

Original Article

Carcinogen-induced Thyroid Proliferative Lesions in Wistar Hannover GALAS Rats with Thyroid Dysplasia

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Abstract: Incidences and morphological features of thyroid proliferative lesions induced by carcinogens in Wistar Hannover GALAS rats (GALAS rats) showing normal growth with or without thyroid dysplasia were examined. All thyroid tissue samples were obtained from our recently conducted study using male GALAS rats treated with 5 carcinogens according to the medium-term multiorgan carcinogenicity bioassay protocol (called DMBDD treatment). In the DMBDD-treated rats, thyroid dysplasia was found in 9 out of 114 rats. Follicular cell adenomas were found in 5 out of 9 rats with thyroid dysplasia and in 7 out of 105 rats without thyroid dysplasia. The incidence of adenoma was significantly increased in rats with thyroid dysplasia (55.6%) compared with that in rats without thyroid dysplasia (6.7%). Adenomas in rats with thyroid dysplasia were observed as single or multiple nodules, well demarcated and composed of variously sized vacuolated cells or unvacuolated cells. These histopathological features and staining profiles of luminal colloid for PAS and thyroglobulin, together with PCNA-positive cells, were fundamentally similar to those of rats without thyroid dysplasia. On the other hand, the luminal colloid in adenomas of rats with thyroid dysplasia had a tendency to be poorly stained for T₄ compared with that of rats without thyroid dysplasia. From these findings, it appears that dysplastic thyroids of rats showing normal growth are more sensitive to carcinogens than normal thyroids. In addition, the morphological features of carcinogen-induced thyroid proliferative lesions in GALAS rats with thyroid dysplasia were fundamentally similar to those of rats without thyroid dysplasia, except for the vacuoles and T₄ staining profile. (DOI: 10.1293/tox.25.11; *J Toxicol Pathol* 2012; 25: 11–17)

Key words: multiorgan carcinogenesis model, thyroid dysplasia, thyroid proliferative lesion, Wistar Hannover GALAS rats

Introduction

Wistar Hannover GALAS rats (GALAS rats) have recently been used in toxicity studies in Japan because of their beneficial characteristics such as a higher survival rate and a lower body weight than other ordinary strains, such as Sprague-Dawley and Fisher 344¹. In GALAS rats, it has been reported that vacuolar change of thyroid follicular cells sometimes occurs as a spontaneous lesion². Histopathologically, this lesion is characterized by huge vacuoles in the follicular cells, which are distributed diffusely within the thyroid². The vacuole formation is attributed to the dilation

of rough endoplasmic reticula (r-ERs) that are present at the basal site of the follicular cells². The vacuolar change of thyroid follicular cells has been called ‘thyroid dysplasia’³ and confirmed as an autosomal recessive hereditary disorder^{3,4}. Homozygous animals with thyroid dysplasia exhibit dwarfism due to hypothyroidism. These thyroids show little colloid formation and decreased follicular size, and the serum values of growth hormone (GH), triiodothyronine (T₃) and thyroxine (T₄) are markedly low in the dwarf rats^{3,4}. On the other hand, heterozygous animals exhibit thyroid dysplasia without external abnormality or endocrinological derangement²⁻⁴. There are several reports regarding spontaneous thyroid proliferative lesions in GALAS rats showing normal growth with thyroid dysplasia³ and in dwarfs⁵. However, few reports have been published that deal with chemical-induced thyroid proliferative lesions in these rats.

We observed the development of thyroid proliferative lesions in male GALAS rats showing normal growth with thyroid dysplasia in a recently conducted study⁶. That study was based on the medium-term multiorgan carcinogenicity bioassay protocol featuring a combined treatment with 5 chemical carcinogens⁷⁻⁹. In the present study, we evaluated

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the incidences of carcinogen-induced thyroid proliferative lesions in GALAS rats with or without thyroid dysplasia, and examined the histopathological, histochemical and immunohistochemical features of these thyroid proliferative lesions.

