

Autoradiographic analysis of [^{14}C]deoxy-D-glucose in thyroid cancer xenografts: A comparative study with pathologic correlation

Masahiro KUBOTA,*† Takatoshi TSUDA,*†† Takashi MINASE,** Kunihiro NAKADA***
and Masayori FURUDate***

*Department of Radiology, School of Medicine, Sapporo Medical University

**Clinical Laboratory of NTT Sapporo Hospital

***Department of Nuclear Medicine, Faculty of Medicine, Hokkaido University

† Department of Radiology, Teine Keijinkai Hospital

†† Department of Radiology, Kushiro Chuo Hospital

An experimental model of thyroid cancer was prepared for evaluating the accumulation of [^{14}C]deoxy-D-glucose ([^{14}C]DG) in thyroid cancer xenografts (AC2). A continuous cell line established from a biopsy specimen of a metastatic thyroid carcinoma possessed the ability to synthesize the cellular protein without increase in cell division after adding bovine TSH *in vitro*. The histological sections of the xenografts resected from the ^{131}I treated nude mice mainly consisted of structures showing follicular and trabecular growth. Immunohistochemically the cytoplasm of the tumor cells was positive for human thyroglobulin(hTg). These observations provide strong evidence that the AC2 cell originates in the thyroid follicular epithelium. By comparing autoradiographic accumulation patterns of [^{14}C]DG and histopathological examinations, it was found that the uptake of [^{14}C]DG was higher in the granulation tissues surrounding necrosis than in viable tumor cells of trabecular growing and follicle forming tissues.

It is suggested that the degree of [^{14}C]DG content reflects not only tumor cell viability and proliferation but also the inflammatory and degenerative reaction accompanying tumor cell growth.

Key words: thyroid carcinoma xenograft, [^{14}C]deoxy-D-glucose ([^{14}C]DG), autoradiography

INTRODUCTION

INCREASED GLYCOLYSIS is one of the most important characteristics of cancer cells. Studies on the glucose metabolism of experimental tumors have been reported by many authors.¹⁻³

Clinically, it is reported that glucose consumption measured by [^{18}F]fluoro-2-deoxy-D-glucose (FDG) with PET was found to correlate well with the viability of cancer cells and the prognosis of cancer patients.⁴⁻⁸ However, these previous studies focused on evaluating the viability and growth activity of cancer cells, which are nonspecific characteristics independent of the cancer type. Cancer cells occasionally express the phenotypes of their

original tissues, and the degree of expression may represent the degree of differentiation. A clinical study on the metabolic heterogeneity of thyroid carcinoma carried out with FDG suggests that metastases that accumulate FDG, but not ^{131}I may behave more aggressively than metastases that accumulate ^{131}I , but not FDG.⁹

We investigated the accumulation of [^{14}C]DG in athymic nude mice with thyroid cancer xenografts to analyze the correlation with the degree of differentiation and glucose consumption. In this present study, we report a distinctive pattern of uptake of [^{14}C]DG by the secondary inflammatory reaction cells in a thyroid cancer model in mice.

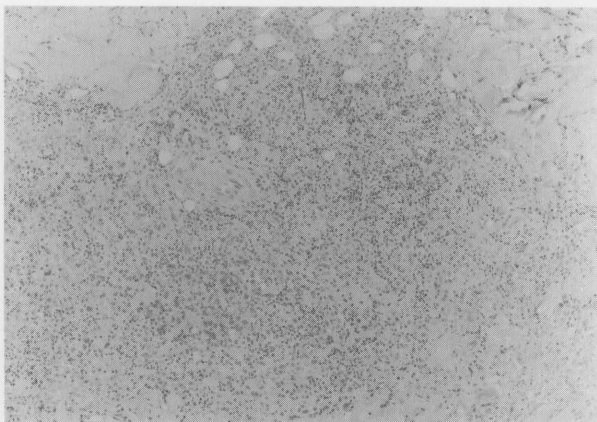
MATERIALS AND METHODS

Thyroid carcinoma

A 58-year-old Japanese male patient was referred to the Sapporo Medical College Hospital in 1984. Metastasis was found in the right supraclavicular lymph nodes. Two courses of radioiodine therapy with 7.2 GBq of ^{131}I were

Received October 21, 1992, revision accepted November 24, 1993.

