ras gene is known to be frequently mutated in colorectal and ovarian mucinous adenocarcinomas, whereas p53 is rarely mutated [24–26]. It has also been reported that serous adenocarcinomas showing micropapillary architecture commonly have a mutated K-ras gene and respond poorly to chemotherapy [3]. Interestingly, we here found that serous adenocarcinomas showing papillary architecture frequently express GlcNAc6ST-2. These observations together suggest that the ectopic expression of GlcNAc6ST-2 by ovarian adenocarcinomas, including papillary serous adenocarcinomas, may correlate with both their poor response to chemotherapy and the presence of K-ras gene mutations.

At present, CA125 is the most commonly used antigen for the serodiagnosis of ovarian cancers and its usefulness in this respect has been proven. However, only 50–60% of patients with stage I ovarian cancer are positive for the serum CA125 test [7]. Moreover, the substantial pseudo-positives associated with this test remain a problem for the early detection of ovarian cancer. Of the cases studied in this report, 70% of the patients with mucinous adenocarcinomas (60% of patients at stage I) and 40% of patients with mucinous adenomas had a CA125 serum level of over 30 units/ml. In addition, an immunohistochemical analysis showed that only 50% of mucinous adenocarcinomas express sTn (data not shown), another tumor marker for ovarian mucinous adenocarcinomas [27]. Thus, GlcNAc6ST-2, which is expressed in 100% of the mucinous adenocarcinomas and none of the mucinous adenomas, may be a better ovarian tumor marker than CA125 and sTn. Interestingly, in the clear cell adenocarcinomas cases, GlcNAc6ST-2 was expressed in all the Stage I cases (six out of 6) but in none of the Stage III cases (zero out of 3). This suggests that only the early stages of clear cell adenocarcinomas may express GlcNAc6ST-2, unlike mucinous adenocarcinomas, which seem to express GlcNAc6ST-2 regardless of the tumor stage (seven cases in stage I and three cases in stage III).

In this report, we demonstrated that GlcNAc6ST-2 is ectopically expressed by not only colonic mucinous

adenocarcinomas but also ovarian mucinous adenocarcinomas. Investigation of the mucinous carcinomas derived from other organs will be required to determine whether ectopic expression of GlcNAc6ST-2 is a common characteristic of all mucinous carcinomas. Nevertheless, our results indicate that GlcNAc6ST-2 may be a useful clinical marker for ovarian mucinous, papillary serous, and clear cell adenocarcinomas.

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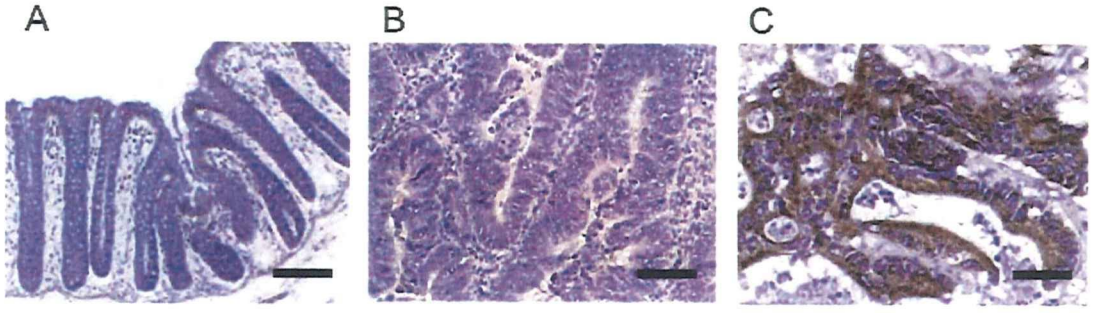

Fig. 2 Immunohistochemical staining for GlcNAc6ST-2 in colon tissue sections. A, normal colon mucosa; B, colonic differentiated adenocarcinoma; C, colonic mucinous adenocarcinoma. (Scale bars: A, 500 μm; B and C, 100 μm).

