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Original Article

Hyperglycemia contributes to the development of Leydig cell hyperplasia in male Spontaneously Diabetic Torii rats

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Abstract: Spontaneously Diabetic Torii (SDT) rats are a well-known animal model of non-obese type 2 diabetes mellitus. Although this animal model has been studied extensively over the last decade, the incidence rates of Leydig cell hyperplasia and tumors in this model have not been reported. In this study, pathophysiological analyses of the testes were performed on male SDT rats, to understand the effect of insulin treatment on the development of Leydig cell hyperplasia and tumors and the expression of integrins and extracellular matrix proteins. Testicular Leydig cell hyperplasia and tumors were observed in SDT rats at 64 weeks of age but were rarely identified in Sprague-Dawley (SD) rats of the same age. Insulin treatment decreased plasma glucose and HbA1c levels, and interestingly, decreased the number of hyperplastic Leydig cell foci and Leydig cell tumors in treated animals. A similar reduction in the expression of Ki67 in these Leydig cell foci was also observed. In addition, insulin treatment decreased the expression of integrin α5, integrin β1, integrin αvβ3, fibronectin, and vitronectin in hyperplastic Leydig cell foci. These results suggest that insulin might decrease the incidence of Leydig cell hyperplasia by reducing Leydig cell proliferation and the expression of integrins and extracellular matrix proteins through the reduction of serum glucose concentrations in these animals. (DOI: 10.1293/tox.2019-0088; J Toxicol Pathol 2020; 33: 121–129)

Key words: Leydig, hyperplasia, tumors, hyperglycemia, insulin, Spontaneously Diabetic Torii (SDT) rats

Introduction

Hyperplasia of Leydig cells (LCH) is a benign condition that is characterized by small, multifocal, and frequently bilateral, testicular nodules[1, 2]. Because the sex hormones secreted from Leydig cells and Sertoli cells interact with each other, an imbalance between Sertoli and Leydig cells could induce changes in Leydig cell function and multiplication. Thus, Leydig cells have the potential to transition from hyperplasia to tumors[3, 4].

In rodents, Leydig cell tumors (LCT) are particularly common in F344 rats, but are less frequent in SD rats[5]. Spontaneously Diabetic Torii (SDT) rats, an inbred strain of Sprague-Dawley (SD) rats, are a well-known non-obese type 2 diabetic model that achieve diabetic status with high plasma glucose levels by 42 weeks of age[6]. The occurrence of LCH/LCTs in SDT rats has not been reported. However, our preliminary examination revealed a relatively high rate of occurrence for these anomalies (LCH/LCTs) in aged SDT rats (over 50-week-old) (data not shown).

In humans, LCTs represent 1–3% of all testicular tumors, and 10–15% of these are malignant[7]. LCTs develop at any age in male patients, but there are 2 peak risk periods: age 5–10 years and 25–35 years. As in germ cell tumors, the route of spread is hematogenous and lymphatic through the retroperitoneal lymph nodes. Unlike germ cell tumors, LCTs have relatively low sensitivity to radio- and chemotherapy agents[8]. The treatment of LCH/LCTs consists of surgical inguinal orchidectomy with a high ligature of the spermatic cord. LCH or LCTs are hard to diagnose because of the similar physiological events accompanying this phase of growth. Men with LCH or LCTs show signs of hypogonadism, most frequently gynecomastia, and have low serum testosterone[9–11].

Type 2 diabetes is associated with an increased risk of various cancers, including liver, pancreatic, endometrium, colorectal, breast, and bladder cancers[12, 13]. Several pathophysiological mechanisms for this association have been

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suggested, including insulin resistance, cell adhesion, oxidative stress, endoplasmic reticulum stress, aberrant metabolic state, and inflammation[14–16]. In addition, congenital LCH is observed in infants from diabetic mothers as a result of hyperstimulation by the mother's human chorionic gonadotropin[17]. However, no relationship between hyperglycemia and LCH/LCTs in animal models has been previously described. Moreover, it remains unclear whether diabetes may be associated with increased risk for LCH/LCTs. In tissues from diabetic humans and rats, the expression of integrins and extracellular matrix (ECM) proteins have been shown to be increased[18–20]. Moreover, it has been reported that integrin α5β1 mediates Leydig cell binding to fibronectin and enhances Leydig cell proliferation[21] and an antagonist of integrin αvβ3 inhibits the growth of LCTs in rats[22]. Therefore, it was hypothesized that hyperglycemia might contribute to the occurrence of LCH/LCTs through aberrations in the regulation of integrin and ECM expression. In this study, we investigated the occurrence of LCH/LCTs and assessed whether the prevention of hyperglycemia by insulin treatment may affect the development of LCH/LCTs and the expression of integrins and ECM proteins in aged SDT rats.

