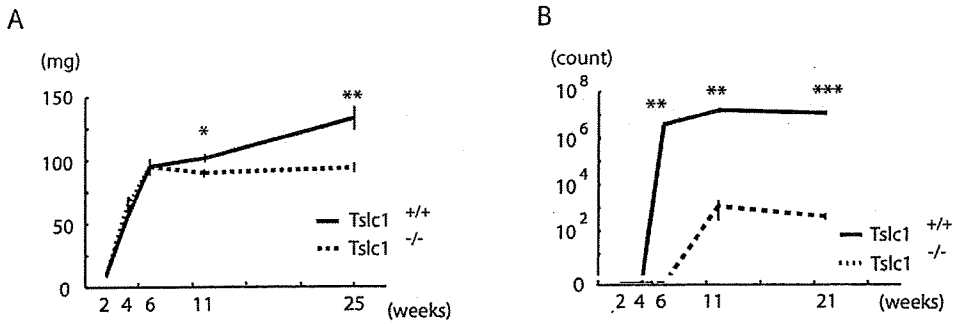

FIG. 8. Weights of testes and numbers of normal sperm during postnatal development of *Tslc1*$^{+/+}$ and *Tslc1*$^{-/-}$ mice. (A) Weights of testes. (B) Numbers of normal sperm. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

spermatids began to slough, vacuoles appeared at the basal portions of the seminiferous tubules in the *Tslc1*$^{-/-}$ testes. This occurred in mice around 8 weeks of age and developed more prominently by the time the mice were 11 weeks of age. Testicular weight showed a significant reduction in *Tslc1*$^{-/-}$ mice compared with that in *Tslc1*$^{+/+}$ mice at 11 weeks of age (Fig. 8A). It should be noted, however, that very few elongated spermatids and even spermatozoa could be found among the degenerated round cells in the *Tslc1*$^{-/-}$ testes of 5-week-old mice and in the epididymides of 11-week-old mice, respectively. Therefore, maturation arrest of the spermatids caused by a TSLC1/IGSF4 deficiency did not seem to be complete (Fig. 8B).

Electron microscopic examination of spermatogenic cells and Sertoli cells. To further elucidate the pathological defect, electron microscopic analysis was carried out using *Tslc1*$^{-/-}$ mice as well as *Tslc1*$^{+/+}$ mice. In the testes, the morphology of the spermatids in step 5 was essentially unaffected (Fig. 9A and D). On the other hand, sloughing of the spermatids from the Sertoli cells of *Tslc1*$^{-/-}$ mice was clearly seen in steps 7 and 8. Vacuoles in the cytoplasm were preferentially observed in these sloughing spermatids compared with those in *Tslc1*$^{+/+}$ testes (Fig. 9B, C, E, and F). Furthermore, spermatids in step 10 and later were very rare and, when present, showed more drastic morphological changes, including morphologically abnormal nuclei and a large amount of residual cytoplasm (Fig. 9G). Intercellular boundaries were not clear in some spermatids (Fig. 9H). Compared with those in the testes, sloughed spermatids observed in the epididymides were more severely degenerated, with numerous vacuoles, even in step 7 or 8 (Fig. 9I). On the other hand, Sertoli cells from *Tslc1*$^{-/-}$ mice contained numerous phagocytes and a considerable number of large vacuoles in the cytoplasm (Fig. 9J). However, the Sertoli cell-Sertoli cell junction, which separates the spermatogonia and the spermatocytes from the luminal side up to the preleptotene phase, was unaffected in *Tslc1*$^{-/-}$ mice (Fig. 9K). In addition, ectoplasmic specialization, a junctional apparatus between the spermatids and the Sertoli cells, was unaffected as long as the spermatids were present and attached to the Sertoli cells (Fig. 9L).

Expression profile of testes from *Tslc1*$^{-/-}$ mice. To elucidate the molecular mechanisms underlying the TSLC1/IGSF4 deficiency and the defect in spermatogenesis, we carried out expression profiling of testes from *Tslc1*$^{-/-}$ mice and *Tslc1*$^{+/+}$ mice, using an oligonucleotide microarray (MG-U74A v.2; Affymetrix) containing a total of 18,400 transcripts. A search for genes whose expression in the testes from *Tslc1*$^{-/-}$ mice showed a >1.3-fold increase or decrease compared with the testes from *Tslc1*$^{+/+}$ mice in two independent experiments yielded 33 and 130 genes, respectively. It should be noted that some transcripts of the *Tslc1/Igsf4* gene were listed as the most down-regulated genes, indicating that the experimental system works well (Table 3).

Among the 163 up- and down-regulated genes, we selected 6 up-regulated and 15 down-regulated genes on the basis of the amount of expression as well as their possible biological significance and then confirmed the differences in the amounts of these transcripts by quantitative RT-PCR analysis. As shown in Table 3, significant differences in gene expression were detected in four up-regulated and six down-regulated genes, including *Tslc1/Igsf4*. Among the gene products, Gas6 (growth arrest-specific factor 6) was shown not to be expressed in the spermatids but expressed in the spermatogonia as well as Sertoli and Leydig cells, and this protein functions to prevent apoptotic cell death in an auto- or paracrine manner with its group of receptors, Tyro3 family molecules (2). Therefore, Gas6 might play a role in enhanced apoptosis of the spermatogenic cells in *Tslc1*$^{-/-}$ mice. On the other hand, the transcripts of Oaz3 and Ppp2r2b were shown to be specifically expressed in the spermatids but not in the spermatogonia or the spermatocytes (6, 16). Therefore, a decrease in the number of these transcripts in testes from *Tslc1*$^{-/-}$ mice might be caused by a reduction in the spermatid cell population.

Similar findings were obtained by Western blotting, in which the amount of alpha-tubulin decreased dramatically in the testes from *Tslc1*$^{-/-}$ mice in comparison with those from *Tslc1*$^{+/+}$ or *Tslc1*$^{+/-}$ mice (see Fig. S1A in the supplemental material), although no significant difference was detected in the numbers of transcripts by RT-PCR analysis (see Fig. S1B in the supplemental material). Immunohistochemical studies revealed that alpha-tubulin was preferentially expressed in mature spermatids but not in other cells in the testes (see Fig. S1C and D in the supplemental material), indicating that the marked reduction in the amount of alpha-tubulin protein was caused by a reduction in the population of mature spermatids in the testes from *Tslc1*$^{-/-}$ mice.

