IMMUNOHISTOCHEMICAL STUDIES OF TSH-PRODUCING CELLS IN THE PITUITARY AND EXPRESSION OF GROWTH FACTORS IN THYROIDAL PROLIF- ERATIVE LESIONS IN RATS TREATED WITH THIOUREA AND EXCESS VITAMIN A

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ABSTRACT — Changes of TSH-producing cells in the pituitary and thyroid expression of the growth factors, transforming growth factor α (TGF α) and epidermal growth factor receptor (EGFR), as well as cyclin D1, were investigated immunohistochemically in order to clarify their contribution to the enhancing effects of excess vitamin A (VA) on thyroidal carcinogenesis induced by thiourea (TU). Male rats were allocated to 4 groups, control, TU, VA, and TU+VA, respectively, receiving no treatment, water containing 0.2% TU, diet containing 0.1% VA, and both for 10 or 19 weeks after a single s.c. injection of DHPN (2800 mg/kg) for initiation. Immunohistochemistry using antibodies against TSH demonstrated enlargement of TSH-producing cells in the TU+VA group as compared to the TU group, supporting our conclusion that enhanced TSH stimulation is mainly responsible for promoting the effects of excess VA. Since the expression of TGF α , EGFR, and cyclin D1 in thyroid proliferative lesions did not exhibit any differences between the TU and TU+VA groups in the present study, these factors are unlikely to participate in VA enhancement of carcinogenesis.

KEY WORDS: Rat, Vitamin A, TSH cell, TGF α , EGFR, Cyclin D1

INTRODUCTION

Goitrogens such as thiourea (TU) are known to induce follicular cell hyperplasia and neoplasia in the thyroid via sustained thyroid-stimulating hormone (TSH) stimulation through the hypothalamic negative feedback system (Haschek and Rousseaux, 1991; Hill *et al.*, 1989; McClain, 1992). We previously reported that simultaneous treatment with excess vitamin A (VA) enhanced the thyroidal carcinogenesis induced by

TU, as evidenced by elevated cell proliferation in focal proliferative lesions (Mitsumori *et al.*, 1996; Takegawa *et al.*, 1997). The enhancing effect was presumed due to a rise in TSH, since induction of T4-uridine diphosphate glucuronosyltransferase (T4-UDPGT) activity in the rat liver was enhanced in the case treated with TU and excess VA simultaneously (Takegawa *et al.*, 1997).

The inhibition of thyroid hormone synthesis was reported not to be related to the enhancing effect of excess VA on the circulating TSH, since no enhance-

ment by excess VA of depression of the iodine uptake or organification was recognized in the other mechanistic study (Okuno *et al.*, 1996).

Although TSH is well known to be the most important modulator of thyroid follicular cell growth, other factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF) have also been suggested to be involved (Polychronakos *et al.*, 1986; Smith *et al.*, 1986; Westermarck *et al.*, 1983). The fact that rat thyroid hyperplasia induced by propylthiouracil is associated with increased numbers of receptors for IGF (Polychronakos *et al.*, 1986) and the finding that TSH cannot stimulate DNA synthesis in isolated thyroid follicular cells in the absence of other growth factors (Smith *et al.*, 1986) are indications that the proliferative response may be controlled by complicated interactions between signals and signaling pathways. To assess the possibility that growth factors in proliferative lesions induced by TU might be altered by simultaneous treatment with excess amounts of VA, the present study was conducted. In addition to a morphometric investigation of pituitary TSH-producing cells of rats treated with TU alone and TU+VA after N-bis(2-hydroxypropyl)nitrosamine (DHPN)-initiation, the expression of transforming growth factor α (TGF α), epidermal growth factor receptor (EGFR), and cyclin D1 in thyroid proliferative lesions was immunohistochemically examined for this purpose.

MATERIALS AND METHODS

Male F344 rats, four weeks old, were obtained from Charles River Japan Inc. (Atsugi, Japan) and housed five to a plastic cage with wood chips as bedding in an air-conditioned animal room (room temperature, $23 \pm 2^\circ\text{C}$; relative humidity, $60 \pm 5\%$; lighting cycle, a 12h light/12h dark). Pulverized diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and distilled drinking water were available ad libitum during the acclimatization period of two weeks. TU was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and VA was purchased as Dry Vitamin A Acetate, type 325 L (vitamin A powder: 3,250,000 IU/g) from F. Hoffman La Roche & Co., Ltd. (Basle, Switzerland). DHPN was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

A total of 40 rats without any abnormal findings was allocated to 4 groups consisting of 10 rats each so that the initial body weights of each group were approximately equal. All rats were given a single subcutaneous injection of 2800 mg DHPN/kg. From a week later, rats in the control group received tap water

and basal diet while those in the TU, VA, and TU+VA groups received water containing 0.2% TU, diet containing 0.1% VA, and 0.2% TU+0.1% VA, respectively, for 10 weeks. The doses of TU and VA were the same as those applied in our previous studies (Mitsumori *et al.*, 1996), where enhanced cell proliferation in hypertrophic follicular epithelia, hyperplasias and neoplastic lesions was observed, along with decreased serum T3 and T4 and increased serum TSH in the TU+VA-treated group. The treatment period of 10 weeks was chosen in order to observe TSH-producing cells during the term in which elevated serum TSH levels caused by TU are considered to be maintained, on the basis of our finding that values peak at around 4 weeks and continue to be higher for up to 10 weeks (Shimo *et al.*, 1994).