Materials and Methods

Animals

All experiments were performed in compliance with institutional Laboratory Guidelines for Animal Experimentation and were approved by the Animal Care and Use Committee of the Central Pharmaceutical Research Institute, Japan Tobacco Inc. Male SDT rats (SDT/Jcl, CLEA Japan Inc., Tokyo, Japan) aged 6 weeks were used. Age-matched SD rats (Crj:CD (SD), Charles River Laboratories Japan Inc., Kanagawa, Japan) were used under similar conditions as the normal control (n=8). The SDT rats were divided into untreated (SDT, n=10) and insulin treated (SDT + insulin, n=10) groups at 20 weeks of age. The rats were housed in a climate-controlled room at a temperature of 23 ± 3°C, humidity of 55 ± 15%, and a 12 h light/dark cycle. Basal diet (CRF-1, Charles River Laboratories Japan Inc.) and water were provided ad libitum. To control plasma glucose levels in insulin-treated SDT rats, an insulin pellet (Linshin Canada Inc., Ontario, Canada) was implanted subcutaneously, while the animals were subjected to light anesthesia between 20 and 44 weeks of age. Insulin was released from these pellets at a maximum rate of 2 U/24 h/implant. Plasma glucose levels were measured once a week (9:00 to 11:00 AM), and additional insulin pellets were implanted if the plasma glucose levels in the SDT rats in the treated group reached >250 mg/dL. All rats used in these experiments were euthanized by exsanguination following isoflurane anesthesia.

Measurement of body weight and biological parameters

Body weight was measured at 20–64 weeks of age at 8–12 weeks intervals. Blood samples were collected in heparinized tubes from the tail vein. Part of the blood samples were then centrifuged at 10,000 × g at 4°C for 5 min to obtain plasma samples. Plasma concentrations of glucose and HbA1c were determined. The plasma glucose levels were measured using the hexokinase method on an automatic biochemical analyzer (Model 7180, Hitachi High-Technologies Corporation, Tokyo, Japan). The total hemoglobin and HbA1c concentrations were determined by the colorimetric method and a turbidimetric inhibition immunoassay, respectively, on an automatic biochemical analyzer. The HbA1c levels were calculated using the following formula of the instruction manual:

HbA1c (%) =

0.94 × HbA1c (g/dL)/total hemoglobin (g/dL) × 100 + 1.63

Tissue sampling and histopathology

The testes of male SDT rats at 64 weeks of age were harvested and fixed in Bouin's solution. The fixed testes were trimmed transversally for cross sectioning including the rete testis. Next, the specimens were embedded in paraffin and cut into 4 μm sections. Subsequently, the sections were stained with hematoxylin and eosin (HE) and immunostained. Images were captured using a fluorescence microscope (BZ-9000, Keyence Corp., Osaka, Japan), and analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA). Outlines of the seminiferous tubules were traced and then measured. The top and bottom 10th percentile values for size were excluded. The average of the remaining values was used as the size of normal seminiferous tubules. The diagnosis of LCT versus LCH was primarily based on whether the size of the Leydig cell mass exceeded the size of three normal seminiferous tubules or not, as described in INHAND[23, 24]. Foci larger than 0.01 mm^2 and smaller than or equal to three times the size of normal seminiferous tubules were classified as LCH and those over three times the size of normal seminiferous tubules were classified as LCTs and counted.

Immunofluorescent microscopy was performed on testis sections. The slides were dewaxed, rehydrated, and subjected to antigen retrieval using antigen retrieval buffer (ab93678, Abcam plc., Cambridge, UK). The sections were blocked with 5% bovine serum albumin and then incubated at 4°C with the anti-Ki67 (NB110-89717, Novus Biologicals, LLC., Centennial, CO, USA), anti-integrin α4 (ab202969, Abcam plc.), anti-integrin α5 (ab150361, Abcam plc.), anti-integrin β1 (ab179471, Abcam plc.), anti-integrin αvβ1 (bs-2016R, Bioss Antibodies Inc., Woburn, MA, USA), anti-integrin αvβ3 (bs-1310R, Bioss Antibodies Inc.), anti-fibronectin (ab2413, Abcam plc.), anti-vitronectin (ab45139, Abcam plc.), and anti-tenascin C (ab108930, Abcam plc.) antibodies overnight. Following washing, goat anti-rabbit IgG H&L Alexa Fluor 488 (ab150077, Abcam plc.) was applied. Slides were then mounted for viewing. Ki67 staining was quantified using a fluorescence microscope and BZ-II Analyzer software (Keyence Corp.) and then calculated as the percentage of positively stained areas within the LCH/LCT foci. Images of integrin and ECM staining were captured using a fluorescence microscope; Leydig cell foci (detected

by fluorescence intensity) and normal seminiferous tubules (used as an internal standard) were quantitatively evaluated using Image J software. Arbitrary units (AU) for the Leydig cell foci and normal seminiferous tubules per unit surface area were calculated. AU (Leydig cell foci/normal seminiferous tubules) were calculated using the following formula:

AU (Leydig cell foci/normal seminiferous tubules) = AU in Leydig cell foci/AU in normal seminiferous tubules

Statistical analysis

Results are expressed as the mean ± standard deviation. Differences between the SD and SDT rats were tested for statistical significance using the F test, followed by the Student *t* test for equal variances or the Aspin-Welch *t* test for unequal variances. The differences between the SDT and insulin-treated SDT rats were evaluated using the F test, followed by the Student *t* test for equal variances or the Aspin-Welch *t* test for unequal variances. StatLight 2000 (C) (Yukms Corp., Kanagawa, Japan) was used for statistical analysis.

Results

Body weight and biological parameters

Body weight was significantly lower in SDT rats compared to SD rats, and was significantly higher in insulin-treated SDT rats compared to SDT rats from at 28 weeks of age (Fig. 1A). Plasma glucose levels in SDT rats were already elevated at 20 weeks of age. In insulin-treated SDT rats, plasma glucose levels decreased, and the levels were similar to those in SD rats from 24 weeks of age (Fig. 1B). HbA1c levels were significantly higher in SDT rats than in SD rats at 64 weeks of age, and similar between the insulin-treated SDT and SD rats (Fig. 1C). Thus, we confirmed that SDT rats develop diabetes without obesity, as reported by Shinohara *et al.* [6].

Histopathological and Leydig cell proliferation analyses

Histopathological changes in the testes are shown in Table 1 and Fig. 2. A high incidence of LCH/LCTs was observed in the testes of SDT rats, but not in the testes of the SD rats (Fig. 2A–D). The mean area of the LCH foci was lower in insulin-treated SDT rats than in SDT rats (Fig. 2G). Insulin treatment also decreased the number of LCH foci (Fig. 2H). The number of LCTs decreased slightly in the insulin-treated SDT rats, but this effect was not significant (Fig. 2I). Moreover, insulin treatment decreased the expression of Ki67 (Fig. 3). Altogether, these results suggest that insulin treatment decreases the number of LCH/LCT foci by inhibiting Leydig cell proliferation in SDT rats.

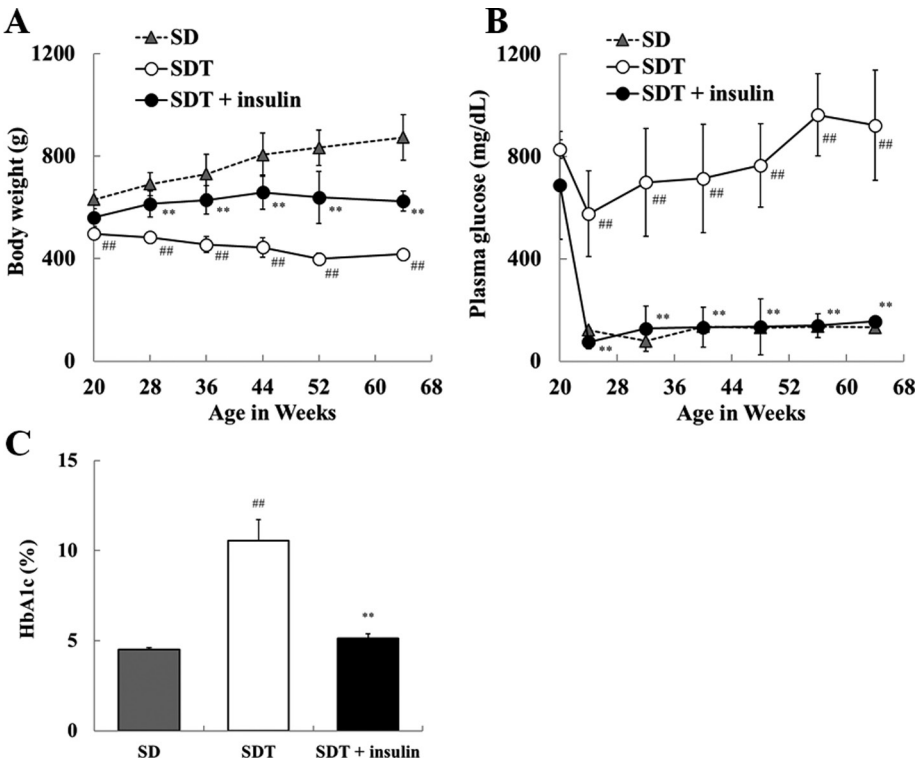

Fig. 1. Body weight and other biological parameters of SD, SDT, and SDT + insulin rats. A: Body weight. B: Plasma glucose levels. C: HbA1c levels at 64 weeks of age. Data represent the mean ± S.D. (SD: n=6–8, SDT: n=4–10, SDT + insulin: n=4–10). ## $p<0.01$: significantly different from SD rats (Student *t* test or Aspin-Welch *t* test). ** $p<0.01$: significantly different from SDT rats (Student *t* test or Aspin-Welch *t* test).