Materials and Methods

Thyroid tissue samples

All thyroid tissue samples were obtained from our recently conducted medium-term multiorgan carcinogenicity bioassay using male Wistar Hannover GALAS (BrlHan:WIST@Jcl, GALAS) rats with an externally normal appearance⁶. Briefly, 6-week-old rats provided by CLEA Japan, Inc. (Tokyo, Japan) were subjected to a combined treatment with 5 carcinogens targeting different organs (the DMBDD treatment: a single intraperitoneal administration of 100 mg/kg body weight of *N*-nitrosodiethylamine at commencement, 4 intraperitoneal administrations of 20 mg/kg body weight each of *N*-methylnitrosourea during the first 2 weeks, 4 subcutaneous administrations of 40 mg/kg body weight each of 1,2-dimethylhydrazine during the second 2 weeks and continuous administrations by admixing into the drinking water *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine at a concentration of 0.05% for the first 2 weeks and 2,2'-dihydroxy-di-*n*-propylnitrosamine at a concentration of 0.1% for the second 2 weeks) or their vehicles during the initial 4-week period. Subsequently, all rats were given a basal diet or a diet containing test substances (copper gluconate and green tea catechins) for 25 weeks and then sacrificed under light ether anesthesia to obtain major organs/tissues including the thyroids. These were fixed in a 10% neutrally buffered formalin solution, embedded in paraffin and sectioned to prepare 4- μ m-thick specimens. Because none of the used test substances alone exerted carcinogenic or toxicological effects on the thyroid in the rats with or without the DMBDD treatment, we considered that there was no effect of the test substance treatment on the thyroids and handled the DMBDD-treated rats as a one group in this study. As a result, thyroid tissue samples were available from 114 DMBDD-treated rats. These experiments were conducted according to the Guidelines for Animal Experimentation, Japanese Association for Laboratory Animal Science, 1987.

Histological, histochemical and immunohistochemical examinations

Histological examination was performed on the thyroid specimens processed by a routine hematoxylin and eosin (H.E.) staining procedure. We diagnosed the thyroid proliferative lesions according to the criteria of Boorman and Elvell¹⁰ and counted their numbers from bilateral thyroid glands on one section. The thyroid specimens for which proliferative lesions were observed were sectioned additionally and used for the following examinations. For histochemical examination, the specimens were processed for the periodic acid-Schiff (PAS) reaction. For immunohistochemical examinations, the specimens were stained for thyroglobulin (polyclonal antibody, Dako Japan Inc., Kyoto, Japan; 100-

fold dilution), L-thyroxine (T_4) (polyclonal antibody, Sigma-Aldrich, Inc., St. Louis, MO, USA; 100-fold dilution) and calcitonin (polyclonal antibody, Dako; 100-fold dilution) using a labeled polymer method (Histofine Simple Stain Rat MAX-PO (MULTI), Nichirei Biosciences Inc., Tokyo, Japan) and for proliferating cell nuclear antigen (PCNA) (monoclonal antibody, clone PC10, Dako; 50-fold dilution) using an avidin-biotin complex method (Vectastain ABC Kit, Vector Laboratories Inc., Burlingame, CA, USA).

Statistics

Fisher's exact test was performed for intergroup differences of lesion incidences between rats with thyroid dysplasia and rats without thyroid dysplasia. For intergroup differences of lesion multiplicities, the F-test was performed, followed by the Aspin-Welch test when there was a significant difference or by the Student's t-test when there was no significant difference. The level of significance was set at $P < 0.05$.

Results

The incidences of thyroid dysplasia and proliferative lesions in the DMBDD-treated rats are listed in Table 1. Thyroid dysplasia was found in 9 out of 114 rats. Follicular cell hyperplasia was found in 1 out of 9 rats with thyroid dysplasia and 6 out of 105 rats without thyroid dysplasia. There was no significant difference in the incidence of hyperplasia in rats with thyroid dysplasia (11.1%) compared with that of rats without thyroid dysplasia (5.7%). Follicular cell adenomas were found in 5 out of 9 rats with thyroid dysplasia and 7 out of 105 rats without thyroid dysplasia. The incidence of adenoma was significantly increased in rats with thyroid dysplasia (55.6%) compared with that of rats without thyroid dysplasia (6.7%). There was no significant difference in the multiplicity of adenoma in rats with thyroid dysplasia ($4.4 \pm 5.5/\text{rat}$) compared with that of rats without thyroid dysplasia ($1.4 \pm 0.79/\text{rat}$).