At the end of the treatment period, all animals underwent complete autopsies. Pituitary and thyroid tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, serially sectioned at 4-5mm, and stained with hematoxylin and eosin for microscopic examination. Thyroid proliferative lesions were classified on the basis of the diagnostic criteria proposed by Mitsumori *et al.* (1996). Follicular cell hyperplasia was divided into hypertrophic and small cell types, and neoplasias were classified into two types with adenomatous growth pattern and solid growth patterns. Lesions demonstrating invasion into the capsule were diagnosed as intracapsular proliferative foci (IPF).

Immunohistochemical staining using antibodies against TSH was performed on the pituitaries of all animals. The area occupied by TSH-positive cells was measured with the aid of an Image Processor for Analytical Pathology (IPAP) (Sumika Technos Corp., Osaka, Japan). The rate of the positive area to the total area observed of the pituitary was calculated. In addition, the expression of TGF α and cyclin D1 in the thyroid was immunohistochemically examined for serial sections next to HE specimens. For each proliferative lesion, the intensity of TGF α and cyclin D1 staining was recorded. The rates of moderately or intensely stained lesions were calculated for each type of lesion classified on HE specimens. Rabbit monoclonal antibodies against TSH (HAC-RT29-01RBP86, Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Gunma, Japan), and mouse monoclonal antibodies for human TGF α (Oncogene Science, Inc., NY, U.S.A.) and human cyclin D1 (IBL, Gunma, Japan) were applied at dilutions of 1/8000, 1/17, and 1/1000, respectively. Biotinylated secondary antibodies and avidin-biotin peroxidase complex (ABC) method were applied using 3,3'-diaminobenzi-

dine (DAB) as a chromogen.

An additional two-stage carcinogenicity study was conducted in order to examine the expression of EGFR in thyroidal proliferative lesions in rats treated with TU and/or VA. Male F344 rats, four weeks old, were obtained from Charles River Japan Inc. and housed under the same conditions as described above. After a two-week acclimatization, a total of 16 rats was allocated to 4 groups consisting of 5 rats each for the TU and TU+VA groups and 3 rats each for the VA and control groups, again so that the initial body weights of each group were approximately equal, and maintained for 19 weeks, starting one week after a single subcutaneous injection of 2800 mg DHPN/kg. The treatment period was selected on the basis of more apparent enhancement observed for cell proliferation of follicular cells in thyroidal proliferative lesions after 19 weeks of the treatment rather than 10 weeks (Mitsumori *et al.*, 1996; Takegawa *et al.*, 1997). At the end of the treatment period, thyroid tissues were frozen and preserved at -80°C until use. These frozen tissues were serially sectioned at 5 mm, and stained with hematoxylin and eosin for microscopic examination. Immunohistochemical stain-

ing using sheep polyclonal antibodies against human EGFR (Seikagaku Corp., Tokyo, Japan; 1/200 dilution) was performed for the semi-serial frozen section of each animal. Biotinylated secondary antibody and ABC methods were applied using DAB as a chromogen.

As to the rates of the area occupied by TSH-positive cells in the pituitary, mean and standard deviation values were generated for each group, and inter-group differences were then analyzed with the Student's *t*-test.

RESULTS

In immunohistochemistry, cells positive for TSH in the pituitary could be divided into intensely and weakly stained cells, the former predominating in the control rat (Photo 1, a). Morphometrically, the areas occupied by intensely stained cells were significantly decreased in the TU group as compared to the control. Decrease was also evident in the TU+VA group, although it was not statistically significant (Photo 1, b). On the other hand, the area occupied by weakly stained cells was significantly increased in the TU+VA case, as compared to the control and also to the TU alone case

Photo 1. TSH-producing cells stained immunohistochemically. $\times 165$.
 a: Pituitary from a control rat. Many intensely stained cells (arrows) are observed.
 b: Pituitary from a rat simultaneously treated with thiourea and excess vitamin A for 10 weeks after DHPN initiation. Intensely stained cells are rare, but weakly stained enlarged cells (arrows) can be recognized.

(Table 1).