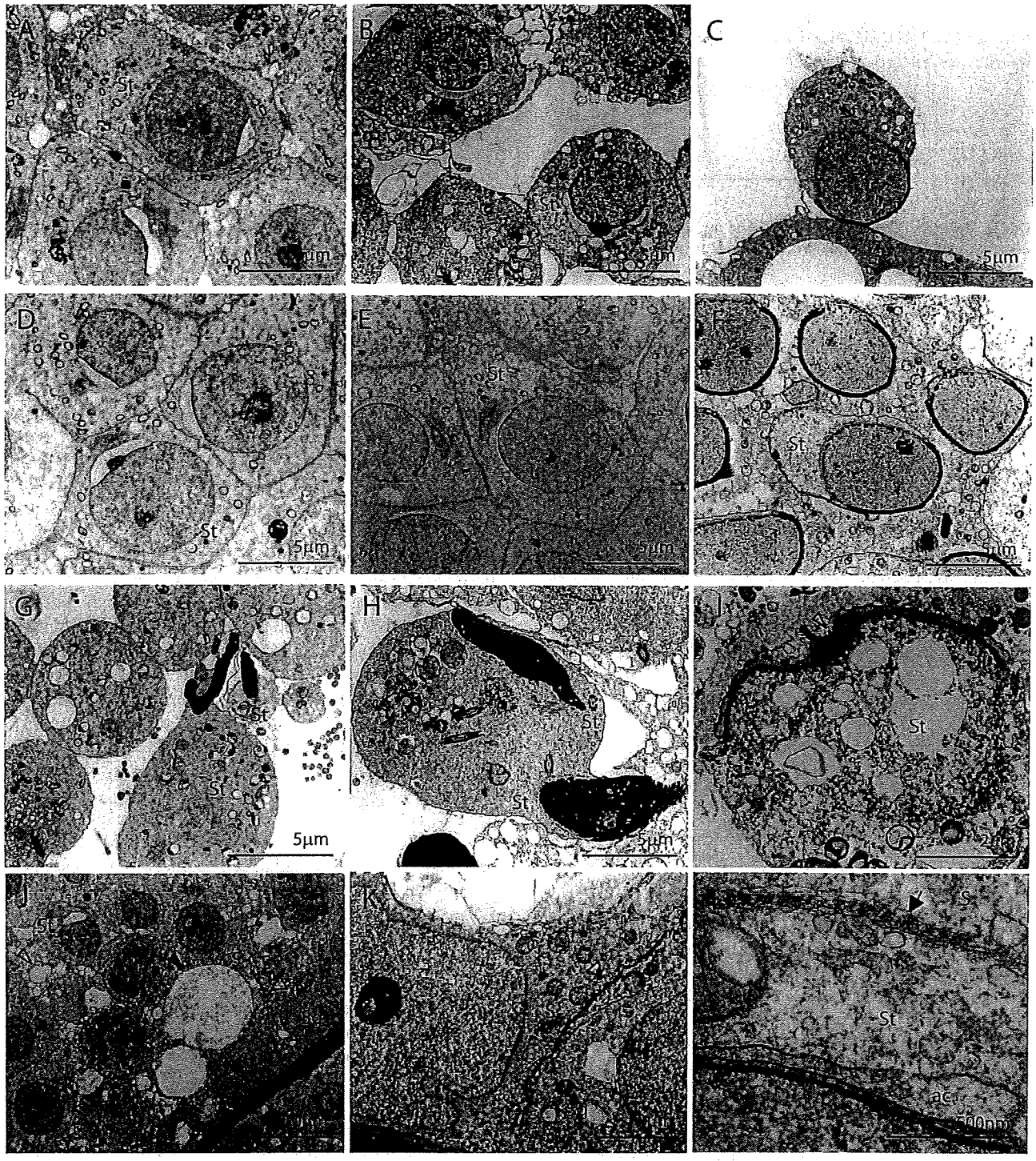

FIG. 9. Electron microscopic analysis of spermatogenic cells and Sertoli cells from *Tslc1*$^{+/+}$ and *Tslc1*$^{-/-}$ mice. (A to H) Spermatids from *Tslc1*$^{-/-}$ mice (A to C, G, and H) and *Tslc1*$^{+/+}$ mice (D to F). Spermatids in step 5 (A and D), step 7 (B and E), step 8 (C and F), and step 10 or later (G and H) are demonstrated. (I) A degenerated cell in the epididymis from a *Tslc1*$^{-/-}$ mouse. Densely staining materials corresponding to the acrosome (open arrowhead), as well as numerous degenerated vacuoles, were observed. (J to L) Numerous figures of phagocytosis (open arrowhead in panel J) and vacuolization (closed arrowhead in panel J) were observed within the Sertoli cells. Note that the Sertoli cell-Sertoli cell junction (open arrows in panels J and K) and ectoplasmic specialization (closed arrow in panel L) were unaffected in the testes from *Tslc1*$^{-/-}$ mice. S, Sertoli cell; Sg, spermatogonium; Sc, spermatocyte; St, spermatid; ac, acrosome.

TABLE 3. Genes up- and down-regulated in testes from *Tslc1*$^{-/-}$ mice

Gene product	Accession no.	Microarray analysis results			Quantitative RT-PCR analysis results[b]		*P* value[c]
		Signal intensity[a]		Ratio of *Tslc1*$^{-/-}$ value/*Tslc1*$^{+/+}$ value	*Tslc1*$^{-/-}$ value	*Tslc1*$^{+/+}$ value	
		Tslc1$^{-/-}$	*Tslc1*$^{+/+}$				
Up-regulated genes in *Tslc1*$^{-/-}$ testes							
Phospholipase A2, group XIIA (Pla2g12a)	AI845798	13,102	771	17.0	11 ± 2.1	4.1 ± 0.8	0.03
Purine-nucleoside phosphorylase (Pnp)	U35374	1,629	410	4.0	6.1 ± 0.7	2.5 ± 0.6	0.02
Mus musculus cDNA 5′ end clone	AA874329	706	272	2.6			
FUS interacting protein 1 (Fusip1)	AF060490	695	282	2.5			
Hemoglobin, beta adult major chain (Hbb-b1)	J00413	1,446	636	2.3			
DnaJ homolog, subfamily A, member 2 (Dnaja2)	AA763945	1,833	860	2.1	470 ± 160	300 ± 120	NS
Vascular cell adhesion molecule 1 (Vcam1)	U12884	2,321	1,546	1.5	110 ± 5.8	38 ± 2.5	0.0002
Guanylate kinase 1 (Guk1)	U53514	3,225	2,294	1.4	23 ± 12	4.4 ± 0.1	NS
Angiopoietin-like 4 (Angpt14)	AF110520	6,143	4,823	1.3	3.1 ± 0.2	1.2 ± 0.2	0.002
Down-regulated genes in *Tslc1*$^{-/-}$ testes							
Immunoglobulin superfamily 4 (Igsf4/Tslc1)	AF061260	444	3,943	0.11	0 ± 0	100 ± 18	0.001
Immunoglobulin superfamily 4 (Igsf4/Tslc1)	AB021966	690	3,739	0.18			
Platelet/endothelial cell adhesion molecule 1 (Pecam1)	L06039	181	799	0.23	1.6 ± 0.2	1.2 ± 0.3	NS
Mus musculus cDNA 3′ end clone	AW047207	241	895	0.27			
Peroxiredoxin 2 (Prdx2)	U20611	281	892	0.31	16 ± 3.0	11 ± 0.6	NS
Dehydrogenase/reductase X chromosome (Dhrsx)	AI846822	4,509	11,760	0.38	11 ± 1.6	12 ± 2.1	NS
Ornithine decarboxylase antizyme 3 (Oaz3)	AB016275	17,735	44,962	0.39	94 ± 6.8	150 ± 9.2	0.007
Splicing factor, arginine/serine-rich 16 (Sfrs16)	AF042799	430	1,030	0.42			
Protein phosphatase 2, regulatory subunit B, beta isoform (Ppp2r2b)	AW048155	6,134	14,358	0.43	9.2 ± 1.9	23 ± 1.5	0.004
Mouse endogenous murine leukemia virus modified polytopic provirus DNA	M17327	789	1,769	0.45			
Deleted in polyposis 1-like 1 (Dplll)	AA755260	14,224	29,836	0.48	340 ± 30	970 ± 130	0.008
Lysophospholipase 1 (Lypla1)	U89352	6,971	14,600	0.48	7.8 ± 0.4	5.6 ± 1.6	NS
Suppressor of K^+ transport defect 3 (Skd3)	U09874	7,709	15,612	0.49			
S100 calcium binding protein A13 (S100a13)	X99921	773	1,430	0.54			
Protamine 1 (Prm1)	Z47352	18,600	31,700	0.58	12,000 ± 2,200	16,000 ± 1,700	NS
RAN GTPase activating protein 1 (Rangap1)	U20857	4,960	8,480	0.58	72 ± 33	110 ± 47	NS
Growth arrest-specific protein 6 (Gas6)	X59846	1,120	1,820	0.61	80 ± 8.2	170 ± 35	0.04
Bcl2-associated athanogene 1 (Bag1)	AF022223	3,412	5,546	0.61	0.1 ± 0.1	1.1 ± 0.4	NS
Cyclin-dependent kinase inhibitor 1C (Cdkn1c)	U22399	1,090	1,650	0.66	2.3 ± 0.2	1.8 ± 0.1	NS
Mothers against decapentaplegic homolog 6 (Smad6)	AF010133	2,070	2,930	0.70	79 ± 11	94 ± 8.4	NS
Sperm mitochondrion-associated cysteine-rich protein (Smcp)	M88463	29,700	38,900	0.76	430 ± 43	930 ± 81	0.002