Fig. 3

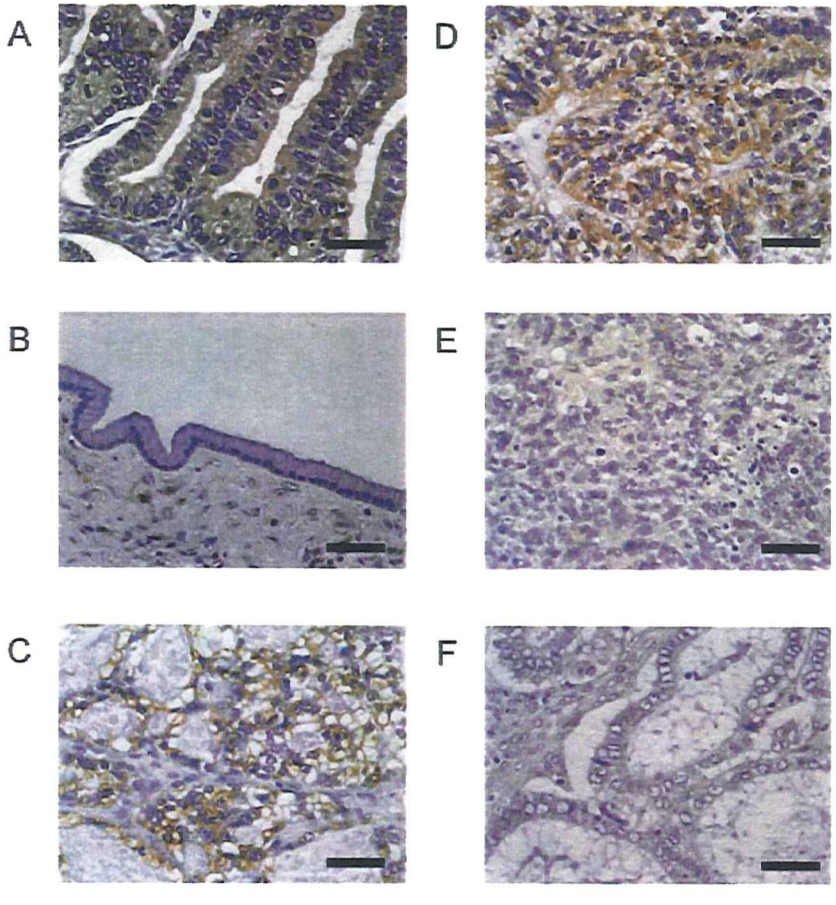

Fig. 5

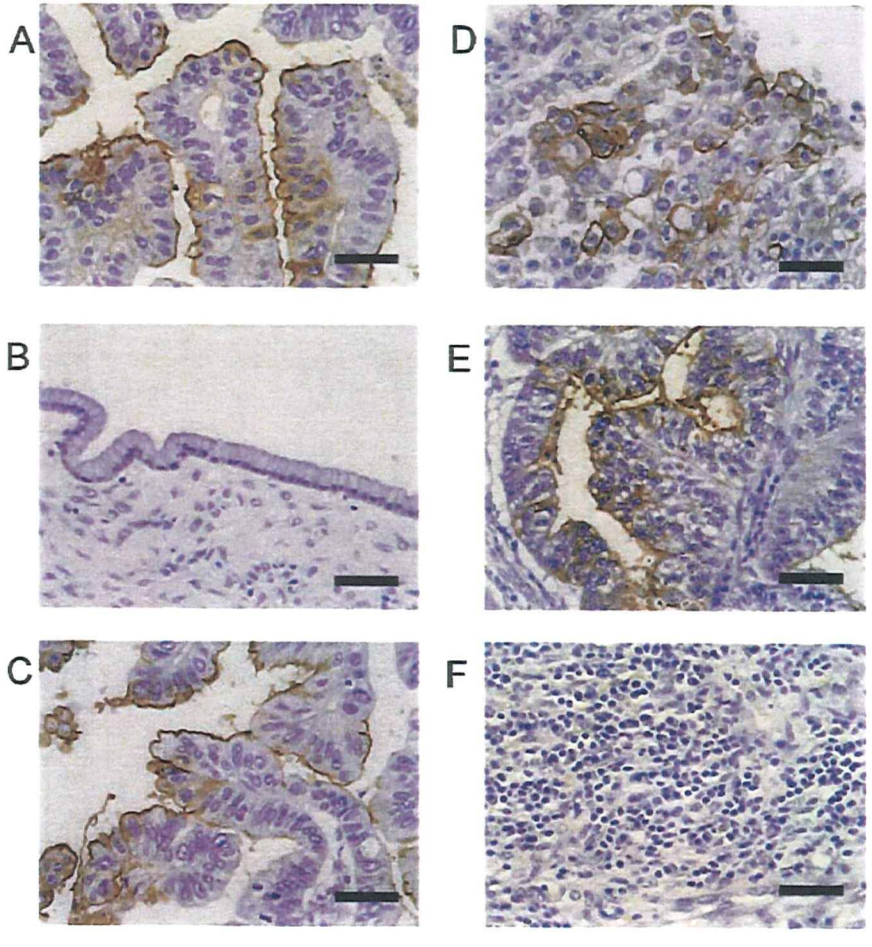

Fig. 3 Immunohistochemical staining for GlcNAc6ST-2 in ovarian tissue sections. A, mucinous adenocarcinoma; B, mucinous adenoma; C, clear cell adenocarcinoma; D, papillary serous adenocarcinoma; E, solid serous adenocarcinoma; F, endometrioid adenocarcinoma. (Scale bars, 50 μm).

Fig. 5 Immunohistochemical staining with antibodies specific for 6-sulfo-LacNAc (AG107; A, B, D-F) or 6-sulfo-Lewis X (AG223; C). A and C, mucinous adenocarcinoma; B, mucinous adenoma; D, clear cell adenocarcinoma; E, papillary serous adenocarcinoma; F, solid serous adenocarcinoma. (Scale bars, 50 μm).

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—Original—

Reduction of Primordial Follicles Caused by Maternal Treatment with Busulfan Promotes Endometrial Adenocarcinoma Development in Donryu Rats

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Abstract. Ovarian dysfunction leading to hormonal imbalance plays a crucial role in uterine carcinogenesis in rats as well as women. However, the effects of a reduction in primordial follicles at birth on uterine adenocarcinoma development have hitherto not been determined. The present study was therefore conducted using female Donryu rats, a high incidence rat strain of uterine adenocarcinoma. The animals were maternally exposed to 2.5 or 5.0 mg/kg of busulfan on gestation day 14 to reduce primordial follicles, and were then initiated by intrauterine treatment with *N-ethyl-N'-nitro-N- nitrosoguanidine* at 11 weeks of age. Both busulfan treatment doses caused earlier occurrence of persistent estrus, with dose-dependence as compared to controls. At 15 months of age, the rats were euthanized. The incidence of uterine adenocarcinomas and multiplicity of uterine neoplastic lesions were significantly increased by the 5.0 mg/kg, but not the 2.5 mg/kg busulfan treatment. Morphologically, the ovaries exposed to busulfan treatment exhibited severe atrophy, with few or no follicles and corpus lutea. Serum 17β-estradiol (E2), progesterone, and inhibin levels were significantly decreased in the busulfan treatment groups, with a clear dose-relation. Interestingly, only the 5.0 mg/kg busulfan treatment elevated the E2/progesterone ratio. These results provide evidence that the reduction of primordial follicles promotes uterine adenocarcinoma development in rats in association with an earlier occurrence of the persistent estrus status.

Key words: Busulfan, Rat, Reduction of follicles, Uterine cancer

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Ovarian hormones are crucial for uterine carcinogenesis in humans and rats, and atrophic ovaries characterized by atretic and/or cystic follicles and a lack of corpus lutea are known to be associated with an increased blood 17β-estradiol (E2)/progesterone ratio and a high risk for uterine as well as mammary cancer [1, 2]. Many aspects of the aging process in the ovary remain unclear, but changes in follicles presumably play a central role [1, 3, 4]. Studies have provided morphological evidence that a reduction in the number of primordial follicles accelerates follicle growth in intact mice and mice treated with ovotoxic agents [5], or rats that were unilaterally ovariectomized [6].

Treatment with busulfan, an alkylating agent, during the period of germ cell proliferation reduces the number of oogonia in rats [7], and consequently reduces the number of primordial follicles formed in the ovary [8]. Hirshfield found an inverse correlation between the number of primordial

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follicles in the ovary and the rate at which they moved into the growing pool [8]. In addition, Shirota *et al.* [9] have demonstrated that a reduction in the number of primordial follicles resulting in decrease in the number of follicles entering the growing phase, a major source of circulating inhibin in the neonatal and infantile ovary. A consequent elevation in circulating FSH may accelerate follicular development and cause early puberty in rats treated with busulfan maternally. However, it is not known how this impacts on aging in the uterus and uterine adenocarcinoma development.

Uterine cancers in the corpus, the majority being endometrial adenocarcinomas, are relatively prevalent in developed countries and constitute a leading cause of cancer deaths [1]. In rats, naturally occurring endometrial adenocarcinomas are rare, but Maekawa *et al.* found a high incidence of such lesions with morphological and biological similarities to human tumors in aged animals of the Donryu strain [10]. They further demonstrated that endometrial adenocarcinoma development was remarkably linked to an age related ovarian hormonal imbalance that resulted in an increase in the serum estrogen/progesterone ratio [11, 12]. Using this rat strain, they have also established a 2-stage uterine carcinogenesis model to detect promoting or preventive effects of test-chemicals [13, 14].