Table 1. Histopathological Findings in the Testes

LCH/LCT count	Group	SD					SDT			SDT + insulin				
	Rat No.	1	2	3	4	5	1	2	3	1	2	3	4	5
Number of LCH foci		N.D.	N.D.	N.D.	N.D.	N.D.	53	52	32	7	21	28	29	4
Number of LCTs		N.D.	N.D.	N.D.	N.D.	N.D.	0	0	2	0	0	0	1	0

N.D.: not detected.

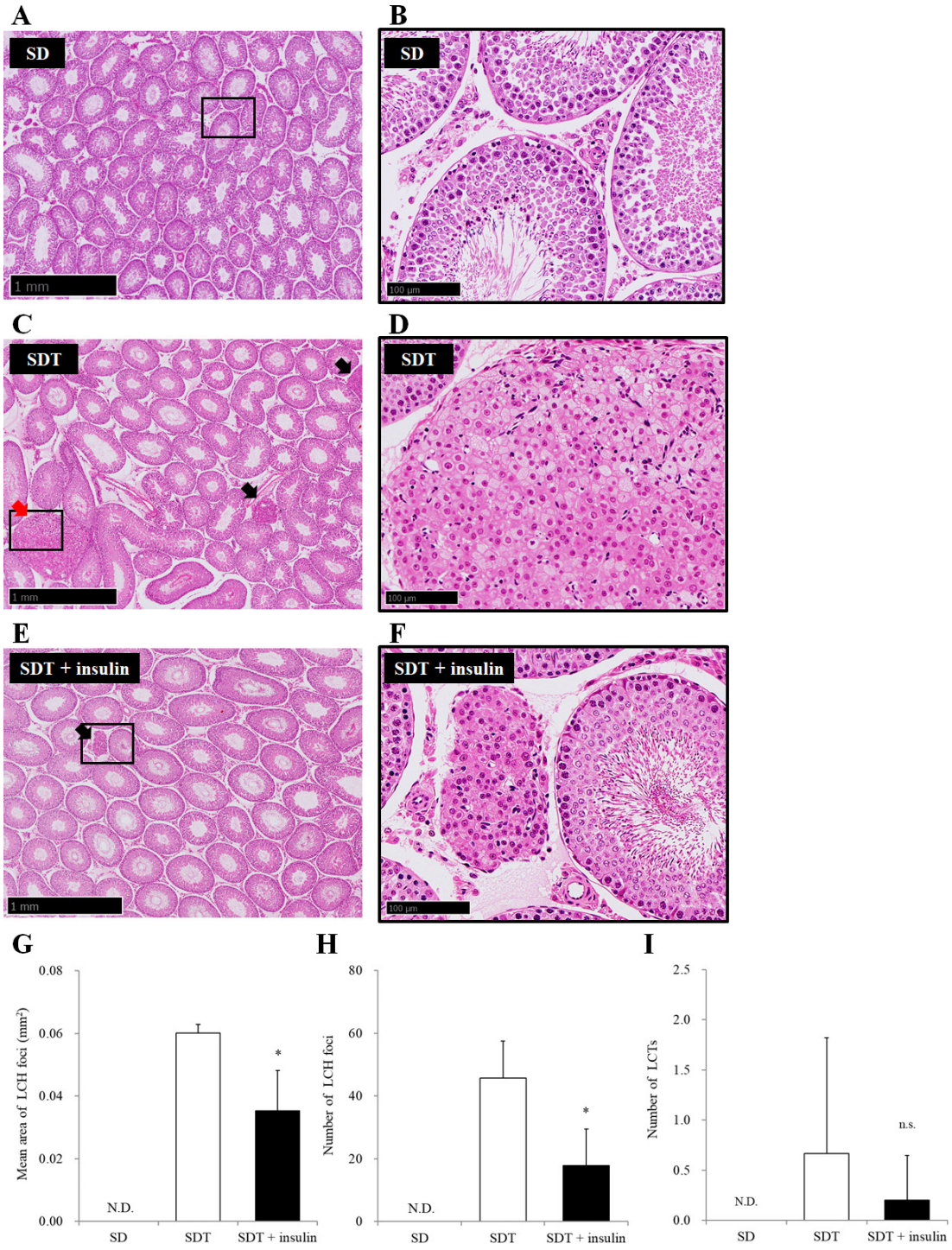

Fig. 2.