For reprint contact: Masahiro Kubota, Department of Radiology, Teine Keijinkai Hospital, Maeda 1-12, Teine-ku, Sapporo 006, JAPAN.



Biopsied specimen

HE x120

Fig. 1 Light micrograph of the biopsied specimen: The sections of the original tumor show a trabecular growth pattern with microfollicular structures (HE stain; original magnification $\times 120$).

carried out after thyroidectomy. The primary lesion was pathologically diagnosed as papillary carcinoma. Widespread metastases occurred three months after second ^{131}I therapy. At this time, a metastatic tumor of the anterior chest wall was biopsied. Histological examination confirmed metastatic thyroid carcinoma with anaplastic transformation. Thyroid carcinoma was obtained from the biopsied specimen (Fig. 1).

Establishment of AC2 cell line

The biopsied tissue was cut as finely as possible, and cells obtained from the tumor pieces were suspended in 20 ml of Eagle's medium (Nissui Pharma, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah). The cells were cultured in 25 cm² flasks (Falcon 3013, Falcon Plastics, Oxnard, CA) at 37°C in a humidified atmosphere of 5% CO₂ in air. During the subsequent 4 weeks of primary culture, colonies of small epitheloid cells attached to the flasks were observed and grew slowly. The tumor cell colonies were surrounded by a considerable number of fibroblastic cells. During a further 5 weeks of primary culture, it became apparent that a homogeneous population of small epitheloid cells had gradually propagated and reached confluency. The cell line was passaged with 0.25% trypsin and 0.02% EDTA in PBS.

Radiolabeling of proteins in thyroid cell culture

[^{35}S]methionine (41.8 TBq/mmol; EIDupont de Nemours Inc, N. Billerica, MA) was added to the medium (10 $\mu\text{Ci}/\text{ml}$; Modified Eagle's MEM) 2 hours before the end of the culture with bovine thyroid stimulating hormone (bTSH) (100 $\mu\text{IU}/\text{ml}$) or without bTSH. Cultured cells were extensively but quickly washed with PBS, then isolated by centrifugation and stored at -80°C . The cells were homogenized (10×10^6 cells/0.6 ml) in PBS and mixed with

hot trichloroacetic acid (TCA). Then the mixture was centrifuged at 27000 r.p.m. for 5 minutes. The precipitated protein fractions were trapped on a 0.5 μm glass filter and washed with cold 10% TCA solution. Their radioactivity was measured in a scintillation counter.

Animal model

Ten 4-week-old nude mice (BALB/cAJcl-nu) were subcutaneously inoculated with 1×10^7 to 1×10^8 AC2 cells. Four weeks before the cell inoculation, five mice were given 3.7 MBq of ^{131}I for their thyroid ablation. Four to eight weeks after the inoculation, tumors in the treated nude mice were resected for light microscopical and autoradiographic examinations. Five other mice without ^{131}I treatment were used for histopathological examination only.

Histopathological examination

The extirpated xenograft was fixed with 10% formalin in PBS, embedded in paraffin, and stained with hematoxylin and eosin (H-E) for light microscopic examination. For immunohistochemical analysis of human thyroglobulin (hTg), tissue sections were deparaffinized in xylene, washed with PBS, and rehydrated. They were then applied for the detection of hTg. Immunostaining was achieved by the avidin-biotin peroxidase complex (ABC) method. Endogenous peroxidase was removed by incubation in a mixture of 100 ml absolute methanol and 1 ml 30% hydrogen peroxide for 30 minutes. The slides were also incubated with normal horse serum to avoid non-specific binding. Subsequently, anti-human 27S, 19S, and 12S thyroglobulin (hTg) mouse monoclonal antibody (Sanbio Bv-Biological Products, Uden, The Netherlands) was applied to the sections. Goat biotinylated anti-mouse antisera and ABC were then successively placed on the tissue section for 30 minutes, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Peroxidase activity was examined by staining with 0.1% diaminobenzidine tetrahydrochloride (Sigma Chemical Co, St Louis, MO) in Tris-HCl buffer (50 mM, pH 7.6) containing 0.02% hydrogen peroxide. Control sections were incubated with PBS in place of the monoclonal antibodies.