The present study was conducted to clarify the effects of a reduction of primordial follicles on uterine carcinogenesis in rats maternally treated with busulfan. Busulfan was used as an agent since this chemical has been reported to reduce the number of oogonia during the period of germ cell proliferation and to consequently reduce the number of primordial follicles formed in the ovary in rats [8, 9]. In addition, the endocrinological status of the treated rats was analyzed.

Materials and Methods

Animals and housing conditions

Thirty pregnant female Crj:Donryu rats were purchased from Charles River Japan (Kanagawa, Japan) on gestation day 2. The animals were maintained in an air-conditioned animal room under constant conditions of 24 ± 2 C and 55 ± 10% humidity with a 12-h light/dark cycle (light, 8:00–20:00; dark, 20:00–8:00), and housed individually in cages until weaning. Offspring were also maintained in the same conditions and housed 3 or 4 to a cage. A commercial pellet diet (CRF-1, Oriental Yeast, Kanagawa, Japan) and drinking tap water were available *ad libitum* for dams and offspring. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Chemicals and selection of a busulfan dosage

Busulfan (Sigma, St Lous, MO) was weighed to give doses of 2.5 and 5.0 mg/kg body weight of dams, suspended in a small amount of corn oil (Wako Pure Chemical, Osaka, Japan) and the concentration was adjusted for use at a constant volume of 5 ml/kg body weight of the dams.

Treatment and maintenance of animals

2.5 and 5.0 mg/kg of busulfan were administered intraperitoneally (ip) to 10 pregnant females per group on day 14 of gestation, and the females were then allowed to deliver spontaneously. The size of each litter was standardized to ten on day 4 after birth, and offspring were weaned on day 21 after birth. Control offspring were maternally treated with the corn oil vehicle on day 14 of gestation in the same manner. The numbers of offspring obtained were 27, 24, and 24 females for the controls, 2.5, and 5 mg/kg busulfan treated groups, respectively.

Uterine carcinogenesis and histopathological examination

For initiation, the female offspring were treated with a single dose of 20 mg/kg *N-ethyl-N'-nitro-N-nitrosoguanidine* (ENNG, Nacalai Tesque, Kyoto, Japan) into one of the uterine horns via the vagina using a stainless steel catheter at 11 weeks of age. This initiation is known to exert no carcinogenic effects in other organs [13]. At 15 months of age, all surviving animals were decapitated and necropsied, and organs and tissues of reproductive and endocrine systems, including the uterus, ovaries, adrenals, liver, kidneys, brain, and spleen were weighed and fixed in 10% neutral buffered formaldehyde solution. These tissues and the pituitary, thymus, mammary gland, brain, vagina, bone with bone marrow and macroscopic abnormalities were fixed and routinely processed for histopathological examination. The upper, middle and lower parts of each uterine horn and

the uterine cervix were cross-sectionally cut into 3 pieces to evaluate uterine neoplastic lesions, and classified into three degrees of atypical hyperplasia (slight, moderate, or severe) and adenocarcinomas, according to criteria described previously [11–13]. In addition, adenocarcinomas were subdivided into well, moderately and poorly differentiated types, and also classified as to the degree of invasion: limited to the uterus, invading into the serosa and/or surrounding adnexae, and with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers [15]. Animals found dead or euthanized when moribund were also examined in the same manner. The tissues and/or organs fixed were routinely processed, paraffin embedded and stained with hematoxylin and eosin for histopathological examination. Throughout the experiment, all animals were checked for growth, clinical signs, and their estrous cyclicity by vaginal cytology. Estrus lasting for continuous for 4 days or more was defined as persistent estrus (PE).

Radioimmunoassays

Using serum obtained after decapitation, concentrations of follicle-stiumulating hormone (FSH), lutenizing hormone (LH), inhibin, E2, and progesterone were determined using double-antibody radioimmunoassays and ^{125}I-labelled radio-ligand. National Institute of Diabetesand Digestive and Kidney Disease (NIDDK) radioimmunoassay kits were employed for rat FSH and LH (NIAMDD, NIH, Bethesda, MD), as described by Taya *et al.* [16] and Watanabe *et al.* [17]. Immunoreactive inhibin in the serum was analyzed using rabbit anti-serum, TNDH-1[18]. Serum concentrations of E2 and progesterone were also measured, as described by Taya *et al.* [19].

Statistical analysis

Values for incidences were statistically analyzed using the Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between the treated and control groups were made using the Dunnett's *t*-test. P values of less than 0.05 were considered to be statistically significant.

Results

Growth and estrous cyclicity

The busulfan treatment did not affect the body weights and clinical status of the animals. Regarding estrous cyclicity, all animals showed regular 4-day cycling during first 3 months after commencement of the experiment. Thereafter some animals treated with 5.0 mg/kg busulfan showed an estrus stage that lasted 2 or 3 days. Consequently, the incidence of persistent estrus (PE) in this group began to increase at 4 months of age, and most of the animals showed PE at 6 months of age, 4 months earlier than in the controls (Fig. 1). In the 2.5 mg/kg group, PE was observed 2 months earlier than in controls.

Effects of busulfan on uterine carcinogenesis

Incidences of uterine neoplastic lesions, including atypical hyperplasias and adenocarcinomas, and data concerning their multiplicity, which was indicated as total number of the neoplastic lesions per rat, are shown in Table 1. The incidence of adenocarcinomas was significantly increased in the 5.0 mg/kg busulfan-treated animals compared with the controls, but not in the 2.5 mg/kg busulfan group. Similarly, the multiplicity was significantly increased at the high dose, but not at the low dose. Histologically, almost all uterine adenocarcinomas were of a well-differentiated type limited to the uteri, without invasion or metastasis to other organs, and morphological or biological malignancy was not influenced by either dose of busulfan.

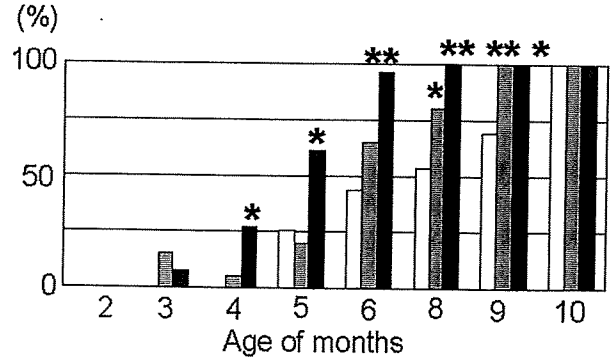

Fig. 1. Sequential changes in incidences of persistent estrus in the controls (□), 2.5 mg/kg busulfan (▩), and 5.0 mg/kg busulfan treated (■) groups. *, ** Significantly different from the controls at P<0.05, and P<0.01, respectively.