Histopathologically, follicular cell hyperplasia, which was detected in one rat with thyroid dysplasia, was observed as one small proliferative follicle lined by a single layer of epithelium with small vacuoles that partly formed a short papillary projection into the lumen (Figs. 1A and B). Follicular cell adenomas of rats with thyroid dysplasia were observed as single or multiple nodules in unilateral or bilateral thyroid glands. They were well demarcated, slightly compressed their adjacent tissue and lacked a capsule (Fig. 2A). These follicles were filled with colloid, but one adenoma lacked luminal colloid, and the follicles contained eosinophilic debris (Fig. 2A, nodule i). Adenomas were composed of follicles with various sizes and were principally lined by a single layer of epithelial cells (Fig. 2A, nodules f, g, h and i). Occasionally, they formed irregular papillary projections into the lumen (Fig. 2B, high magnification of nodule h). The follicular cells of adenomas showed several different characteristics such as a high columnar epithelium with huge vacuoles (Fig. 2C lower half, high magnification of nodule f), a

Table 1. Incidences of Thyroid Dysplasia and Proliferative Lesions in the DMBDD-treated Rats

Proliferative lesion	Total number of rats	114	
	Number of rats	Rats with thyroid dysplasia 9 (7.9%) ^a	Rats without thyroid dysplasia 105 (92.1%)
Follicular cell hyperplasia			
Number of rats		1 (11.1%)	6 (5.7%)
Number of lesions ^b		1	11
Multiplicity ^c		1.0	1.8 ± 0.75 ^d
Follicular cell adenoma			
Number of rats		5 (55.6%)*	7 (6.7%)
Number of lesions		22	10
Multiplicity		4.4 ± 5.5	1.4 ± 0.79
Follicular cell carcinoma			
Number of rats		0 (0.0%)	1 (1.0%)
Number of lesions		0	1
Multiplicity		0.0	1.0

* Significantly different from the incidence of adenomas of rats without thyroid dysplasia ($P < 0.05$). ^a Values in parentheses show incidences. ^b Number of proliferative lesions was counted from bilateral thyroid glands on one section. ^c Mean number of proliferative lesions per rat. ^d Mean ± SD.