Immunohistochemical examination of the expression of growth factors in thyroid proliferative lesions revealed no alteration by excess VA, with the possible exception of IPF (see Table 2,3).

Normal epithelia of follicles in the control group

did not show any TGF α expression. Hypertrophied epithelia induced by TU displayed a moderate positive reaction, also apparent in the TU+VA case. Most hyperplasias of both hypertrophic and small cell type were stained, but the intensity was very varied.

Table 1. Areas of TSH-positive cells in the pituitaries of rats treated with thiourea and/or vitamin A for 10 weeks after DHPN initiation.

Group	The rates (%) of the area occupied by TSH cells to the whole pituitary area	
	Intensely stained	Weakly stained
Control	2.30 $\pm$ 2.34 ^{a)} (10) ^{b)}	3.22 $\pm$ 2.13 (10)
0.2% TU	0.55 $\pm$ 0.51 (10) *	3.83 $\pm$ 3.98 (10)
0.2% TU+0.1% VA	1.65 $\pm$ 1.59 (10)	11.00 $\pm$ 3.82 (10) **, #
0.1% VA	2.98 $\pm$ 0.99 (10)	3.14 $\pm$ 1.07 (10)

TU: thiourea, VA: vitamin A

a): Mean $\pm$ S.D. b): No. of animals

*, **: Significantly different from the control value at P<0.05 or 0.01, respectively.

#: Significantly different from the 0.2% TU group value at P<0.01.

Table 2. Percentages of the proliferative lesions immunohistochemically staining for transforming growth factor α (TGF α) in the thyroids of rats treated with thiourea and/or vitamin A for 10 weeks after DHPN initiation.

Group	The percentage of positive lesions									
	HH		HS		NA		NS		IPF	
	+	++	+	++	+	++	+	++	+	++
0.2% TU	57.6	15.9	52.2	5.4	31.3	68.8	0	100	100	0
0.2% TU + 0.1% VA	87.9	5.2	60.4	12.5	29.4	64.7	NL	NL	0	100

TU: thiourea, VA: vitamin A.

HH: hyperplasia of hypertrophic cell type, HS: hyperplasia of small cell type.

NA: neoplasia of adenomatous growth pattern, NS: neoplasia of solid growth pattern.

IPF: intracapsular follicular cell proliferative lesion.

NL: No lesions were observed.

+: moderately positive, ++: intensely positive.

Table 3. Percentages of the proliferative lesions immunohistochemically staining for cyclin D1 in the thyroids of rats treated with thiourea and/or vitamin A for 10 weeks after DHPN initiation.

Group	The percentage of positive lesions									
	HH		HS		NA		NS		IPF	
	+	++	+	++	+	++	+	++	+	++
0.2% TU	64.6	32.3	65.6	22.2	25.0	75.0	0	100	0	100
0.2% TU + 0.1% VA	84.2	15.8	89.6	11.5	35.3	58.8	NL	NL	0	100

TU: thiourea, VA: vitamin A.

HH: hyperplasia of hypertrophic cell type, HS: hyperplasia of small cell type.

NA: neoplasia of adenomatous growth pattern, NS: neoplasia of solid growth pattern.

IPF: intracapsular follicular cell proliferative lesion.

NL: No lesions were observed.

+: moderately positive, ++: intensely positive.

Neoplasms with adenomatous or solid growth pattern generally revealed more intense staining than hyperplasias, although strongly positive reactions were frequently limited to focal areas of cells (Photo 2, a, b). In some cases these limited areas had features suggestive of malignancy such as nuclear atypia. Nuclei were sometimes stained, particularly in foci of apparently positive cells.

As to cyclin D1, normal epithelium in the control and VA groups hardly showed any positive reaction, whereas nuclei of hypertrophied follicular epithelia of the TU and TU+VA cases showed scattered weak staining. Hyperplasias of hypertrophic and small cell type revealed positive staining, positive nuclei being more frequently observed than in surrounding hypertrophied follicular epithelia, but without strong intensity. In most of the neoplasms with either adenomatous or solid growth pattern, intense positive reactions were recognized in many nuclei, particularly in morphologically atypical cells positive for TGF α (Photo 2, c).

With regard to the expression of EGFR, positive staining was evident on the cell membranes of normal follicular epithelium in control rats. Thus, many spots were usually located on the surfaces facing the follicular lumen. In the hypertrophied follicular epithelia induced by TU or the epithelia of proliferative lesions in the TU and TU+VA groups, the same staining pattern was observed (Photo 3). No difference in expression levels was apparent.