[a] Average value of two independent experiments.
[b] The Amount of Igsf4/Tslc1 in the *Tslc1*$^{+/+}$ testis was assigned a value of 100. Data are average values ± SE of three to five independent experiments.
[c] NS, not significant.

DISCUSSION

The present study clearly demonstrates that TSLC1/IGSF4 is essential for spermatogenesis. Although the TSLC1/IGSF4 protein is expressed in the brain, lungs, testes, and various other tissues, no overt defects other than male infertility are observed. These findings suggest that the function of TSLC1/IGSF4 is mostly compensated for by other molecules, although detailed analyses of brain function as well as disease susceptibility, including that to cancer, are being conducted. In addition, it would be valuable to compare the phenotypes of other *Tslc1*$^{-/-}$ mice, because the *neo* cassettes might affect the phenotype of *Tslc1*$^{-/-}$ mice in the present study. Based on the observations described above, we propose that a defect in spermatogenesis in *Tslc1*$^{-/-}$ male mice is caused by delayed maturation from spermatocytes to spermatids, the sloughing of spermatids in steps 7 to 9 from the seminiferous epithelia into the lumen, and the apoptosis and mature arrest of spermatids in step 10 and later. A small but significant number of spermatocytes were also sloughed from the seminiferous tubules. As a result, mature spermatozoa were scarcely observed in semens from *Tslc1*$^{-/-}$ mice. These findings mostly agree with the observation by immunohistochemistry that the TSLC1/IGSF4 protein was expressed in spermatogenic cells from intermediate spermatogonia to early pachytene spermatocytes and from step 7 spermatids to step 16 residual bodies. It is noteworthy, however, that no sloughing was observed in spermatogonia expressing a considerable amount of the TSLC1/IGSF4 protein. The Sertoli cell-Sertoli cell junction may prevent the spermatogonia from sloughing from the seminiferous epithelium. Indeed, the Sertoli cell-Sertoli cell junction was unaffected when examined by electron microscopic analysis. For Sertoli cells from *Tslc1*$^{-/-}$ mice, however, numerous images of phagocytosis and vacuoles were observed, although the TSLC1/IGSF4 protein was not expressed in Sertoli cells from

Tslc1$^{+/+}$ mice. These morphological changes in Sertoli cells were likely caused by a secondary effect in response to sloughing of the seminiferous epithelial cells because in *Tslc1*$^{-/-}$ mice, large vacuoles emerged at 8 weeks of age, whereas sloughing of the spermatids occurred at 5 weeks of age. It is also interesting that multinucleated giant cells and abnormal sperm with multiple tails were found in *Tslc1*$^{-/-}$ mice, suggesting that the intercellular cytoplasmic bridge between sister spermatids could also be disrupted by sloughing. These observations suggest that TSLC1/IGSF4 is essential for adhesion between the spermatogenic cells and between the spermatogenic cells and Sertoli cells. However, the presence of a scant amount of mature sperm implies that these functions might be compensated for by other molecules in a very small population of spermatogenic cells.

Tslc1$^{+/-}$ male mice were fertile and gave offspring with the expected Mendelian ratio when crossed with *Tslc1*$^{+/-}$ or *Tslc1*$^{-/-}$ female mice. These findings indicate that all haploid spermatids differentiated normally into functional spermatozoa, although half of them had lost the *Tslc1/Igsf4* gene in *Tslc1*$^{+/-}$ male mice. It should be noted that neither the maturation of spermatogenic cells nor the expression pattern of the TSLC1/IGSF4 protein in these cells from *Tslc1*$^{+/-}$ mice was affected in comparison with *Tslc1*$^{+/+}$ mice. In fact, as seen in testes from *Tslc1*$^{+/+}$ mice, the TSLC1/IGSF4 protein was detected even in the spermatids in step 7 to the residual bodies in step 16 from *Tslc1*$^{+/-}$ mice, although the *Tslc1/Igsf4* gene had been lost in one-half of these spermatids. The discrepancy between the absence of the gene and the presence of the TSLC1/IGSF4 protein in spermatids at step 7 and later can be explained if the Tslc1/Igsf4 mRNA that was transcribed in the diploid spermatogonia was transmitted into the spermatids and then translated into the protein in the spermatids at step 7 and later.

Our findings are consistent with those given in a previous report by Wakayama et al. regarding the discrepancy in the expression of SgIGSF4/IGSF4 mRNA and its protein in mouse spermatogenic cells. They found that SgIGSF/IGSF4 mRNA was detected in the spermatogonia and the early premeiotic spermatocytes that were situated adjacent to the basement membrane of the seminiferous tubules in the preleptotene to zygotene stages, whereas no mRNA signal was detected in the series of spermatids (19). On the other hand, they later observed that the SgIGSF/IGSF4 protein was expressed in spermatogenic cells from the intermediate spermatogonia to the spermatocytes and from the spermatids at step 7 to the residual bodies in step 16 (18). Thus, they speculated that the transcription of SgIGSF/IGSF4 mRNA terminates in the early steps in spermatocytes but that the translation of SgIGSF/IGSF4 restarts in the round spermatids at step 7 and later using the remaining mRNA. The SgIGSF/IGSF4 mRNA might possibly be stored in the cytoplasm as a ribonucleoprotein complex until it is recruited to the translation machinery several days later (14). Therefore, the long half-life of Tslc1/Igsf4 mRNA could prevent the spermatids lacking the *Tslc1/Igsf4* gene from sloughing from the seminiferous epithelia in *Tslc1*$^{+/-}$ mice.