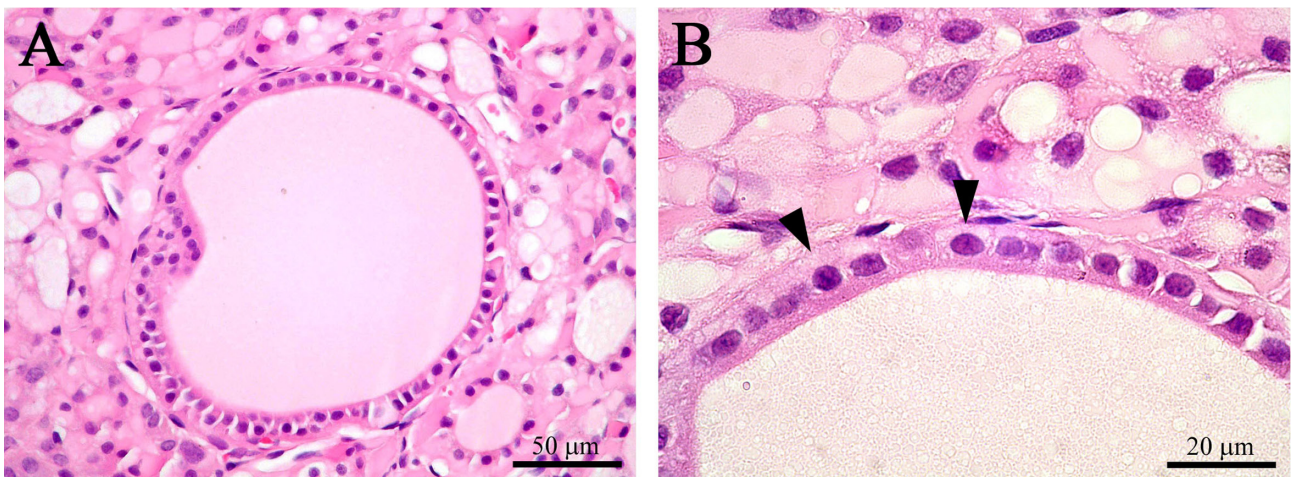


Fig. 1. Follicular cell hyperplasia in the DMBDD-treated rats with thyroid dysplasia (H.E. staining). The follicle is lined by a single layer of epithelial cells (A) with small vacuoles (arrowheads) (B).

low columnar epithelium with small vacuoles (Fig. 2D, high magnification of nodule g) and a basophilic epithelium without vacuoles (Fig. 2C upper half, high magnification of nodule h). All adenomas in rats with thyroid dysplasia showed a low degree of cellular atypia and no invasion or metastasis regardless of being composed of vacuolated or unvacuolated cells. In addition, the histopathological features of hyperplasias and adenomas of rats with thyroid dysplasia were fundamentally similar to those of rats without thyroid dysplasia except for the vacuoles.

The staining properties for follicular cell adenomas in the DMBDD-treated rats with or without thyroid dysplasia are listed in Table 2. The staining profiles of luminal colloid for PAS (Fig. 3A) and thyroglobulin (Fig. 3B), together with PCNA-positive cells in adenomas seen in rats with thyroid dysplasia, were fundamentally close to those of the counterparts seen in rats without thyroid dysplasia (Table 2). On

the other hand, the luminal colloid of all adenomas in rats without thyroid dysplasia was minimally or slightly positive for T_4 , while about one-quarter of that in rats with thyroid dysplasia were negative for T_4 (Fig. 3C and Table 2). The luminal colloid of surrounding follicular cells in rats with or without thyroid dysplasia was slightly positive for PAS, thyroglobulin and T_4 . In addition, the luminal colloid of a carcinoma in a rat without thyroid dysplasia was moderately positive for PAS but negative for thyroglobulin and T_4 , and the PCNA-positive cells were detected moderately. The materials of intracellular vacuoles were negative for PAS, thyroglobulin and T_4 in follicular cells regardless of hyperplasia, adenoma or their surrounding follicular cells in rats with thyroid dysplasia. All proliferative lesions in the thyroids examined in this study were negative for calcitonin, while C-cells in the adjacent tissues demonstrated a positive signal (Fig. 3D).