Estimation of bromodeoxyuridine (BrdU) labeling index

In a BrdU distribution study, 50% ethanol fixed tissue sections from the ^{131}I treated mice were deparaffinized in xylene, washed in PBS, and rehydrated. Anti-BrdU mouse monoclonal antibody (MBL CO, Tokyo) was used. Staining was also performed by the ABC method described above. The positive nuclear staining rate per 1000 tumor cells was then found. The labeling index (L.I.) was estimated as an indicator of the tumor cell proliferative activity under the various light-microscopic fields.

Deoxy-D-glucose distribution

Tumor bearing athymic nude mice treated with ^{131}I were given an intraperitoneal injection of $10\text{ }\mu\text{Ci}$ of [^{14}C]deoxy-D-glucose ([^{14}C]DG) (E I Du Pont de Nemours Inc, N. Billerica, Ma) and sacrificed 1 hour after the injection. For macro-autoradiographic studies, the xenografts and the selected tissue sections were applied to Fuji RX X-ray film and exposed for 8 weeks. Micro-autoradiography was performed according to the previously reported method.¹⁰ Slides bearing HE stained sections were dipped twice in a 1% solution of celloidin. After drying overnight at room temperature, the celloidin coated slides were smeared with a small amount of egg albumine : glycerine solution (1 : 1), except over the section itself. In a completely light-proof dark room the slides were dipped for 1–2 seconds in the melted NTB2 nuclear emulsion (Eastman Kodak, USA) and then allowed to dry in a vertical position. The slides were exposed in black plastic boxes for 3 weeks.

RESULTS

Effect of TSH in vitro

A kinetic study on the growth of the cultured cells is shown in Figure 2. From the curve, the population doubling time of TSH treated and untreated cells was estimated to be 1.7 days and 1.8 days, respectively. TSH had no effect on cell division or cellular morphology (data not shown).

The [^{35}S] activity of cells cultured for 2 hours was significantly increased by TSH, and this is shown in Table 1. The mean uptake of cells treated with bTSH was about twice as great as that of the cells without bTSH ($p < 0.001$).

Histology of xenografts

Subcutaneous transplantation of AC2 cells into athymic nude BALB/c mice led to the production of tumors that were clearly visible 3 to 4 weeks later. The solid tumors obtained from the ^{131}I treated and untreated nude mice consisted of tissues showing signs of trabecular growth and small follicle formation. In some areas, the morphology of a typical differentiated adenocarcinoma with follicles was observed. In other parts of the tumors, sheets of either very large cells or small cells were observed. Most tumor cells were viable in the tissues from both the treated and untreated mice. The tumors from treated mice consisted of tissues with more follicle formation than those from untreated mice.

Microscopically, the tissues with follicle formation showed a tendency to grow near the granulation and/or necrotic tissues. Histochemically, more intensely immunoreactive hTg was observed in the cytoplasm of the viable tumor cells from the treated mice than in those from untreated mice (Fig. 3-A, B).

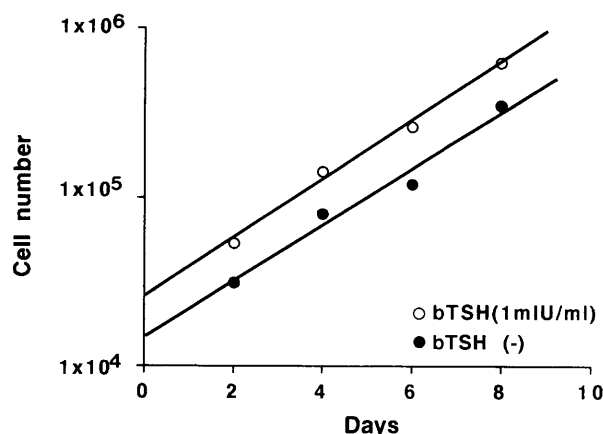


Fig. 2 Long-term effect of TSH on cell growth. The doubling time of the cell population is about 1.8 hours in the logarithmic growth phase without TSH. TSH does not work stimulatory in growth.