Finally, we examined the expression profiles of whole testes from *Tslc1*$^{+/+}$ and *Tslc1*$^{-/-}$ mice. The number of up- and down-regulated genes in the present study was smaller than that reported in most similar studies of other genes. This result could be due to the fact that TSLC1/IGSF4 is a membrane protein and is not directly involved in the transcriptional control of other genes. Among the genes which were significantly up-regulated in the testes from *Tslc1*$^{-/-}$ mice, the *Pla2g12a* gene, encoding a group XIIA secretory phospholipase A2 precursor, showed a 17-fold increase in expression in the *Tslc1*$^{-/-}$ testes. The physiological function of this enzyme has not been determined yet, although other members of this gene family are known to be involved in the metabolism of arachidonic acid. On the other hand, Gas6 is a potentially interesting gene among those down-regulated in *Tslc1*$^{-/-}$ testes because it is a ligand of the Tyro3 family of receptors, consisting of Tyro3, Axl, and Mer, which are known to prevent apoptotic cell death (2, 4, 5, 8). Furthermore, male mice deficient in each of these genes (*Tyro3*$^{-/-}$, *Axl*$^{-/-}$, and *Mer*$^{-/-}$ mice) showed a complete loss of mature sperm owing to the progressive death of differentiating spermatogenic cells (9). The loss of TSLC1/IGSF4 function might enhance the apoptosis of spermatids by modifying the signaling of the Gas6 and Tyro3 cascade in *Tslc1*$^{-/-}$ testes. Sperm mitochondrion-associated cysteine-rich protein (Smcp) is another candidate molecule that could be implicated in the pathogenesis of *Tslc1*$^{-/-}$ testes because Smcp is directly involved in sperm motility. Moreover, *Smcp* homozygous mutant mice showed male infertility due to asthenozoospermia (13). Decreased expression of Smcp might be involved in the low motility of the sperm in *Tslc1*$^{-/-}$ mice. Characterization of these molecules could uncover the physiological function of the TSLC1/IGSF4 cascade, although we must consider the heterogeneity of the seminiferous tubules in terms of stages. *TSLC1/IGSF4* was originally identified as a tumor suppressor gene in lung cancer. No spontaneous tumors, however, have developed in eight *Tslc1*$^{-/-}$ mice over 1 year of age. Studies of chemical carcinogenesis as well as irradiation studies in *Tslc1*$^{-/-}$ mice are being conducted in order to understand the role of TSLC1/IGSF4 in the oncogenesis of various organs.

Most of the genes that have been reported to be essential for spermatogenesis in mice also show a variety of indispensable functions in nongerm tissues. However, no obvious phenotypic abnormality, except that in the testes, was observed in *Tslc1*$^{-/-}$ mice, suggesting that the defects in TSLC1/IGSF4 function are complemented by other molecules in other tissues. This fact is especially interesting because a decreased number or morphological abnormalities of spermatozoa are often the only phenotypes recognized in most infertile human males. Further studies on the function of TSLC1/IGSF4, including carcinogenesis experiments, are required for understanding the physiological and pathological significance of TSLC1/IGSF4.

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Ectopic expression of *N*-acetylglucosamine 6-*O*-sulfotransferase 2 in chemotherapy-resistant ovarian adenocarcinomas

Akira Kanoh · Akira Seko · Hiroko Ideo · Midori Yoshida · Mitsuharu Nomoto · Suguru Yonezawa · Masaru Sakamoto · Reiji Kannagi · Katsuko Yamashita

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Abstract Mucinous and clear cell adenocarcinomas are the major histological types of ovarian epithelial cancer and are associated with a poor prognosis due to their resistance to chemotherapy. A novel tumor marker specific for ovarian mucinous and clear cell adenocarcinomas would be helpful for overcoming these serious diseases. We showed previously by enzymological characterization and RT-PCR that colonic mucinous adenocarcinoma tissues ectopically express GlcNAc6ST-2, a member of the carbohydrate 6-*O*-sulfotransferase family (Seko, A. et al. (2002) *Glycobiology* **12**, 379–388). Here, we prepared a GlcNAc6ST-2-specific polyclonal antibody for immunohistochemical analysis and found that GlcNAc6ST-2 is ectopically expressed by not only colonic mucinous adenocarcinomas but also ovarian mucinous, clear cell and papillary serous adenocarcinomas. In contrast, solid serous adenocarcinomas, endometrioid adenocarcinomas, and mucinous adenomas expressed GlcNAc6ST-2 much less frequently or not at all. RT-PCR analysis confirmed that GlcNAc6ST-2 transcripts are expressed in ovarian mucinous adenocarcinomas but not in mucinous adenomas. In addition, immunohistochemical analysis using sulfated glycan-specific monoclonal antibodies showed that ovarian adenocarcinoma cells express GlcNAc 6-*O*-sulfated glycans, including an L-selectin ligand and its related glycans. These results indicate that GlcNAc6ST-2 would be a novel tumor antigen that is specifically expressed in ovarian mucinous, clear cell and papillary serous adenocarcinomas.

Keywords Ovarian cancer · Tumor marker · Sulfotransferase · Mucinous adenocarcinoma · Sulfated glycan

A. Kanoh
Hanno Discovery Center, TAIHCO Pharmaceutical Co. Ltd., Saitama, Japan

A. Seko · H. Ideo · K. Yamashita (✉)
Innovative Research Initiatives,
Tokyo Institute of Technology, Yokohama; CREST,
Japan Science and Technology Agency, Japan
e-mail: kyamashi@bio.titech.ac.jp
Fax: +81-45-921-4308

M. Yoshida
National Institute of Radiological Sciences, Chiba, Japan

M. Nomoto · S. Yonezawa
Department of Pathology, Kagoshima University, Kagoshima, Japan

M. Sakamoto
Department of Gynecology, Kyoundo Hospital, Tokyo, Japan

R. Kannagi
Department of Molecular Pathology, Aichi Cancer Center, Nagoya, Japan

Abbreviations

Gal	galactose
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
HRP	horseradish peroxidase
LacNAc	*N*-acetyllactosamine

Introduction

Ovarian cancer is one of the most frequent causes of cancer-induced death in women and encompasses several common gynecological malignancies. Ovarian epithelial cancers can be clasified into histological types that also vary in chemosensitivity [1–3]. These include solid serous and endometrioid adenocarcinomas, which are sensitive to *cis*-platinum-based chemotherapy, and mucinous, clear cell and papillary

serous adenocarcinomas, which are chemoresistant. A multivariate analysis showed that patients with solid serous and endometrioid adenocarcinomas have more favorable prognoses for survival than those with clear cell or mucinous adenocarcinomas [4]. Thus, an antigen specific for mucinous, clear cell or papillary serous adenocarcinomas would be helpful for early diagnosis of the disease, determining the prognosis, and monitoring the disease status. Moreover, a specific antigen could also serve as a novel target molecule in immunotherapy.