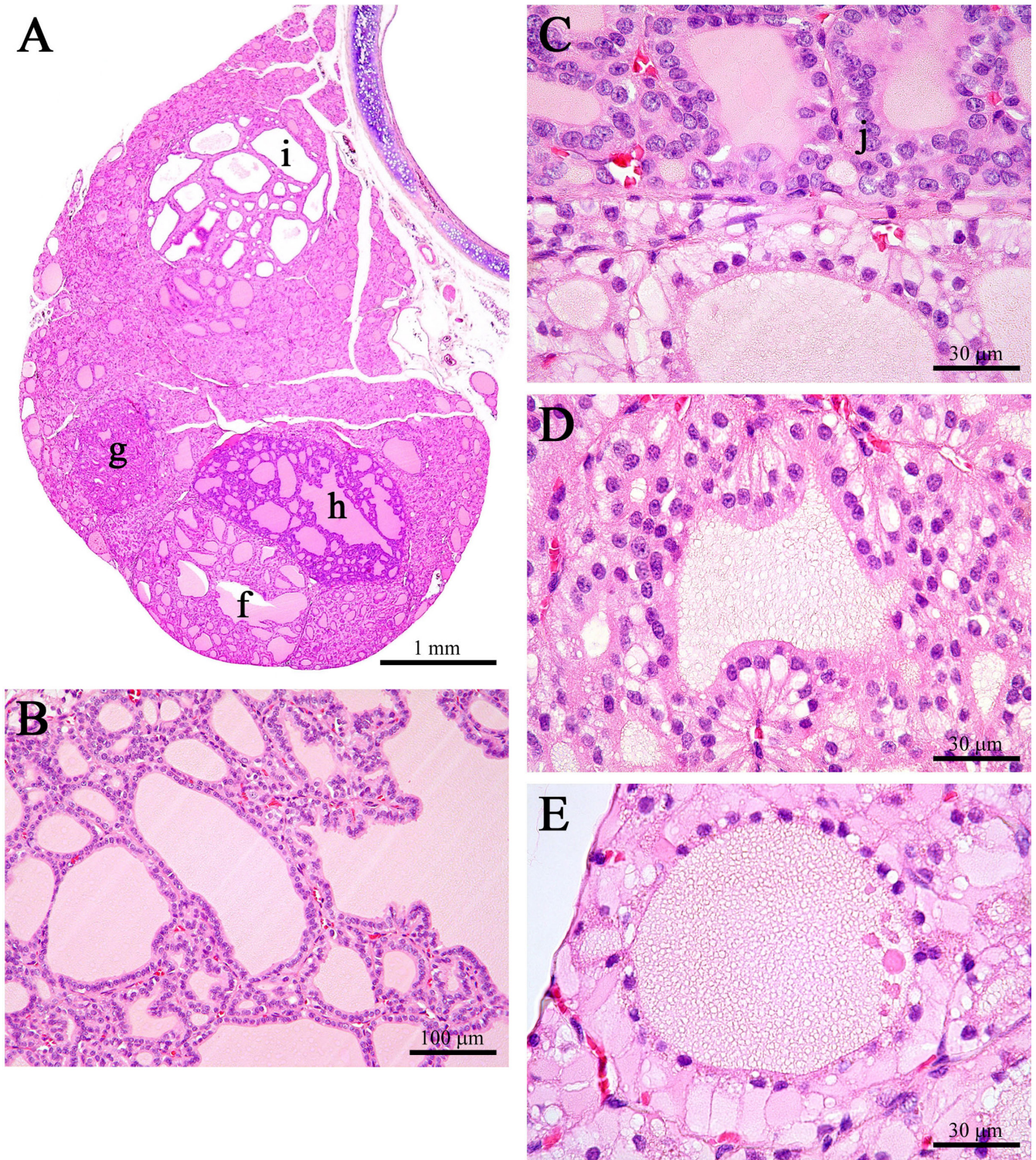


Fig. 2. Follicular cell adenomas in the DMBDD-treated rats with thyroid dysplasia (H.E. staining). The adenomas are observed as multiple nodules, well demarcated and slightly compressing their adjacent tissue (A). They were principally lined by a single layer of epithelial cells (A, nodules f, g, h and i) and occasionally formed irregular papillary projections into the lumen (B, high magnification of nodule h). The follicular cells of adenomas are composed of a high columnar epithelium with huge vacuoles (C lower half, high magnification of nodule f), a low columnar epithelium with small vacuoles (D, high magnification of nodule g) or a basophilic epithelium without vacuoles (C upper half, high magnification of nodule h). The thyroid follicular cells in the adjacent tissue demonstrated thyroid dysplasia (E).