Table 1 Effect of TSH on [^{35}S]methionine incorporation in the monolayer culture

Treatment	[^{35}S]methionine incorporation* (c.p.m./10 ⁶) AC2
None	16308 ± 1703 (5)
bTSH	36156 ± 3388 (5)**

(): the number of assays

*: Mean ± SD

**: Significant difference from the value for the cells incubated in medium alone ($p < 0.001$)

BrdU distribution study

In a comparison of BrdU distribution in the tumor cells and histologic features of the tissue, BrdU L.I. were $19.6 \pm 6.0\%$ in the trabecularly growing tissues and $21.4 \pm 9.5\%$ in the follicle forming tissues, respectively (Fig. 4-A, B).

Autoradiographic study

The macro-autoradiogram of the resected specimens from the ^{131}I treated mice is shown in Figure 5-A. Heterogeneous distribution patterns of silver grains within the tumor were seen. No silver grains could be seen within necrotic tissues in the tumor. A combination with light microscopy revealed that there were more dense grains (Fig. 5-B; arrow, arrowhead) in the follicle forming tumor tissues accompanied with granulation (Fig. 5-C) and/or necrotic tissue (Fig. 5-D), than in viable tumor cells of trabecularly growing tissues (Fig. 5-B; open circle, Fig. 5-E).

Macro-autoradiograms of [^{14}C]DG in formalin and in ethanol fixed sections from four other treated mice showed a similar pattern with a heterogeneous grain density in comparison with portions of the tumor mass (Fig. 6-A–D). In comparison with the H-E stained tissue sections, micro-autoradiograms showed that more dense grains