The levels of various tumor markers in cancer patient sera and tissues are often measured to determine the diagnosis and prognosis. CA125 is the most widely used marker of ovarian epithelial cancers and is considered to be the most useful marker at present [5,6]. However, CA125 has some limitations. In particular, the level of serum CA125 is not elevated in nearly half of all patients with stage I ovarian epithelial cancers [7]. Therefore, a novel tumor marker that is specific for the early stages of ovarian mucinous, clear cell, and papillary serous adenocarcinomas is highly desirable.

We previously demonstrated, using enzymological techniques and RT-PCR, that colonic mucinous adenocarcinomas ectopically express *N*-acetylglucosamine 6-*O*-sulfotransferase-2 (GlcNAc6ST-2) [8], and that non-mucinous adenocarcinomas and normal mucosa do not express this sulfotransferase. GlcNAc6ST-2 (HEC-GlcNAc6ST) belongs to the carbohydrate 6-*O*-sulfotransferase family, members of which transfer sulfate from adenosine 3′-phosphate 5′-phosphosulfate to the C-6 of Gal, GalNAc, or GlcNAc residues in various glycoproteins [9]. In normal human tissues, the expression of GlcNAc6ST-2 mRNA is limited to high endothelial cells of the lymph nodes, pancreas, and liver [10]. GlcNAc6ST-2 expressed in the high endothelial cells of lymphoid tissues is involved in the biosynthesis of the carbohydrate ligand for L-selectin, namely, GlcNAc-6-*O*-sulfated sialyl Lewis X [11,12]. The interaction of this ligand with L-selectin is required for the first step in the process of lymphocyte homing, namely, lymphocyte-endothelial cell adhesion. In colonic mucinous adenocarcinomas, the biological functions in which sulfated glycans synthesized by GlcNAc6ST-2 are involved remain unclear. However, we speculate that the sulfated glycans or the GlcNAc6ST-2 protein itself could serve as tumor markers of mucinous adenocarcinomas in general.

The ectopic expression of GlcNAc6ST-2 in colonic mucinous adenocarcinomas led us to examine whether this gene is expressed in mucinous carcinomas derived from other tissues. We were particularly interested in ovarian mucinous adenocarcinoma because of its poor prognosis. In this report, we used immunohistochemical and RT-PCR methods, and found that chemotherapy-resistant ovarian adenocarcinomas frequently express GlcNAc6ST-2, while benign mucinous adenoma cells and chemotherapy-sensitive ovarian adenocarcinomas express the protein with no or lower frequency. We also found that glycans related to L-selectin ligands and possibly synthesized by GlcNAc6ST-2 are distributed on the cell surface of mucinous, clear cell, and papillary serous adenocarcinoma cells.

Materials and methods

Materials

Fresh samples of normal colonic mucosa and differentiated and mucinous colonic adenocarcinomas were obtained from patients as described in our previous paper [8]. The study was approved by the Kagoshima University Faculty of Medicine Human Investigation Committee (No. H13-4).

Primary human ovarian tumor tissues were obtained, with informed consent, from 66 patients (ranging from 28 to 71 years of age) during surgical operations in Kyoundo hospital and Kagoshima University. The tumor tissues included 10 mucinous carcinomas, 14 clear cell carcinomas, 17 endometrioid carcinomas, 6 papillary serous carcinomas, 14 solid serous carcinomas, and 5 mucinous benign adenomas. Papillary serous carcinoma includes micropapillary architecture over 10% in carcinoma area. For immunohistochemical analysis (see below), the obtained tissues were fixed in 10% formaldehyde, dehydrated in a graded ethanol series, embedded in paraffin, and cut into serial sections using standard methods. For immunohistochemistry with mAb G72, frozen 10-μm thick sections were prepared from the tissues and fixed with acetone at −20°C for 20 min. For analysis of GlcNAc6ST-2 mRNA (see below), the tissues were immediately frozen in liquid nitrogen and later used for the isolation of mRNA.

Antibodies

A cDNA corresponding to the lumenal domain (amino acids 26-386) of GlcNAc6ST-2 was subcloned into the pBlueScript SK vector (Stratagene, La Jolla, CA) by PCR using the following primers: 5′-tttggatccATGTACAGCCACAACATC-3′ (forward) and 5′-tttaagCTTCTCAACCCTCTTAGT-3′ (reverse). The sequence was confirmed by using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The amplified fragments were ligated into the pGEX-6p1 expression vector (Amersham, Piscataway, NJ) between its *Bam* HI and *Sl* I sites. *E. coli* strain BL21 was transformed with the GlcNAc6ST-2/pGEX-6p1 plasmid. Recombinant glutathione &ransferase (GST)-fused GlcNAc6ST-2 was produced and purified using Glutathione Sepharose 4B (Amersham) according to the manufacturer's instructions. An antibody against the truncated GlcNAc6ST-2 protein was then raised in rabbits according to standard procedures. The mouse monoclonal antibodies (mAbs) AG107, AG223, and

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.

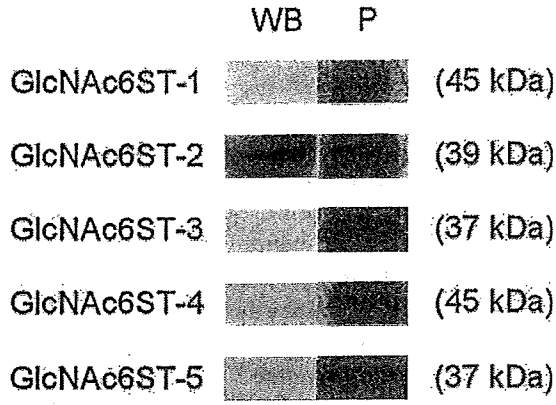

Fig. 1 Confirmation of specificity of anti-GlcNAc6ST-2 polyclonal antibody raised in this study by Western blot analysis using recombinant GlcNAc6STs. WB, Western blot analysis detected by anti-GlcNAc6ST-2 polyclonal antibody; P, Ponceau 3R stain of correspondent bands for five GlcNAc6ST isozymes.