Table 1. Incidence (%) and multiplicity data for uterine endometrial lesions

Group	No. of rats	Atypical hyperplasias				Adc	Multiplicity[a]
		–	+	++	+++		
Control	16	6.3	6.3	43.8	18.8	25.0	1.41 ± 0.80
Busulfan 2.5 mg/kg	18	33.3	5.6	16.7	11.1	33.3	1.21 ± 1.11
Busulfan 5.0 mg/kg	26	11.5	0	23.1	11.5	53.8*	3.03 ± 5.79*

[a] Total number of neoplastic lesions, including atypical hyperplasias and adenocarcinomas, per rat.
–: No lesion; +: Slight; ++: Moderate; +++: Severe
Adc: Adenocarcinoma. * Significant difference from the control group at 5%.

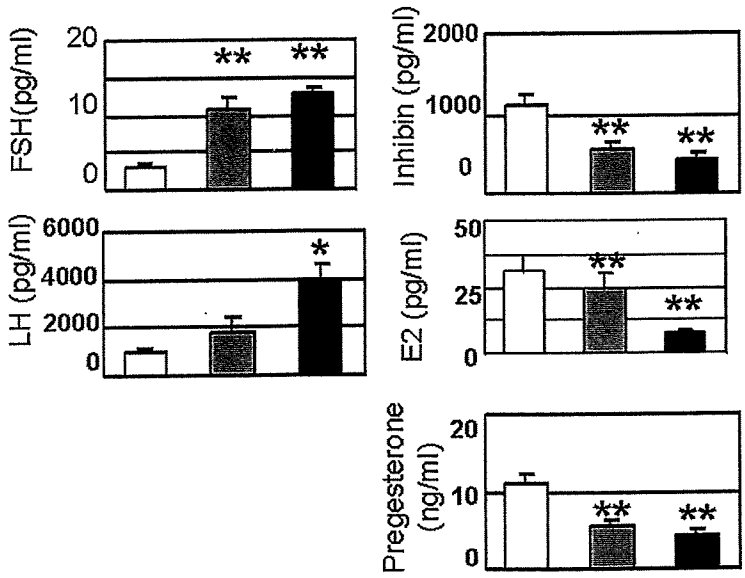

Fig. 2. Hormonal profiles of serum FSH, LH, E2, progesterone and inhibin at the termination of the experiment at 15 months of age in the controls (□), 2.5 mg/kg busulfan (▩), and 5.0 mg/kg busulfan treated groups (■) groups. Results presented are mean ± SE values. *, ** Significantly different from the controls at $P<0.05$, and $P<0.01$, respectively.

Hormone profiles

Data concerning the serum concentrations of E2, progesterone, FSH, LH, and inhibin at 15 months of age are shown in Fig. 2. Inhibin, E2, and progesterone were significantly decreased by the busulfan treatment with dose-dependence. Conversely, serum FSH and LH were increased, although this was not significant for the LH level at 2.5 mg/kg. A remarkable increase in the serum E2/progesterone ratio was evident after the 5.0 mg/kg busulfan treatment, but significance was not achieved due to a high standard deviation (Fig. 3).

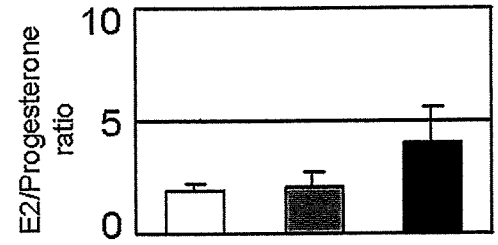

Fig. 3. E2/progesterone ratios at 15 months of age, and at termination of the experiment for the controls (□), 2.5 mg/kg busulfan (▩), and 5.0 mg/kg busulfan treated (■) groups. Results presented are the mean ± SE values.

Organs weights and histopathology

In the uteri, macroscopic lesions such as nodules, hemorrhages, and bead-like horns were found in most animals, including the controls. Relative uterine weights per body weight were decreased in the 5.0 mg/kg busulfan group compared with the control values (Table 2).

Macroscopically, most of the ovaries were small at termination (15 months of age), and weights did not significantly differ across groups. Morphologically, marked atrophy was evident after the busulfan treatment, characterized by very few atretic and/or cystic follicles and a lack of

Table 2. Ovarian and uterine weights

Groups	No. of rats Examined	Final body weight	Uterus	Ovaries
Control	17	357.06 ± 32.26	1297.31 ± 342.05[a]	63.11 ± 44.11
			(361.62 ± 85.44)[b]	(17.59 ± 12.30)
Busulfan 2.5 mg/kg	18	399.81 ± 72.67	1156.62 ± 516.84	58.86 ± 43.28
			(259.54 ± 160.91)	(15.73 ± 12.14)
Busulfan 5.0 mg/kg	23	386.61 ± 46.56	1008.60 ± 405.35*	54.60 ± 47.71
			(268.50 ± 123.84)	(14.60 ± 13.12)

[a] Absolute weight, [b] Relative weights to body weight (mg/kg body weight × 100).
* Significant difference from the control group at 5%.

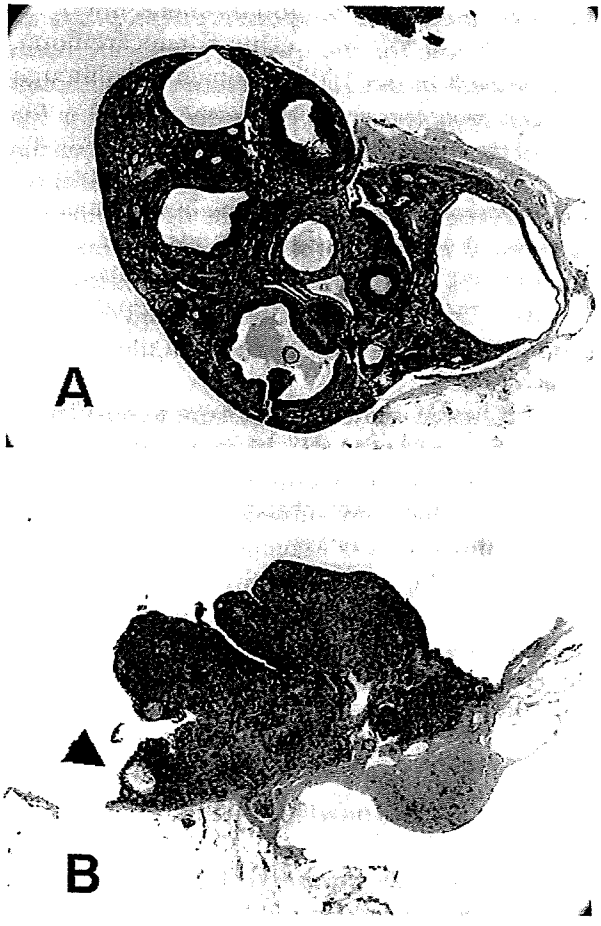

Fig. 4. Microphotographs of ovaries from controls (A) and 5.0 mg/kg busulfan treated animals (B) at 15 months of age. Note several atretic and/or cystic follicles with a lack of corpus and increased interstitial glands in the stroma in the control group (A). Only a small cystic follicle is apparent (arrowhead) in the stroma with 5.0 mg/kg busulfan (B). Hematoxylin and eosin staining. × 20.