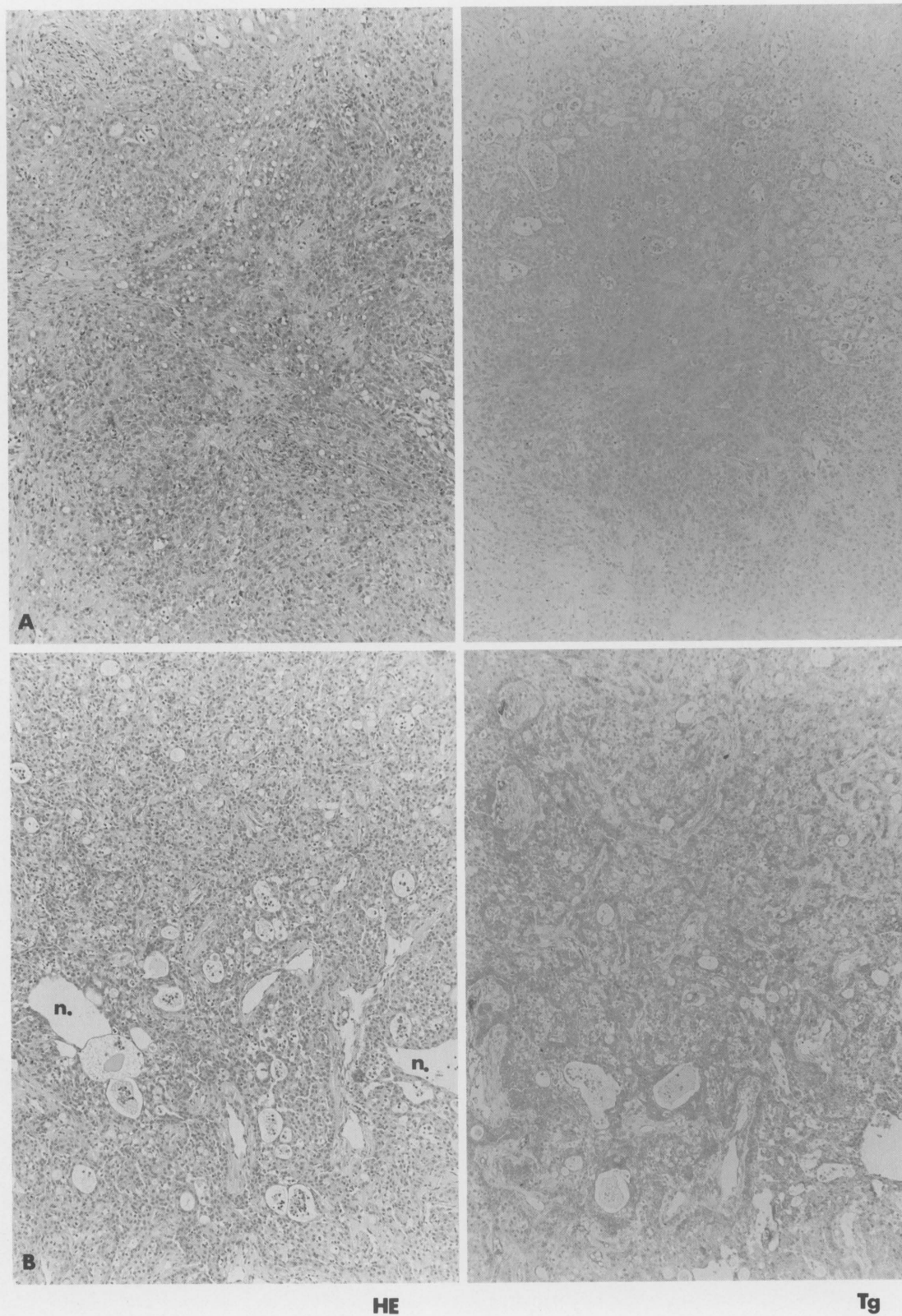


Fig. 3 Histological sections of xenografts in untreated (A) and treated nude mice (B): The strong immunoreaction for hTg in the cytoplasm of the tumor cells. (HE stain, immunostain for hTg [dil.1:5]; original magnification $\times 120$)