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

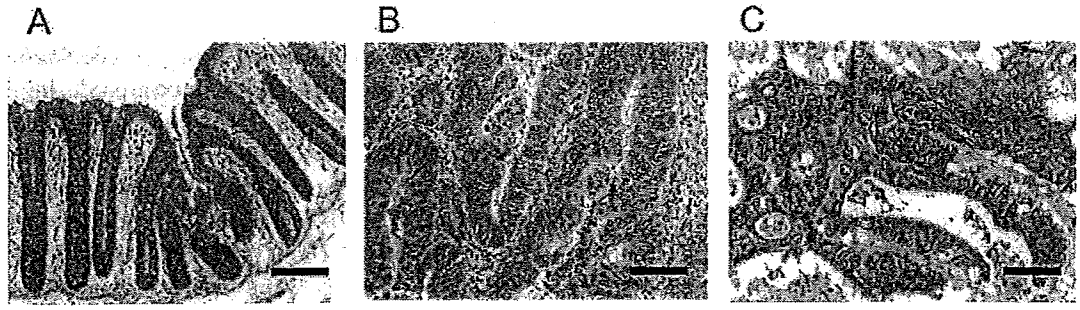

Fig. 2 Immunohistochemical staining for GlcNAc6ST-2 in colon tissue sections. A, normal colon mucosa; B, colonic differentiated adenocarcinoma; C, colonic mucinous adenocarcinoma. (Scale bars: A, 500 μm; B and C, 100 μm).

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%

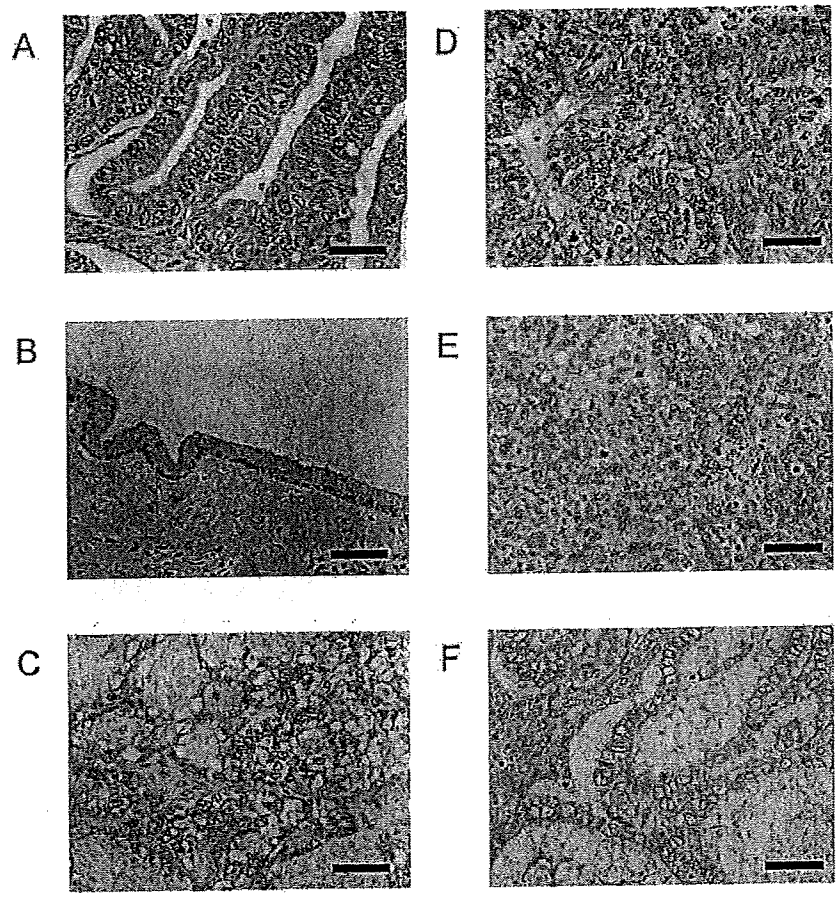

Fig. 3 Immunohistochemical staining for GlcNAc6ST-2 in ovarian tissue sections. A, mucinous adenocarcinoma; B, mucinous adenoma; C, clear cell adenocarcinoma; D, papillary serous adenocarcinoma; E, solid serous adenocarcinoma; F, endometrioid adenocarcinoma. (Scale bars, 50 μm).

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.

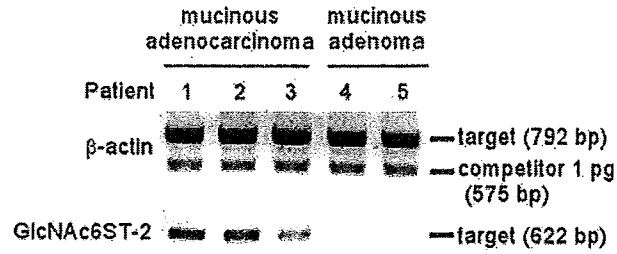

Fig. 4 Analysis of GlcNAc6ST-2 transcripts in mucinous adenocarcinomas and mucinous adenomas. Total RNAs were isolated from tissue blocks obtained from surgical operations and cDNAs were obtained by reverse transcription. The relative amount of cDNA of each sample was estimated by competitive PCR with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

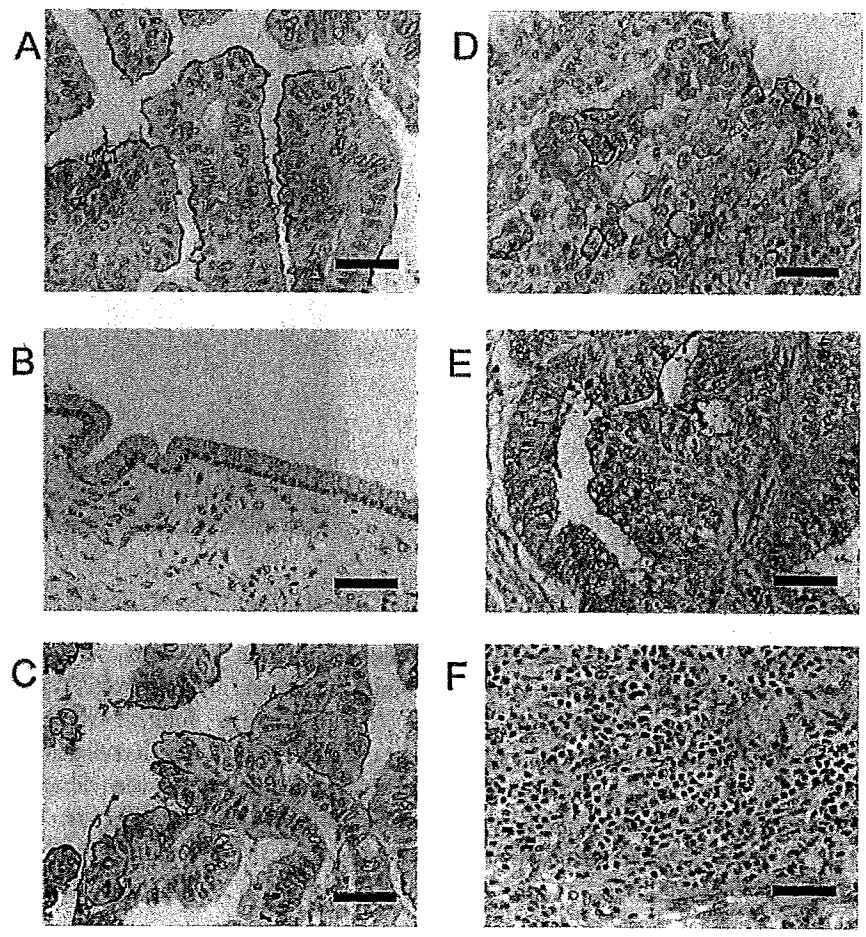

Fig. 5 Immunohistochemical staining with antibodies specific for 6-sulfo-LacNAc (AG107; A, B, D-F) or 6-sulfo-Lewis X (AG223; C). A and C, mucinous adenocarcinoma; B, mucinous adenoma; D, clear cell adenocarcinoma; E, papillary serous adenocarcinoma; F, solid serous adenocarcinoma. (Scale bars, 50 μm).