corpus lutea, whereas the atrophic ovaries in controls demonstrated appreciable numbers of follicles (Fig. 4). In addition, collagen fibers in the ovarian stroma were more evident in busulfan treated rats, although aggregation of lipid containing stromal cells, or so-called interstitial glands, was not prominent. Busulfan, an alkylating agent, is well known to damage bone marrow and lungs [20, 21], but the results of the present study showed no effects of treatment in these body sites. A number of histopathological changes, including age-related ones, were observed in all animals, however there were no treatment-related changes in other organs or tissues.

Discussion

The present study clearly demonstrated that maternal treatment with a high dose of busulfan to enhance uterine carcinogenesis in Donryu rats is associated with an early occurrence of PE and severe ovarian atrophy, with lack of both follicles and corpus lutea. In this rat model, imbalance in ovarian hormones leads to elevation of the serum E2/progesterone ratio, and it was recognized that ovarian atrophy plays an essential role for the endometrial adenocarcinoma development, similar to the case in humans [1, 2, 11]. Using this model, many studies have proven that early and delayed occurrence of PE induced by chemicals promotes and prevents the endometrial adenocarcinoma development, respectively [22, 23]. Therefore, the earlier occurrence of PE in the present study is considered to be crucial for the promoting effect on uterine cancer.

Busulfan is known to accelerate the rate of follicular recruitment, in spite of the smaller

number of growing follicles [8]. Shirota *et al.* have demonstrated that the number of preantral follicles in the ovaries of Sprague-Dawley rats prenatally exposed to 2.5 mg/kg busulfan was comparable to the age-matched control value by day 13 after birth, and that the number of oocytes shed at the first ovulation with 5.0 mg/kg busulfan was also comparable to that in the controls [9]. In general, PE corresponds to an anovulation status, and appears in various situations such as neonatal exposure to high doses of estrogens and/or androgens and with aging in rodents, although the latter greatly varies depending on the strain [3, 4, 24, 25]. Ovaries with PE in rats and in postmenopausal women exhibit a gradual increase in the severity of atrophy, with final appearance as fibrous tissue in the end stage [1, 11, 26]. This is morphologically similar to the atrophy of ovaries treated with busulfan. Our present results suggest that a reduction in follicle resources due to maternal treatment with busulfan leads to earlier occurrence of PE and might accelerate ovarian changes with aging, although no sequential observations of the ovaries could be conducted in the present study.

The hormonal profiles exactly reflected the atrophic ovary status, with marked decreases in E2, progesterone, and inhibin levels, and increases in LH and FSH. Inhibin is a regulatory peptide that inhibits FSH synthesis and release from the pituitary, resulting in regulation of ovulation in mammals [17, 27–30]. Previous studies have indicated that the concentration of inhibin reflects the number of primary and preantral follicles until antral follicle formation in the ovary [9, 31]. Our results provide evidence that the hypothalmo-pituitary-gonadal control system still responds at 15 months of age after busulfan treatment. The observed decrease in uterine weights might be related to lower levels of E2, although a number of uterine lesions were detected in all animals examined at 15 months of age. Interestingly, the 5.0 mg/kg busulfan treatment elevated the serum E2/progesterone ratio, although both E2 and progesterone were markedly decreased. Thus, the results might support the previous finding that elevation of this ratio plays a crucial role in uterine carcinogenesis in our rat model.

In the present study, the possibility remains busulfan, an alkylating agent, might exert direct cytotoxicity damage to the uterus, as well as bone marrow and the lungs [20, 21, 32], but no necrotic changes were observed that suggested cytotoxiciy was increased in the uterus of the busulfan treated groups, nor were they reported in the previous study by Shirota *et al.* [9]. In addition, estradiol receptor mediated responsiveness plays an important role for the uterine adenocarcinoma development in rats [1] and women [2], although estrogen receptors were expressed only in a few areas of the epithelial cells in the fetal uterus on day 15 of gestation, and the receptor mediated responsiveness was absent in the development of the prenatal female reproductive tract in mice and rats [33–35]. Busulfan exerts any estrogenic activity. Therefore, these results suggest that a direct action of busulfan on the fetal uterus might be excluded.

In conclusion, maternal exposure to busulfan at dose of 5.0 mg/kg on day 14 gestation promoted the uterine adenocarcinoma development in Donryu rats that were subsequently initiated with ENNG, and this was associated with an earlier occurrence of PE, severe atrophy of the ovaries, and marked decreases in both serum E2 and progesterone levels. The E2/progesterone ratio, however, revealed an increased trend in the high dose group.

Acknowledgements

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Original

α-Smooth Muscle Actin-positive Stromal Cells Reactive to Estrogens Surround Endometrial Glands in Rats but not Mice

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Abstract: In human endometrium, α-smooth muscle actin (α-SMA)-positive stromal cells (SMA-SCs) surround endometrial glands, and the α-SMA expression is regulated by estrogen. The biological significance of these cells remains to be elucidated, and no information is available with regard to their animal counterparts. The present study, therefore, investigated SMA-SCs in the uteri of female Donryu rats and CD-1 mice. SMA-SCs with morphological similarities to those in the human were detected around the endometrial glands in normal cycling rats, but not in mice. Furthermore, the rat SMA-SCs disappeared after ovariectomy but returned with estrogen replacement in a duration-dependent manner, suggesting the regulatory role of estrogens similar to the human situation. Thus, SMA-SCs are present in rats, but not in mice, with characteristics close to their human counterparts. Their biological significance now needs to be elucidated by comparative studies. (J Toxicol Pathol 2005; **18**: 47–52)

Key words: endometrial stromal cells, α-smooth muscle actin, 17β-estradiol, octylphenol, Donryu rat

Introduction

Actin, a cytoskeletal protein involved in cell contraction, cell movement and cell-to-substrate adhesion[1–6], has been divided into six isoforms: two non-muscle actins (β and γ) known to be cytoplasmic, two smooth muscle actins (α and γ), and two sarcomeric actins (α-cardiac and α-skeletal)[7–9]. The α-smooth muscle actin (α-SMA) is found in smooth muscle cells, pericytes and myoepithelial cells[10–12], and in humans normal endometrial stromal cells around endometrial glands have also been shown to immunostain for α-SMA[13,14]. This α-SMA expression in stromal cells changes during the estrous cycle, greater numbers of positive cells being present in the proliferative than in the secretory phase[13]. Furthermore, in the proliferative phase α-SMA-positive stromal cells (SMA-SCs) can be detected occasionally in the more superficial mucosa and around non-dilated glands as well as in the lower, basal layer of the endometrial mucosa and around dilated or cystic glands, whereas SMA-SCs in the secretory phase are mostly evident in the basal, inactive layer and around single non-secretory glands[14]. This suggests that estrogen influences α-SMA expression in the endometrial stromal cells, although their significance remains to be elucidated. Furthermore, no information is available in the literature about their existence in experimental animals, such as rodents. The present study was thus conducted to determine whether SMA-SCs are a feature of the uteri of rats and mice. Finding them present in rats, we then assessed the reactivity of SMA-SCs to 17β-estradiol and *p-tert*-octylphenol, an endocrine disrupting chemical with estrogenic activity, using ovariectomized rats.