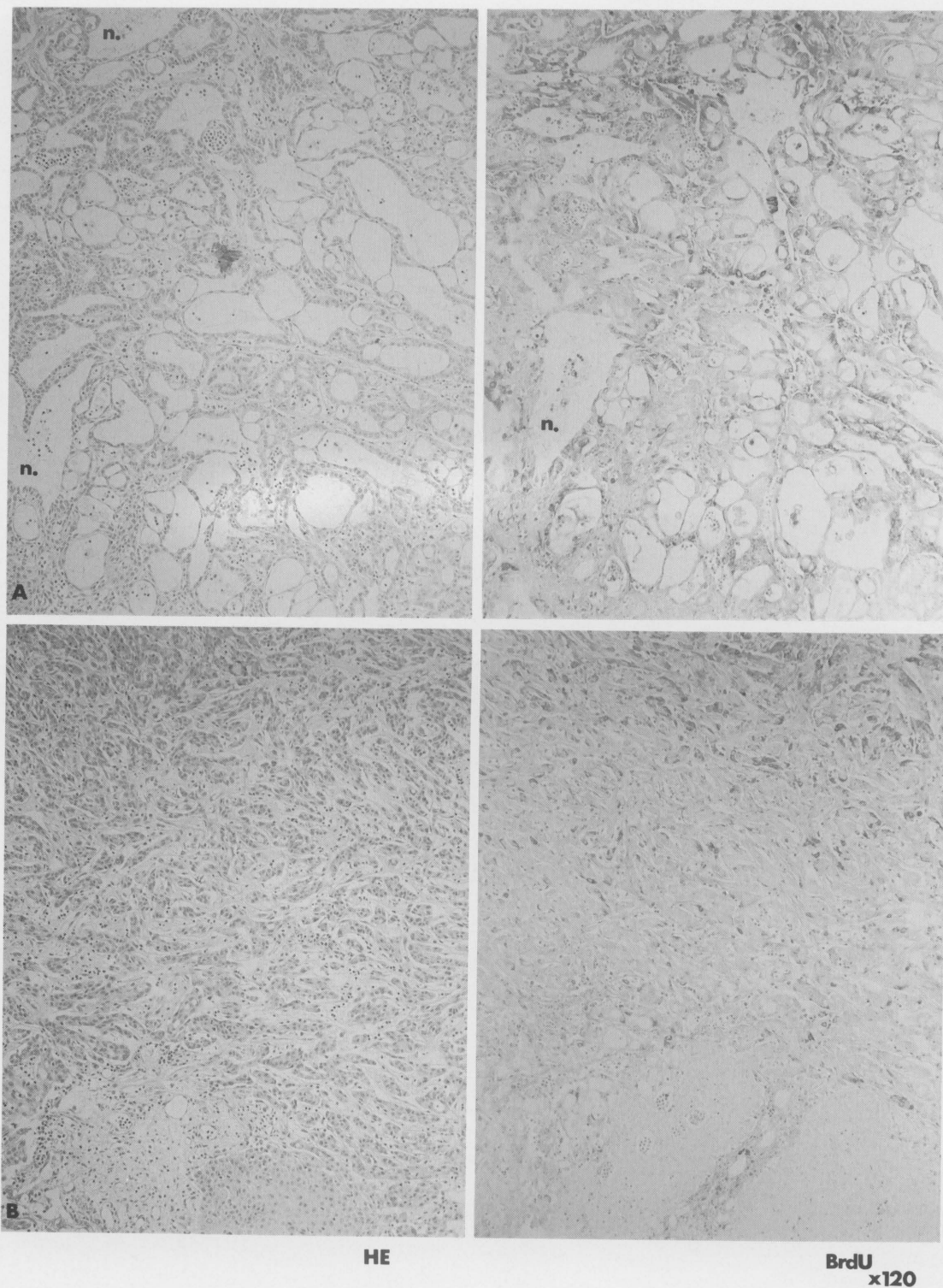


Fig. 4 Distribution of BrdU positive cells in tissues of a xenograft. Labeling indices are $21.4 \pm 9.5\%$ in the follicular growing tissue (A) and $19.6 \pm 6.0\%$ in the trabecular growing tissue (B), respectively. Statistical difference is not observed between the values (unpaired t-test).