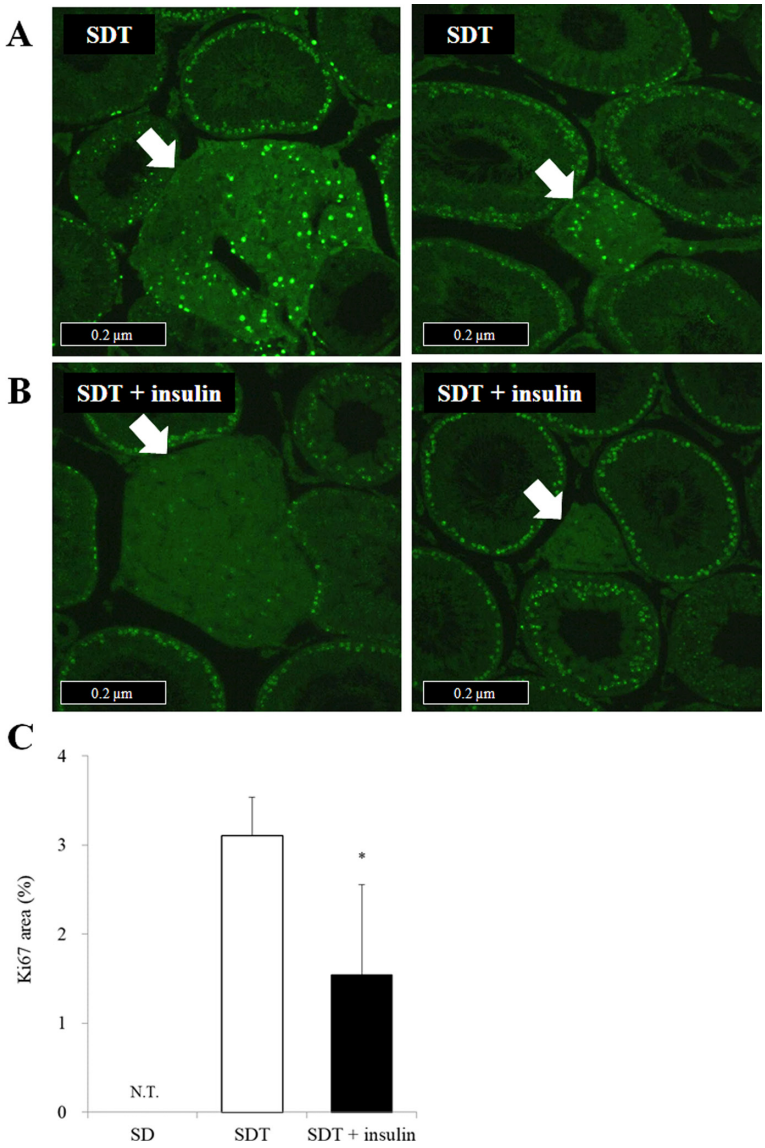

Fig. 3. Cellular proliferation in the testes. A, B: Representative IHC images of proliferating cells visualized using Ki67 (LCH is indicated by the white arrows). C: Quantification of Ki67 staining in LCH/LCTs. Testicular tissues were isolated from 64-week-old SDT rats with and without insulin pellets. Images were captured using a fluorescence microscope (BZ-9000), and BZ-II Analyzer software. Data represent the mean ± S.D. (SDT: n=3, SDT + insulin: n=5). * $p<0.05$: significantly different from the no treatment SDT rats (Student *t* test). N.T.: not tested.

Expression of integrins and extracellular matrix proteins in SDT rats

LCH/LCTs were not observed in the testes of SD rats. Therefore, we evaluated the expression of the integrins and ECM proteins in the Leydig cell foci of SDT rats and insulin-treated SDT rats. The expression levels of both the integrins (α4, α5, β1, αvβ1, and αvβ3) and ECM proteins (fibronectin, vitronectin, and tenascin) were higher in the foci of Leydig cells than those in the normal seminiferous tubules of SDT rats. Insulin treatment decreased the expression of integrin α5, integrin β1, integrin αvβ3, fibronectin, and vitronectin in these foci, but did not significantly affect the expression of integrin α4, integrin αvβ1, and tenascin (Fig. 4).