DISCUSSION

The morphometrical changes in TSH-positive cells of the pituitary observed in the present study are in line with our previous observation of elevated serum TSH in the TU+VA as compared to the TU alone case (Takegawa *et al.*, 1997). Decrease in the area occupied by intensely stained cells in the TU and TU+VA groups can be considered to be caused by depletion of TSH from the pituitary because of increased secretion in response to the negative feedback. Although the nature of the weakly stained cells could not be confirmed, it is likely that they contain a little TSH or precursors. Enlarged TSH-producing cells were also stained weakly. Hypertrophied TSH cells in the pituitary of rats treated with goitrogen demonstrate dilated rough ER in the cytoplasm, suggestive of increased TSH synthesis, at the electron microscope level (Shimo *et al.*, 1995). Therefore, the increase of the area occupied by these weakly stained cells in the TU+VA group presumably reflects elevated TSH synthesis in the pituitary.

Several growth factors have been reported to be overexpressed in tumors in various organs, and considered to be related to autocrine stimulation (Atlas *et al.*, 1992; Bauknecht *et al.*, 1989; Reifengerger *et al.*, 1996). TGF α is one which is known to be overexpressed in many tumors of man and experimental animals (Petride *et al.*, 1990; Walker *et al.*, 1991). Although TGF α may also be expressed in normal tissues (Everitt *et al.*, 1997) including the human kidney (Ambs *et al.*, 1989) and liver (Fukusato *et al.*, 1990), it is considered to be an important factor in organogenesis (Avner, 1990; Humes *et al.*, 1992; Lee *et al.*, 1985) or morphogenesis (Taub *et al.*, 1990) during development. TGF α binds to EGFR, and may thereby regulate the growth of tumor tissues (Atlas *et al.*, 1992). EGFR has also been reported to be overexpressed in various tumor types (Gullick, 1990; Bauknecht *et al.*, 1989; Reifengerger *et al.*, 1996). This has been reported to be associated with malignancy in thyroid tumors (Kashima *et al.*, 1991). In addition, the cyclin D1 gene is often upregulated in neoplasias (Lee *et al.*, 1997; Nishida *et al.*, 1994; Wang *et al.*, 1996; Zhang *et al.*, 1994). However, in the present study, no enhancement by excess VA was recognized for the expression of TGF α , EGFR, and cyclin D1 in thyroid proliferative lesions induced by DHPN and TU, although TGF α and cyclin D1 were overexpressed in general, especially in tumors with features of malignancy. Thus, these growth factors may be important for growth but are unlikely to participate in the enhancing effects of excess VA.

TGF α expression is higher in adenomas or carcinomas than in basophilic foci in the case of DEN-induced hepatocarcinogenesis in mice (Moser *et al.*, 1997), and the factor has been reported to promote carcinogenesis in vitro in rat liver cell lines (Kaufmann *et al.*, 1997). In addition, since regenerative proliferative lesions in rat kidneys are not stained with TGF α , there may be a specific link with transformation rather than just cell proliferation (Everitt *et al.*, 1997). Our finding that the percentages of intensely stained lesions were increased for neoplastic as compared to benign lesions is thus in line with the literature, suggesting that overexpression of TGF α is one marker of malignancy. However, in the present study, staining was not always consistent with this hypothesis, and TGF α is not ubiquitously overexpressed in all hepatocellular adenomas or carcinomas in mice (Moser *et al.*, 1997). The reason for this inconsistency is not clear, but other growth factors might also participate.

Cyclin D1 is intimately associated with tumor

Photo 2. A follicular cell neoplasm demonstrating an adenomatous growth pattern in the thyroid of a rat treated with thiourea and excess vitamin A for 10 weeks after DHPN initiation. $\times 220$.

a: HE staining. Follicular epithelia show papillary growth, and some nuclear atypia is apparent.

b: TGF α immunohistochemistry. Whole lesion is positively stained.

c: Cyclin D1 immunohistochemistry. Note the positive reaction for cyclin D1 in the same area.

Photo. 3. EGFR immunohistochemistry for a frozen thyroid section of a follicular cell neoplasm from a rat treated with thiourea and excess vitamin A for 19 weeks after DHPN initiation. Receptors are recognized as spots located on the cell membrane. $\times 462$.

growth. However, its overexpression is also considered to contribute to cell transformation, based on evidence such as intense immunohistochemical staining in transitional cell hyperplasia or carcinomas of the urinary bladder (Lee *et al.*, 1997). Our present results suggest that neoplastic lesions in the thyroid also tend to demonstrate more intense reaction than hyperplasia. However, whether cyclin D1 might be employed to assess the malignancy of tumors remains to be clarified.

In conclusion, the results obtained in the present examination on the pituitary may support our previous conclusion that the main cause of enhancement of thyroid carcinogenesis by simultaneous treatment with excess VA observed in our previous studies is enhanced TSH stimulation, presumably due to elevated T4-UDPGT activity in the liver in addition to the depressed T3/T4 synthesis by TU. However, further quantitative examinations on the expression of growth factors are necessary for overall understanding on the modulating effect of excess VA on thyroid carcinogenesis.

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