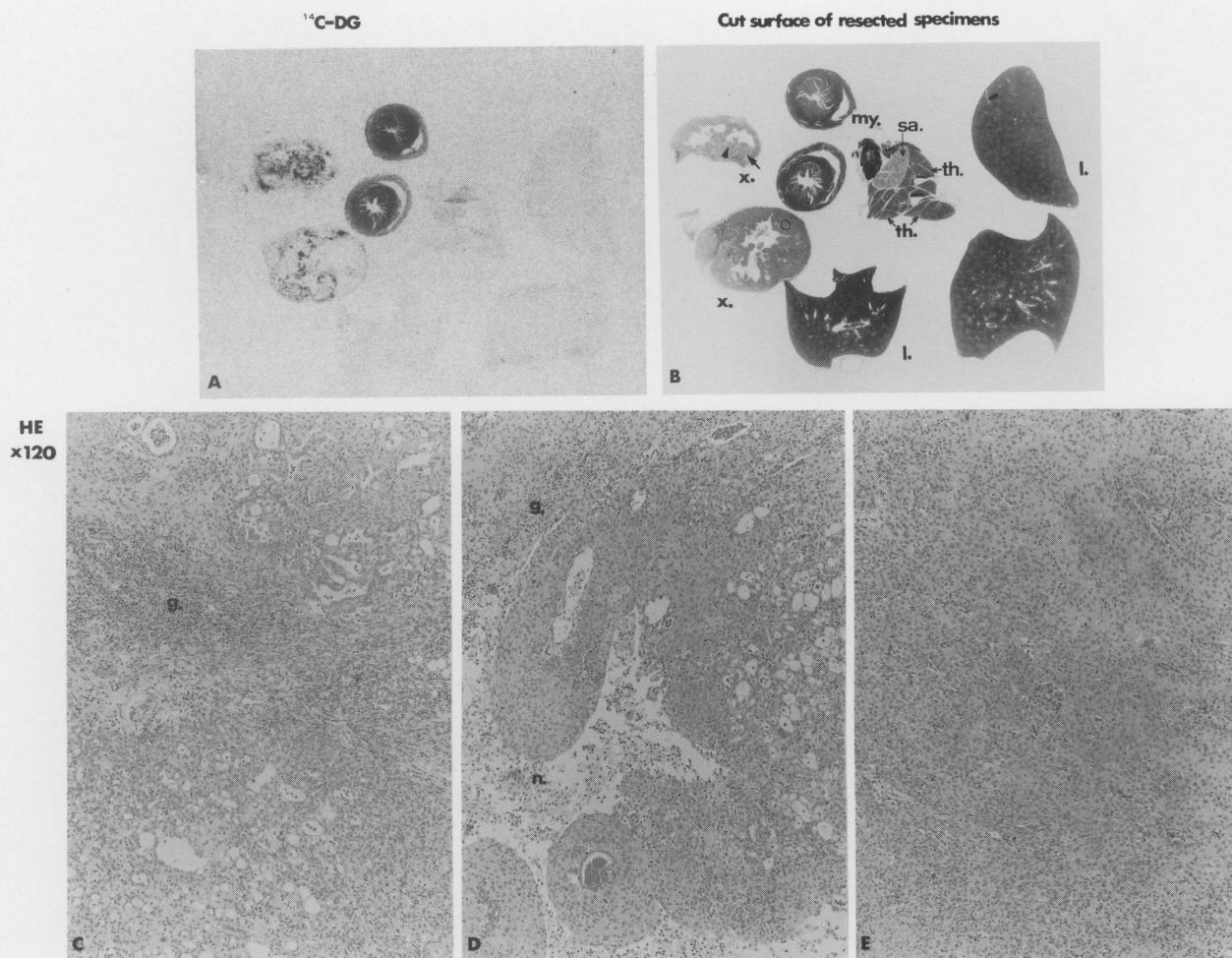


Fig. 5 Comparative macroautoradiographic and histological observations of a xenograft in a treated nude mouse. (A): Macroautoradiogram of the resected xenografts and selected organs in a nude mouse. (B): Cut surfaces (HE stain) of the xenografts and selected organs. (my: myocardium, l: liver, th: thyroid gland, sa: salivary gland, x: xenograft) (C) (D): Microscopic findings of the part (Fig. 5B; arrow, arrowhead) of high accumulation of [^{14}C]DG (HE stain). (n: necrosis, g: granulation) (E): Microscopic findings of the part (Fig. 5B; open circle) of low accumulation of [^{14}C]DG (HE stain). (original magnification $\times 120$)

were seen on the cells of the granulation tissue than on the tumor cells (Fig. 7-A). The grains were localized diffusely on viable tumor cells independent of the histologic features of the tumor tissues (Fig. 7-B).

DISCUSSION

The tumors which originated in thyroid follicular epithelium represent various stages of tumor progression. The benign follicular adenomas are considered to progress at low frequency to the malignant change in follicular carcinomas. In addition, thyroid differentiated cancer sometimes shows anaplastic transformation during its clinical course. Thus, thyroid neoplasms show a wide range of lesions varying from slowly progressive well-differentiated tumors to anaplastic highly malignant neoplasms.¹¹

For practical reasons, [^{18}F]FDG has found widespread use in PET and allowed us to use as the standard radiopharmaceutical for metabolic studies in the brain and various malignant tumors,⁴ such as glioma,⁵ hepatoma,⁶ lymphoma⁷ and lung cancer.⁸ The accumulation mechanism of both FDG and DG as an analogue of glucose into cells is based on the intracellular trapping of DG-6-phosphate,¹² which is the product of the hexokinase reaction of deoxyglucose. Further metabolism of DG-6-phosphate is restricted to tissues devoid of glucose-6-phosphatase due to steric inhibition of enzyme activities. We carried out a correlative functional-histological study by means of autoradiographic imaging of thyroid cancer xenografts with [^{14}C]DG.

TSH significantly increased the total protein synthesis of the AC2 cells in the monolayer culture but was not