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Materials and Methods

Ethical considerations for animal experiments

The animal experiments conducted in this study were approved by the Animal Experimentation Committee of the Sasaki Institute prior to their execution and were conducted under monitoring by the committee in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105 and Japanese

Government Notification on Feeding and Safekeeping of Animals Number 6.

Animals

A total of 30 virgin female Donryu rats (Crj:Donryu, 6 or 10 weeks of age) and 12 virgin CD-1 mice (Crj:CD-1, 7 weeks of age) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed in plastic cages, kept in an air-conditioned animal room (under constant conditions of 24 ± 2°C, 55 ± 10% humidity, and a 12-hour light/dark cycle), and maintained on a basal diet, CRF-1 (Oriental Yeast Inc., Tokyo, Japan) with tap water *ad libitum*.

Experimental design

Experiment I: Twelve animals each of the two species showing normal estrous cyclicity were selected, and vaginal smears were checked every morning. At ages of 12 or 15 weeks (rats) and 9 weeks (mice), 3 animals each were euthanized in the 4 stages of the estrous cycle (proestrus, estrus, metestrus and diestrus), and the uteri were excised for histological and immunohistochemical examination. The uterine horns were fixed in 10% neutrally buffered formalin solution and embedded in paraffin. Appropriate numbers of serial sections at a thickness of 4 μm were then prepared from each specimen, one being routinely stained with hematoxylin and eosin for histological examination. The other sections were processed for immunohistochemical analyses using mouse monoclonal antibodies against α-SMA (clone 1A4, Dakocytomation Japan, Kyoto, Japan, 100-fold diluted) and cytokeratin 14 (CK14; clone LL002, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK, 20-fold diluted), the latter for the rat uteri only, at 4°C overnight, then processed for the labeled polymer method using an Envision Plus kit (Dakocytomation) according to the manufacturer's instructions, and counterstained with hematoxylin. Negative controls were included with primary antibodies omitted.

Experiment II: At 8 weeks of age, 18 rats underwent ovariectomy via the dorsal route under light ether anesthesia. Three weeks after the operation, they were equally divided into 3 groups, given subcutaneous injections of vehicle (dimethylsulfoxide), 5 μg/kg/day of 17β-estradiol (E_2; Wako Pure Chemical Industries Ltd., Osaka, Japan) or 100 mg/kg/day of *p-tert*-octylphenol (OP; Wako), respectively, for 2 or 14 successive days (3 animals each). The animals were euthanized 24 hours after the last administration, and the uteri were excised. The uterine horns were processed in the same manner as for experiment I except that CK14 immunostaining was not performed.

Results

Experiment I

In rats, the endometrial stroma was cellular, especially in the subluminal layer surrounding the glandular epithelium. The stromal cells surrounding the glands were spindly in shape with oval or spindle nuclei, resembling fibroblasts rather than basket-shaped myoepithelial cells. They were arranged in one or several layers around the glands (Fig. 1A) in all stages of the estrous cycle. In mice, the endometrial stroma was also cellular, but stromal cells surrounding the endometrial glands were not very conspicuous (Fig. 2A).

Immunohistochemically, the stromal cells surrounding the endometrial glands in one or several layers were positive for α-SMA in rats (Figs. 1B and 1C). Positive cells were observed through all stages of the estrous cycle, but the cell layers were thickest in proestrus (Figs. 1B and 1C). The stromal cells were negative for CK14 (Fig. 1D). In mice, SMA-SCs were not detected at any stage of the estrous cycle (Fig. 2B). In the uteri of both rats and mice, smooth muscle fibers in the blood vessels and the myometrium were positive for α-SMA (Figs. 1B, 1C and 2B).

Experiment II

In the ovariectomized rats, the uteri were severely atrophic, and the luminal epithelial cells in the endometrium were cuboidal rather than columnar in shape. The endometrial stromal cells and smooth muscle in the myometrium were reduced in size (Fig. 3A). In rats treated with E_2 for 2 days, the size of the uteri recovered remarkably, and the luminal epithelial cells in the endometrium were again columnar in shape. The endometrial stromal cells also recovered, and the myometrium was multi-layered (Figs. 3A and 3B). In rats treated with E_2 for 14 days, the uteri were as large as those of 2-day-treated animals while the endometrial stromal cells were larger (Figs. 3B and 3C) and the myometrium was thicker (Figs. 3B and 3C). In rats receiving OP, the uteri generally demonstrated similar histological findings to those in the rats treated with E_2 for the same term (Figs. 3C and 3D).

Table 1 summarizes the data of the α-SMA immunohistochemistry of stromal cells surrounding the endometrial glands in experiment II. In ovariectomized rats treated with vehicle stromal cells were negative for α-SMA (Fig. 4A), but 2 out of 3 rats treated with E_2 for 2 days exhibited weakly-positive spindle cells around the glands (Fig. 4B). Furthermore, α-SMA-positive cells were observed in all rats injected with E_2 for 14 days (Fig. 4C). Similarly, one out of 3 rats treated with OP for 2 days and all rats receiving OP for 14 days had SMA-SCs (Fig. 4D). Smooth muscle fibers in the blood vessels and the myometrium were positive for α-SMA in rats of all groups (Figs. 4A-D).