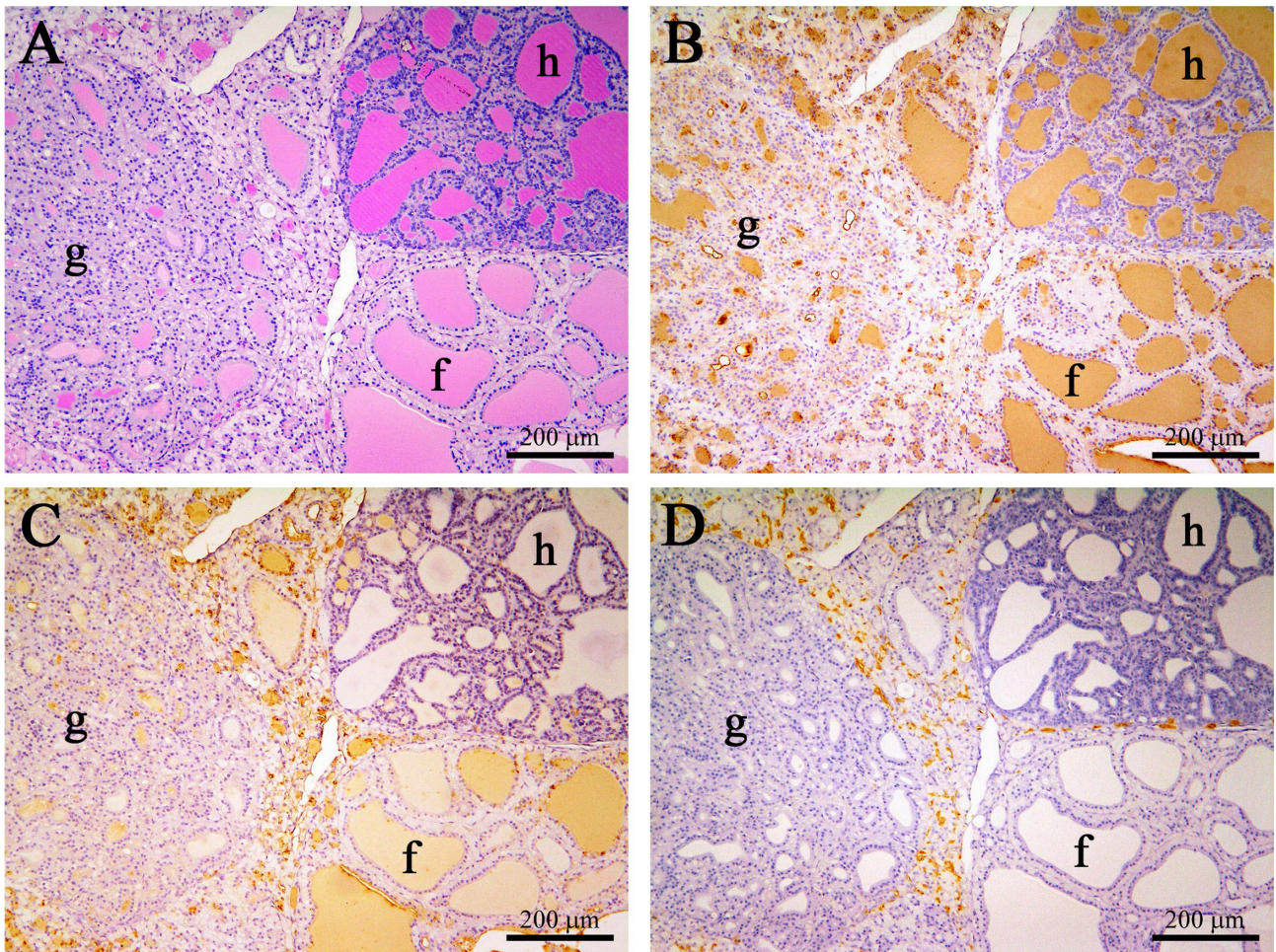


Fig. 3. PAS features (A) and immunostainings for thyroglobulin (B), T₄ (C) and calcitonin (D) of follicular cell adenomas in the DMBDD-treated rats with thyroid dysplasia. Nodules f, g and h are identical to those referred to in Fig. 2A.

Table 2. Staining Properties for Follicular Cell Adenomas in the DMBDD-treated Rats with or without Thyroid Dysplasia

Classification of rats	Number of adenomas ^a	Luminal colloid												Follicular cells			
		PAS				Thyroglobulin				T ₄				PCNA			
		-	±	+	++	-	±	+	++	-	±	+	++	-	±	+	++
Rats with thyroid dysplasia	22	1	0	19	2	1	0	21	0	5	6	11	0	0	8	12	2
Rats without thyroid dysplasia	10	0	1	9	0	0	1	9	0	0	3	7	0	0	0	7	3

Grade signs -, ±, + and ++ represent none, minimal, slight and moderate, respectively. ^a Number of adenomas was counted from bilateral thyroid glands on one section.