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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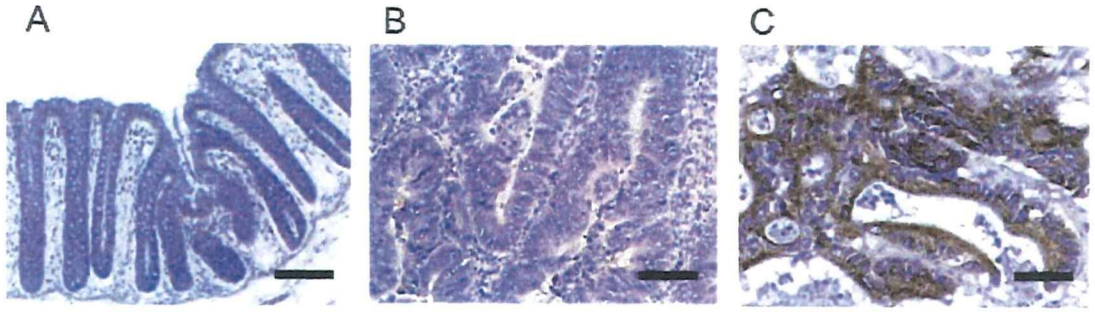

Fig. 2 Immunohistochemical staining for GlcNAc6ST-2 in colon tissue sections. A, normal colon mucosa; B, colonic differentiated adenocarcinoma; C, colonic mucinous adenocarcinoma. (Scale bars: A, 500 μm; B and C, 100 μm).

Fig. 3

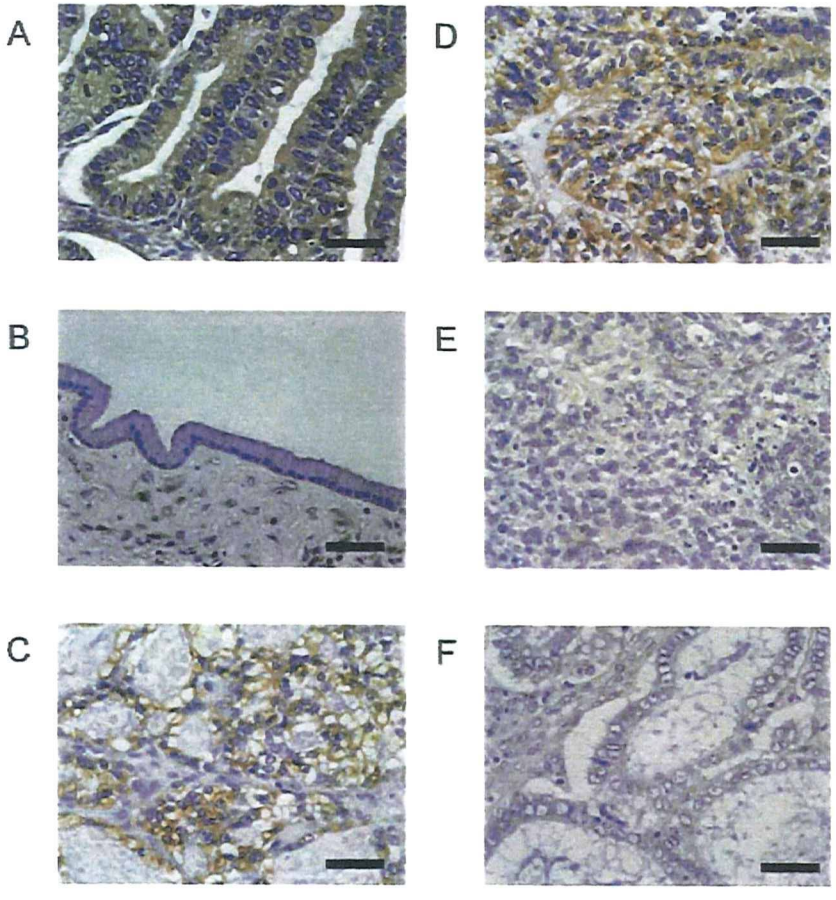

Fig. 5

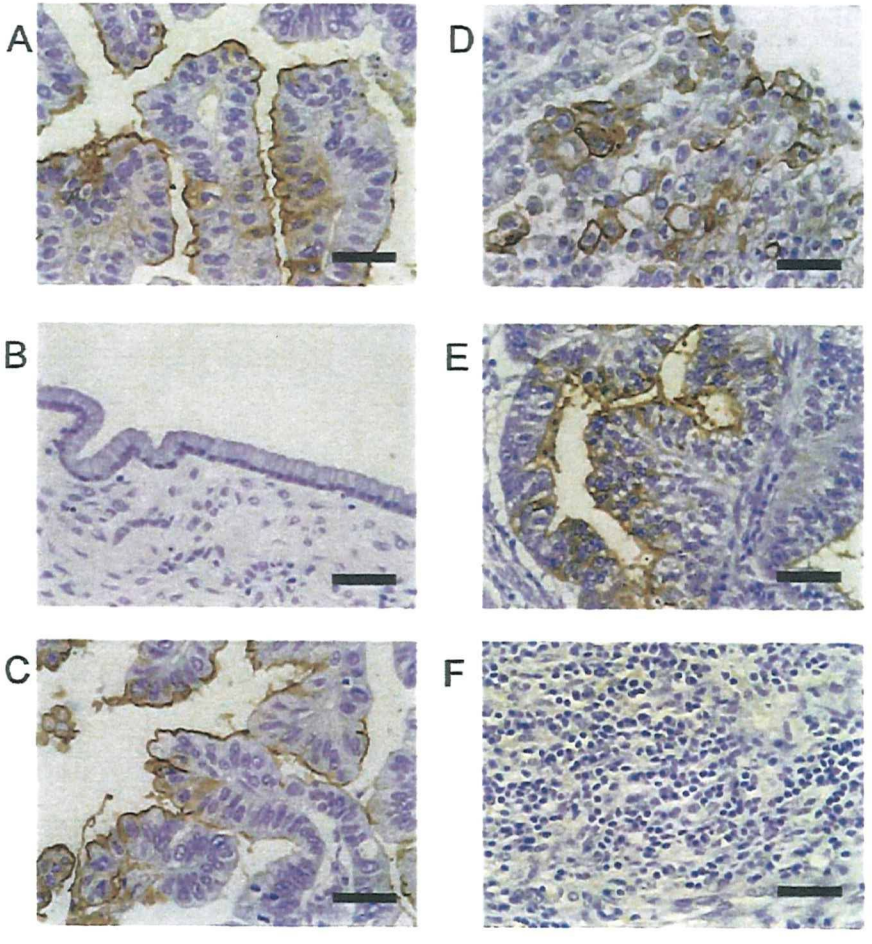

Fig. 3 Immunohistochemical staining for GlcNAc6ST-2 in ovarian tissue sections. A, mucinous adenocarcinoma; B, mucinous adenoma; C, clear cell adenocarcinoma; D, papillary serous adenocarcinoma; E, solid serous adenocarcinoma; F, endometrioid adenocarcinoma. (Scale bars, 50 μm).