Discussion

The present study unequivocally demonstrated the presence of SMA-SCs surrounding endometrial glands in untreated rats but not mice. The SMA-SCs clearly differed from myoepithelial cells, characterized as basket-shaped with a positive CK14 phenotype[15,16], and were similar to SMA-SCs in the human endometrium, negative for

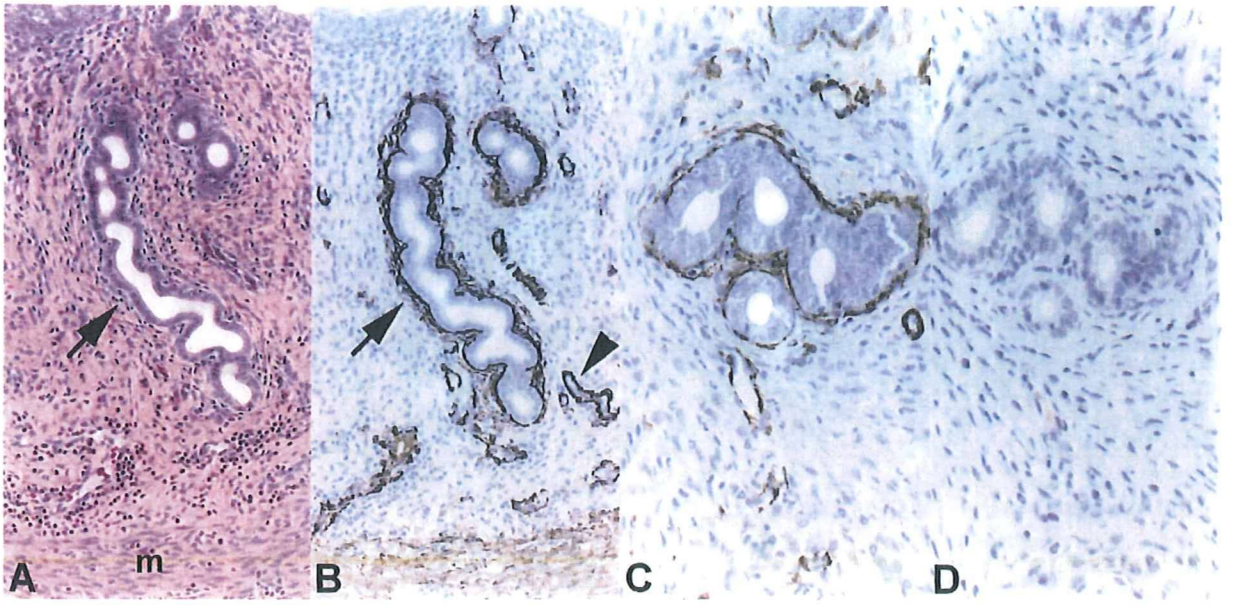

Fig. 1. Uteri of untreated rats (experiment I). (A) Representative histology in proestrus. Several layers of stromal cells surround endometrial glands (arrow). m: myometrium, × 90. (B) Representative α-SMA immunohistochemistry in proestrus. One or several layers of stromal cells surrounding endometrial glands are positive (arrow). Smooth muscle fibers in the blood vessels (arrowhead), as well as in the myometrium, are also positive, × 90. (C) Representative α-SMA immunohistochemistry in metestrus. One or two layers of stromal cells surrounding endometrial glands are positive, × 180. (D) Representative CK14 immunohistochemistry in metestrus. The endometrial stromal cells are negative, × 180.

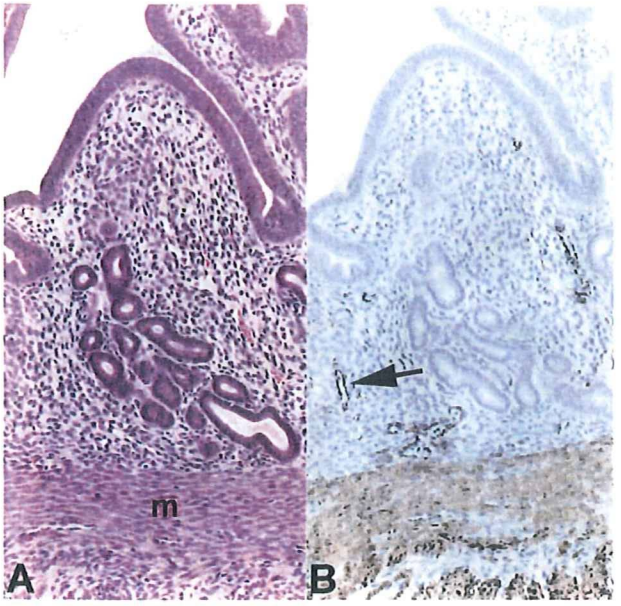

Fig. 2. Uteri of untreated mice (experiment I). (A) Representative histology in proestrus. Endometrial stromal cells are diffusely present throughout the stroma. m: myometrium, × 90. (B) Representative α-SMA immunohistochemistry in proestrus. The endometrial stromal cells are negative. Smooth muscle fibers in the blood vessels (arrow), as well as in the myometrium, are positive, × 90.

Table 1. Grades of α-SMA Immunohistochemistry for the Stromal Cells Surrounding Endometrial Glands in Ovariectomized Rats (Experiment II)

Group	Treatment		Grade		
	Compound	Period	–	±	+
1	Vehicle	2 days	3	0	0
		14 days	3	0	0
2	E_2	2 days	1	2	0
		14 days	0	0	3
3	OP	2 days	2	1	0
		14 days	0	0	3

Symbols used are: –, negative; ±, weakly positive; +, positive.

cytokeratins and distinguishable from myoepithelial cells[14]. Human SMA-SCs have been suggested to be a subset of myofibroblasts[13,14]. Myofibroblasts are characterized as having features of both smooth muscle cells and fibroblasts[17–19], and can be classified into 4 subtypes based on their differential immunoreactivity with antibodies against vimentin, desmin and α-SMA[20]. Results of the present study indicate that the SMA-SCs present in rats similarly have a myofibroblast origin, judging from the morphological findings.

In the rats, layers of SMA-SCs were apparent in proestrus, when serum E_2 levels are the highest of the estrous cycle. The fact that the endometrial stromal cells surrounding endometrial glands were small in size and negative for α-SMA in ovariectomized rats, with recovery