Fig. 2. Histological analysis of the testes. A–F: HE stained sections showing LCH (indicated by black arrows) and LCT (indicated by a red arrow). A and B: SD rats at 64 weeks of age. C and D: SDT rats at 64 weeks of age. E and F: the insulin-treated SDT rats at 64 weeks of age. G: Mean area values for the LCH foci. H: Number of LCH foci. I: Number of LCTs. Images were captured using a fluorescence microscope (BZ-9000), and analyzed with Image J software. Data represent the mean ± S.D. (SD: n=5, SDT: n=3, SDT + insulin: n=5). * $p<0.05$: significantly different from the SDT rats (Student *t* test or Aspin-Welch *t* test). N.D.: not detected. n.s.: no significant differences.

Discussion

In this study, SDT rats where shown to exhibit hyperglycemia without obesity, as reported by Shinohara *et al*.[6]. In addition, insulin treatment prevented hyperglycemia, suggesting that the onset of hyperglycemia in SDT rats was the result of impaired insulin secretion and not insulin resistance.

Testicular LCH is a benign condition that can result in tumor production[1–4]. In rats, the incidence of LCH/LCTs varies between strains. LCH/LCTs are common in F344 rats (incidence: close to 100%), but are rare in SD rats (incidence: ranging from 1 to 5%)[5]. Although SDT rats are an inbred strain of SD rats, there is a high incidence of LCH/LCTs in SDT rats. Moreover, we demonstrated that preventing hyperglycemia by introducing insulin treatment decreased the number of foci in the LCH/LCTs and Leydig cell proliferation in SDT rats. This suggests that hyperglycemia might contribute to the occurrence of LCH/LCTs in SDT rats.

In both humans and rats, diabetes leads to a higher risk for various cancers including liver, pancreas, endometrium, colorectal, breast, and bladder cancers[12, 13]. Several mechanisms underlying the relationship between diabetes and increased cancer risk have been hypothesized, including