Discussion

There are several reports regarding spontaneous thyroid proliferative lesions in GALAS rats with thyroid dysplasia. Weber *et al.* described that GALAS rats with normal growth but thyroid dysplasia did not exhibit higher incidences of preneoplastic or neoplastic changes in the thyroid gland in oncogenicity studies³. On the other hand, it has been shown that the incidence of thyroid tumor in dwarf rats with thyroid dysplasia is higher than that of normal GALAS rats⁵. In the dwarf rats, little colloid formation and a decreased fol-

licular size are observed in the thyroid, and the serum values of GH, T₃ and T₄ are markedly low^{3,4}. The dwarf rats are thought to be affected by primary hypothyroidism, which induces the hypersecretion of thyroid stimulating hormone (TSH) from the anterior pituitary^{3,4}. The persistent hypersecretion of TSH is involved in the increased incidence of thyroid and TSH-producing pituitary tumor⁵. In the present study, the incidence of adenoma in rats with thyroid dysplasia was significantly greater than that of rats without thyroid dysplasia in the DMBDD-treated rats. Although the plasma hormone levels were not determined in this study, no hy-

peritrophy or proliferative lesions were observed in the pituitary of rats both with and without thyroid dysplasia. From these observations, it appears that dysplastic thyroids of rats showing normal growth are more sensitive to carcinogens than normal thyroids, which is considered to be independent of the persistent TSH hypersecretion. Further studies will be needed to elucidate the mechanisms of high sensitivity to carcinogens in GALAS rats showing normal growth with thyroid dysplasia.

The adenomas induced by carcinogens in rats with thyroid dysplasia were composed of variously sized vacuolated cells or unvacuolated cells. These cellular morphological profiles may be just variation and attributable to the genetic background of GALAS rats with the intracellular vacuoles caused by the dilation of r-ERs. The materials of intracellular vacuoles react positively for thyroglobulin in the dwarfs of GALAS rats^{3,4}. The cause of vacuolation was thought to be a mutation of the thyroglobulin gene resulting in retention of misfolded thyroglobulin in r-ERs similar to the mechanism described for the *rdw* rat, which developed dwarfism by genetic hypothyroidism, and huge vacuoles in the thyroid gland¹¹. In contrast, the vacuoles of GALAS rats showing normal growth with thyroid dysplasia were not stained for thyroglobulin in the present study and other reported works^{2,4}. Thus, it is still not clear what was contained in the dilated r-ERs of GALAS rats showing normal growth with thyroid dysplasia. On the other hand, the histopathological features were fundamentally similar regardless of the presence or absence of thyroid dysplasia, except for the vacuoles.

The luminal colloids in most adenomas were positive for PAS and thyroglobulin in both animals with thyroid dysplasia and without thyroid dysplasia. The expression of thyroglobulin has been reported to disappear in a solid (microfollicular) histological pattern of thyroid follicular cell adenoma and carcinoma¹². It has been considered that the tumors with a solid pattern indicate undifferentiated forms and may be unable to synthesize and store thyroglobulin protein¹². Actually, the luminal colloid of a follicular cell carcinoma with a solid pattern, observed in a rat without thyroid dysplasia, was also negative for thyroglobulin in this study. From these findings, the positive expression of thyroglobulin in the neoplasms observed in the animals with thyroid dysplasia indicates that they are not undergoing malignant transformation. On the other hand, the luminal colloid in adenomas of rats with thyroid dysplasia showed a tendency to decrease in terms of the stainability of T₄ when compared with that of rats without thyroid dysplasia. Few reports have appeared in the literature on the relationships between T₄ and follicular cell tumor. It is unclear why adenomas in rats with thyroid dysplasia have such a tendency, but it might be associated with the genetic background.

In conclusion, we evaluated incidences of thyroid proliferative lesions with or without thyroid dysplasia in our medium-term carcinogenicity study and observed the morphological features of these lesions. It appears that dysplastic thyroids of rats showing normal growth are more

sensitive to carcinogens than normal thyroids. In addition, the morphological features of carcinogen-induced thyroid proliferative lesions in GALAS rats with thyroid dysplasia were fundamentally similar to those of rats without thyroid dysplasia, except for the vacuoles and T₄ staining profile.

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