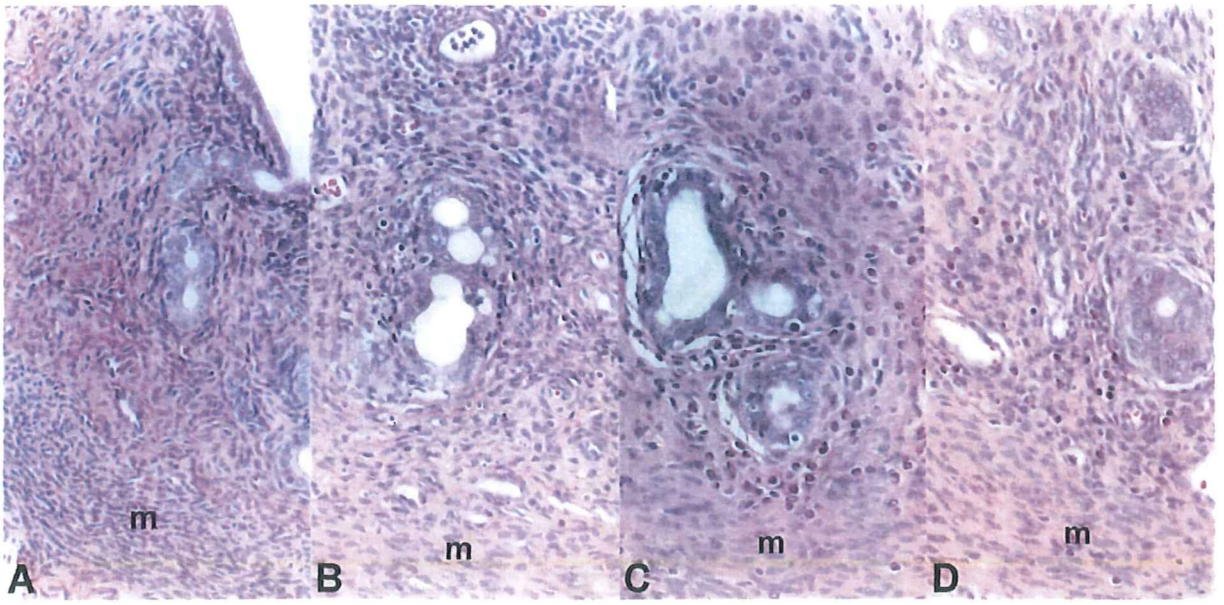

Fig. 3. Representative histology of uteri of ovariectomized rats (experiment II), × 180. (A) Treated with vehicle for 14 days. The endometrial stromal cells and smooth muscle in the myometrium (m) are small in size. (B) After treatment with E_2 for 2 days; the endometrial stromal cells are larger in size than those of the vehicle controls. Smooth muscle in the myometrium (m) is also thicker. (C) After treatment with E_2 for 14 days; the endometrial stromal cells are larger than those of the 2-day-treated rats and the myometrium (m) is thicker. (D) After treated with OP for 14 days; the endometrial stromal cells are large and the myometrium (m) is thick, like those of the rats receiving E_2 for 14 days.

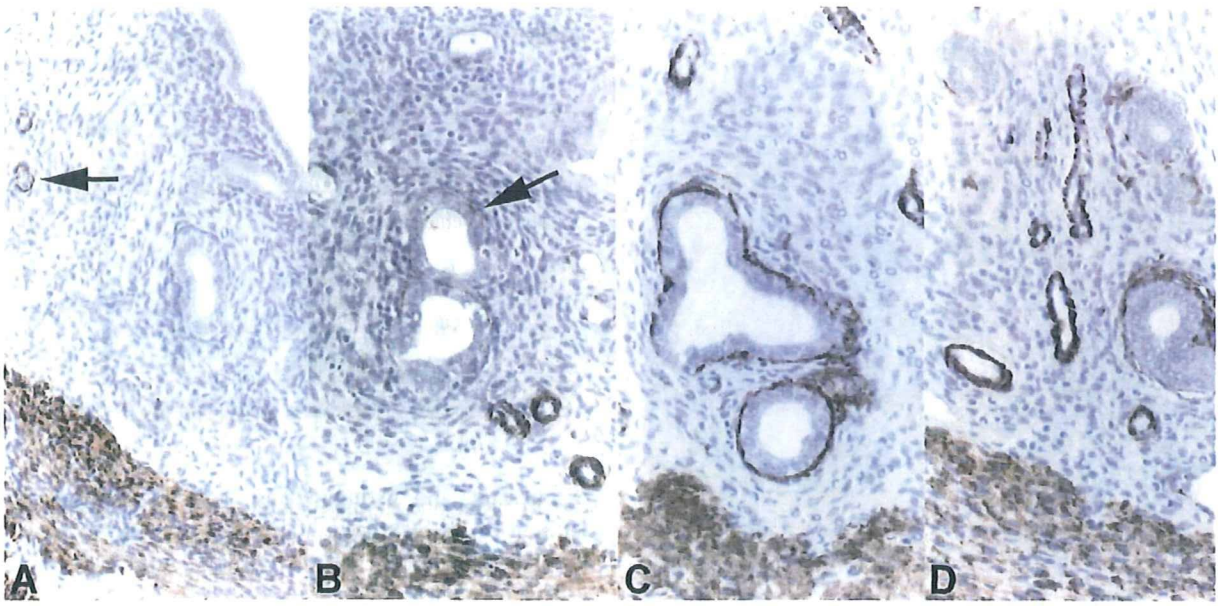

Fig. 4. Representative α-SMA immunohistochemistry of uteri of ovariectomized rats (experiment II), × 180. (A) Treated with vehicle for 14 days. Stromal cells surrounding endometrial glands are negative. Smooth muscle fibers in the blood vessels (arrow), as well as in the myometrium, are positive. (B) Treated with E_2 for 2 days. Some stromal cells surrounding endometrial glands are weakly positive (arrow). (C) Treated with E_2 for 14 days. One layer of the stromal cells surrounding endometrial glands is positive. (D) Treated with OP for 14 days. The stromal cells surrounding endometrial glands are positive.

on treatment with estrogen or OP, in a duration-dependent manner, clearly points to hormone dependence. OP is an endocrine disrupting chemical with estrogenic activity, from *in vitro* and *in vivo* evidence[21–28], and the dose of OP used in this study has been shown to be sufficient to exert estrogenic effects on the female reproductive tract in ovariectomized rats[27]. The results thus suggest that, similar to the human situation[13,14], estrogen modulates α-SMA expression in the

stromal cells surrounding endometrial glands in rats. Although the underlying mechanisms remain largely obscure, estrogen receptors can be immunohistochemically detected in endometrial stroma as well as in epithelium and in myometrium[29], and Hsu and Frankel[30] have demonstrated that mRNA expression of the *smooth muscle actin* gene is up-regulated by estrogens in immature rat uteri.

Myofibroblasts have been proposed as playing crucial roles in the contraction and relaxation of human granulation tissue on the basis of *in vitro* pharmacological reactivity similar to the smooth muscle[2]. α-SMA-positive myofibroblasts are also observed in rat granulation tissue[18] and may act similarly to their human counterparts. α-SMA is also expressed in passaged cultures of chick embryo fibroblasts[10], and stress fibers containing actin have been postulated as playing structural roles in the connection of the cytoplasmic matrix to the substrate rather than being contractile[4]. Thus, SMA-SCs surrounding endometrial glands might either participate in the contraction of glands or in the cell's adhesion to the surrounding substrate. Mechanistic studies are now needed to clarify, for example, the lack of SMA-SCs in mice.

In conclusion, SMA-SCs are present in rats, but not mice, and surround the endometrial glands, exhibiting morphological and endocrinological similarities to their human counterparts. Elucidation of their functional significance now needs to be performed by comparative studies in different species.

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