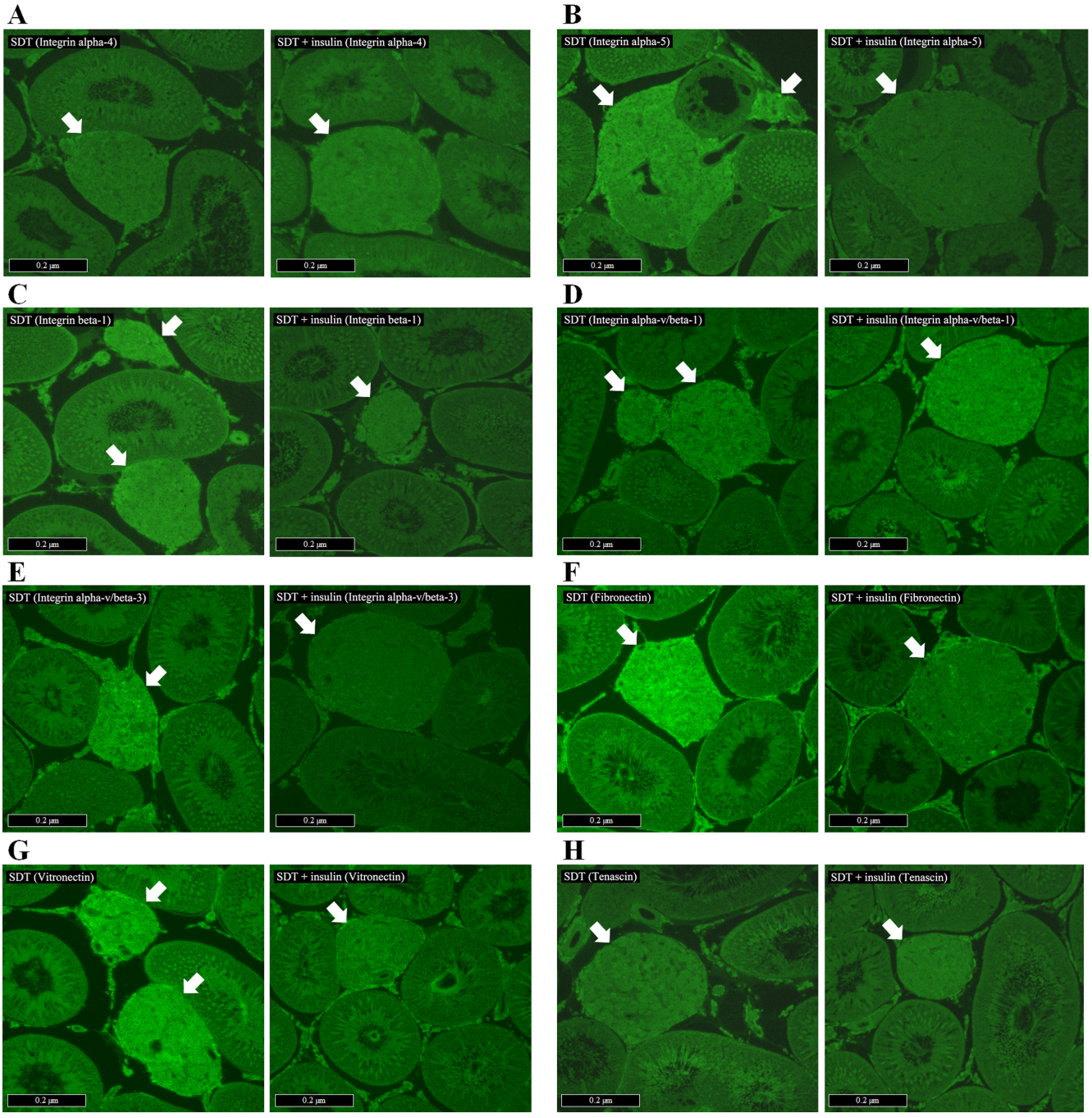

Fig. 4.

increased cell adhesion through upregulated integrin-ECM interactions[14–16]. In fact, the expression of integrins and ECM proteins have been shown to increase in various tissues obtained from diabetic humans and rats[18–20]. Integrins are transmembrane heterodimers that mediate cell-cell and cell-ECM interactions. The integrin family contains at least 12 α subunits and 9 β subunits. It has been reported that integrin α5β1 mediates Leydig cell and fibronectin interactions and enhances Leydig cell proliferation in rats, but it rarely facilitates binding to collagen type I or laminin[21]. Moreover, an antagonist of integrin αvβ3 inhibits the growth of LCTs and the development of malignancy-related hypercalcemia in rats[22]. Integrin α5β1 and αvβ3 bind fibrinogen, vitronectin, and tenascin[25]. In this study, insulin treatment decreased the number of LCH/LCTs and decreased the expression of integrin α5, integrin β1, integrin αvβ3, fibronectin, and vitronectin in Leydig cell foci, suggesting that insulin-induced lowering of plasma glucose levels might decrease integrin and ECM expression, preventing Leydig cell proliferation and reducing LCH/LCT occurrence.

There are some limitations to this study. The incidence of LCTs is much lower in humans than in rodents. The interspecies differences in spontaneous LCT occurrence have always been considered the result of quantitative and qualitative differences in Leydig cell responses to hormonal stimuli. There are several differences between rat and hu-