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

Midori YOSHIDA[1)], Gen WATANABE[2)], Mariko SHIROTA[3)], Akihiko MAEKAWA[1)] and Kazuyoshi TAYA[2)]

[1)]Department of Pathology, Sasaki Institute, 2–2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101–0062, [2)]Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, and [3)]Hatano Research Institute of Food and Drug Safety Centre, Japan

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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Correspondence: M. Yoshida (e-mail: midoriy@sasaki.or.jp)

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.

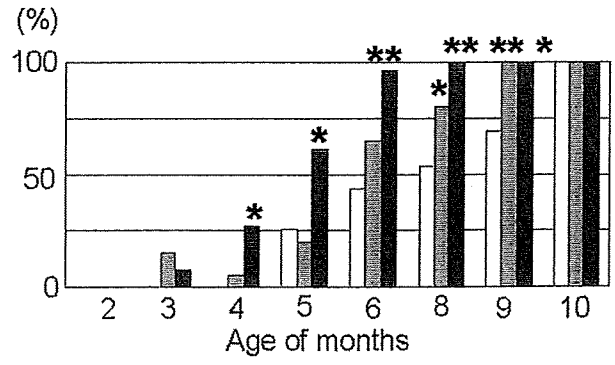

Fig. 1. Sequential changes in incidences of persistent estrus in the controls (□), 2.5 mg/kg busulfan (▨), and 5.0 mg/kg busulfan treated (■) groups. *, ** Significantly different from the controls at P<0.05, and P<0.01, respectively.

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%.

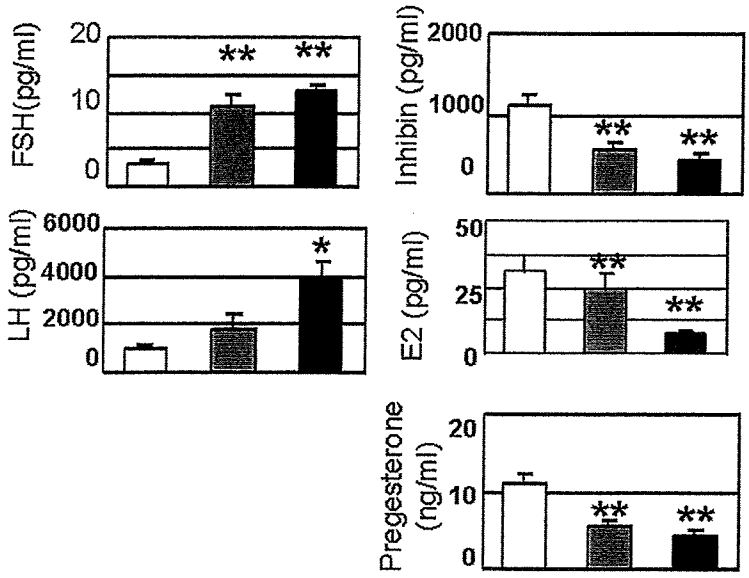

Fig. 2. Hormonal profiles of serum FSH, LH, E2, progesterone and inhibin at the termination of the experiment at 15 months of age in the controls (□), 2.5 mg/kg busulfan (▩), and 5.0 mg/kg busulfan treated groups (■) groups. Results presented are mean ± SE values. *, ** Significantly different from the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

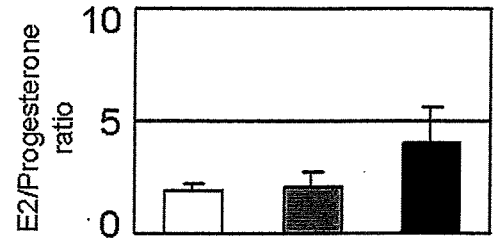

Fig. 3. E2/progesterone ratios at 15 months of age, and at termination of the experiment for the controls (□), 2.5 mg/kg busulfan (▩), and 5.0 mg/kg busulfan treated (■) groups. Results presented are the mean ± SE values.

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] (361.62 ± 85.44)[b]	63.11 ± 44.11 (17.59 ± 12.30)
Busulfan 2.5 mg/kg	18	399.81 ± 72.67	1156.62 ± 516.84 (259.54 ± 160.91)	58.86 ± 43.28 (15.73 ± 12.14)
Busulfan 5.0 mg/kg	23	386.61 ± 46.56	1008.60 ± 405.35* (268.50 ± 123.84)	54.60 ± 47.71 (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%.

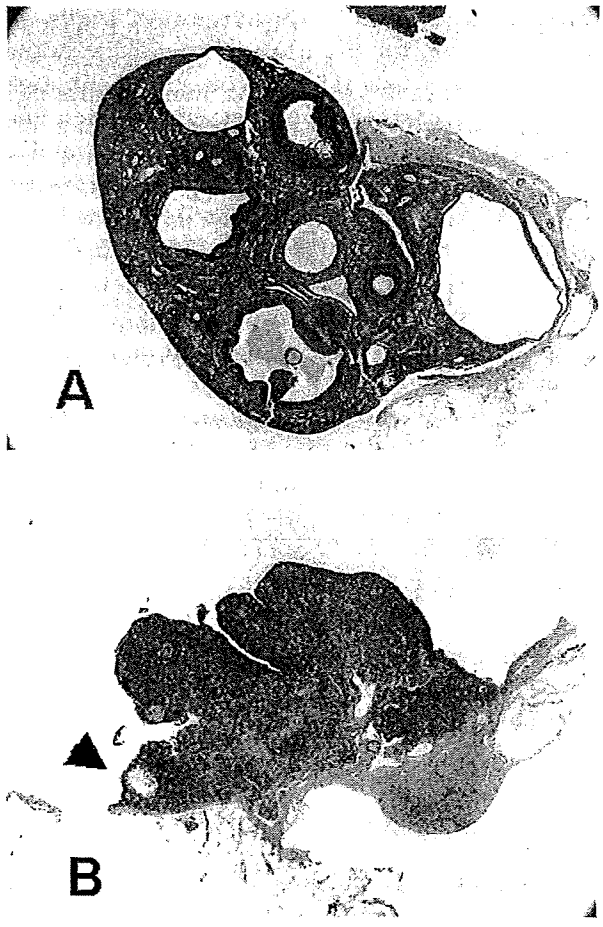

Fig. 4. Microphotographs of ovaries from controls (A) and 5.0 mg/kg busulfan treated animals (B) at 15 months of age. Note several atretic and/or cystic follicles with a lack of corpus and increased interstitial glands in the stroma in the control group (A). Only a small cystic follicle is apparent (arrowhead)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