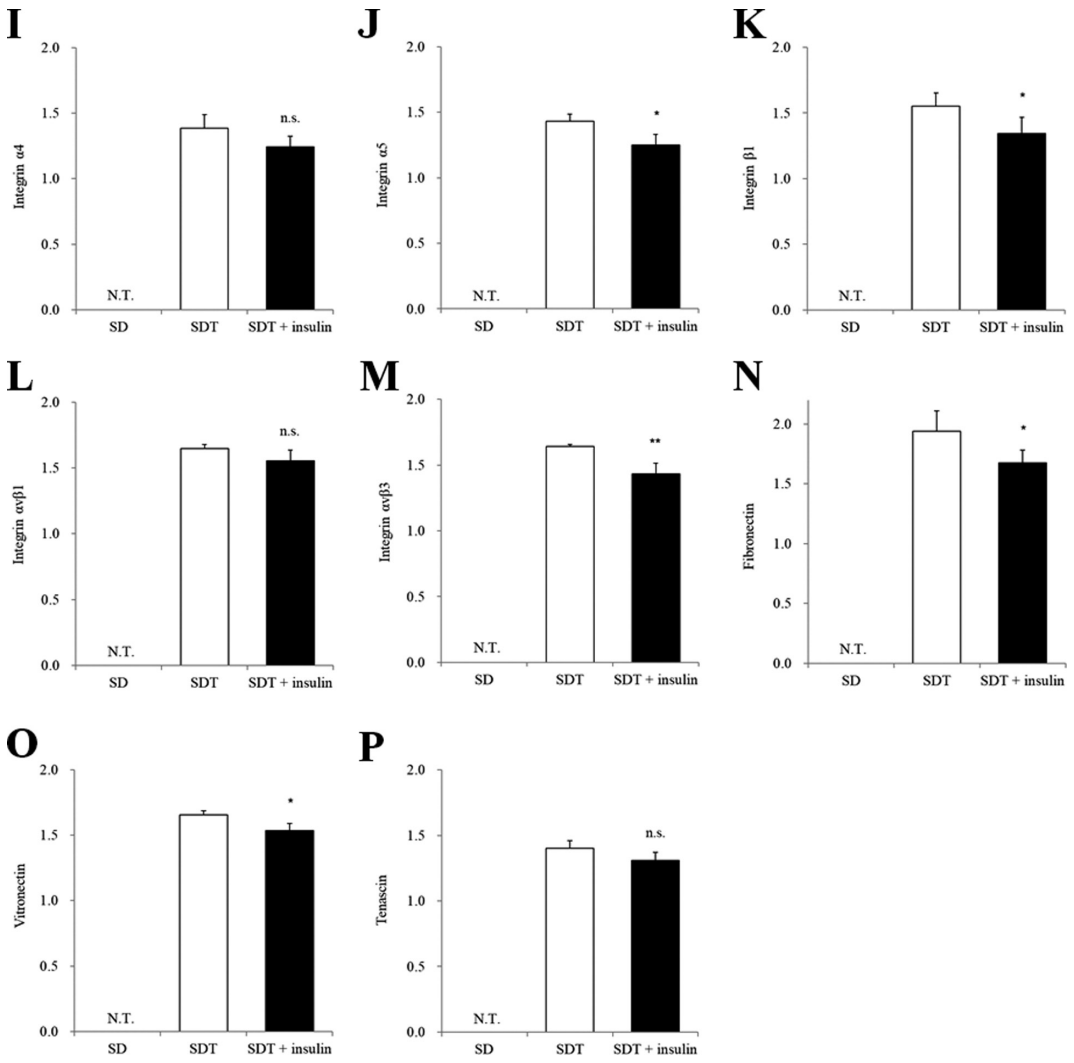

Fig. 4. Immunohistological analysis of testicular samples. A–H: Representative images for each of the proteins characterized in this study. A: Integrin α4. B: Integrin α5. C: Integrin β1. D: Integrin αvβ1. E: Integrin αvβ3. F: Fibronectin. G: Vitronectin. H: Tenascin. I–P: Quantification of expression. I: Integrin α4. J: Integrin α5. K: Integrin β1. L: Integrin αvβ1. M: Integrin αvβ3. N: Fibronectin. O: Vitronectin. P: Tenascin. Testicular sections were isolated from 64-week-old SDT rats with and without insulin pellets. Images were captured using a fluorescence microscope (BZ-9000), and BZ-II Analyzer software. Data represent the mean ± S.D. (SDT: n=3, SDT + insulin: n=5). * $p<0.05$, ** $p<0.01$: significantly different from the no treatment SDT rats (Student *t* test or Aspin-Welch *t* test). N.T.: not tested. n.s.: no significant differences.

man Leydig cells that may contribute to these discrepancies, including differences in the expression levels of some hormone receptors. For instance, gonadotropin-releasing hormone and prolactin receptors are either not expressed or are expressed at very low levels in the testes of humans. Besides, prolonged overexpression of luteinizing hormone (LH) is considered to be important for LCT occurrence in rats[26, 27]. Testosterone administration has been found to decrease LH levels and the occurrence of LCH/LCT foci in F344 rats[28]. In SDT rats, blood testosterone levels decreased following the onset of diabetes[29]. Decreased testosterone levels may contribute to increased LH levels in SDT rats. In addition, the binding of Leydig cells to fibronectin decreases testosterone production in rats[30]. In this study, insulin treatment decreased the expression of fibronectin. Thus, downregulation of fibronectin by insulin might increase testosterone production, and this increase may prevent the formation of LCH/LCTs. However, we did not evaluate the expression of these receptors or hormones in this study. Thus, further studies are needed to examine the effects of insulin treatment on these receptors and hormones in SDT rats and document any species-specific differences in these effects. Because of the small sample size (n=3 in SDT rats), this study may not be adequately sensitive to detect notable changes in some of the parameters, limiting its applicability to larger discussions in the field. However, it is an interesting proof of concept and larger studies should be undertaken to allow for the expansion of these observations.

In conclusion, we show that insulin treatment decreased plasma glucose levels and HbA1c levels in rats, and interestingly, decreased the number of LCH/LCTs and Leydig cell proliferation. In addition, insulin treatment decreased the expression of integrin α5, integrin β1, integrin αvβ3, fibronectin, and vitronectin in hyperplastic Leydig cell foci. These results suggest that insulin lowers the glucose levels and this lowering effects might decrease the incidence of LCH, by reducing Leydig cell proliferation and the expression of integrins and extracellular matrix proteins in SDT rats. SDT rats have several features that make them a useful novel animal model to investigate the relationships between hyperglycemia and the occurrence of hyperplasia and tumors.

Disclosure of Potential Conflicts of Interest: Yoshitomi Nakane, Yusuke Kemmochi, Naoto Ogawa, and Tomohiko Sasase are employees of Japan Tobacco Inc. Takeshi Ohta, Yoshikazu Higami, and Fumio Fukai have no conflict of interest.

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