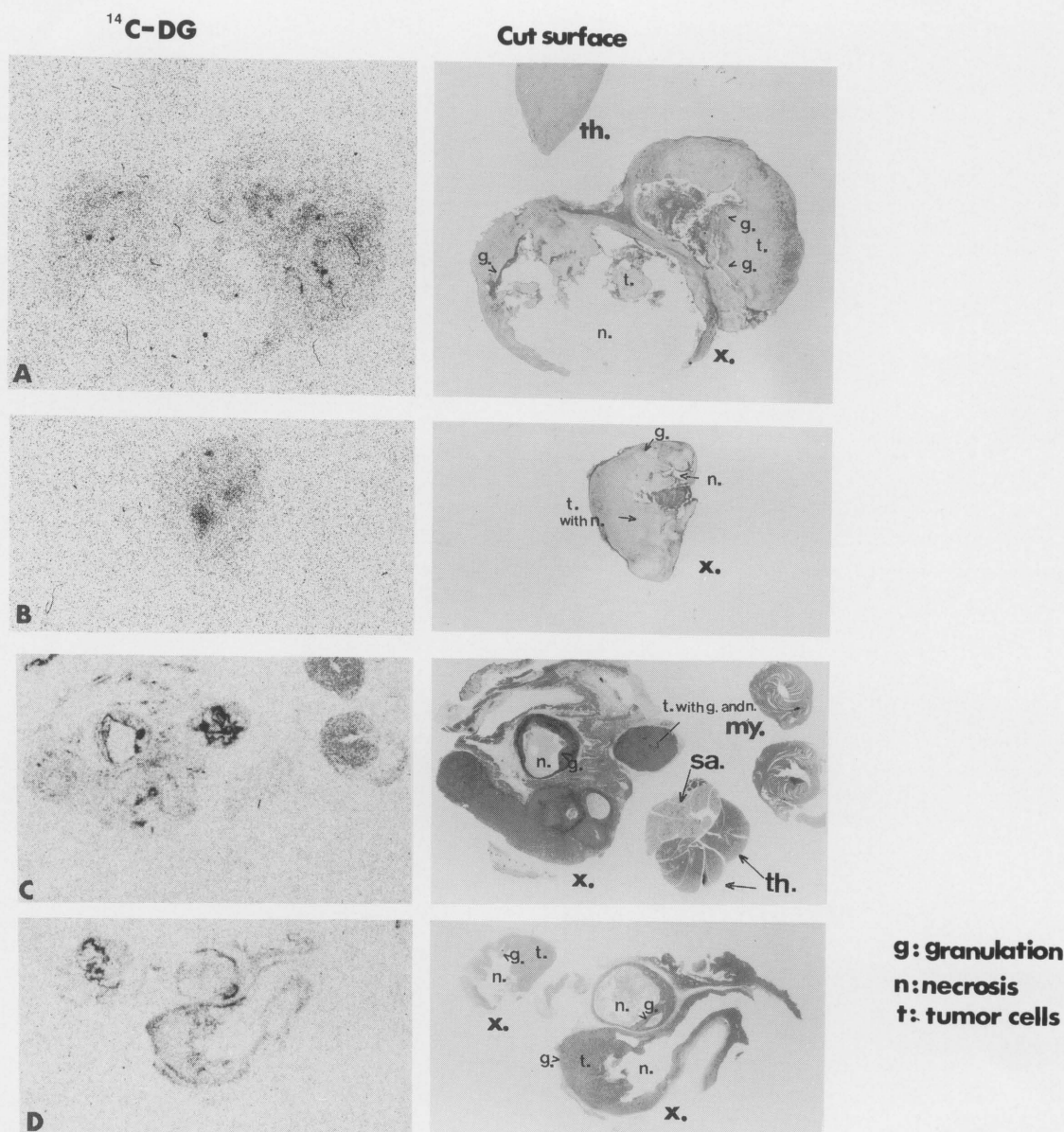


Fig. 6 Comparative macroautoradiographic and histological observations of a xenograft in other 4 treated mice. The pattern of [¹⁴C]DG autoradiograms are similar to that of formalin fixed sections [¹⁴C]DG autoradiograms. (my: myocardium, th: thyroid gland, sa: salivary gland, x: xenograft)
A-B: Ethanol fixed section
C-D: Formalin fixed section

accompanied with cell proliferation. These observation indicated that the established cell line, AC2, has the property of responding to TSH driving total protein synthesis *in vitro*. Recently, we reported that endogenous murine TSH appeared to have an effect on the cell differentiation.¹³ In the present study, the tumors from treated mice consisted of tissues with more follicle formation than those from untreated mice. Histochemically, more intensely immunoreactive 27S, 19S, and 12S hTg were observed in the cytoplasm of the viable tumor cells from the treated mice than in those from untreated mice. These

findings suggest that endogenous murine TSH may have an effect on cell differentiation^{14,15} in AC2 cells *in vivo*.

Watanabe and associates³ studied rat brain tumors and reported a significant correlation between malignancy as measured by the bromodeoxyuridine labeling index and the local cerebral glucose utilization. They suggested that an increase in glucose utilization in these tumors might be due to the requirement for nucleic acid synthesis. Minn and associates¹⁶ also observed a high correlation of FDG uptake with the amount of S-phase-cells. These reports support the hypothesis that the degree of FDG uptake may

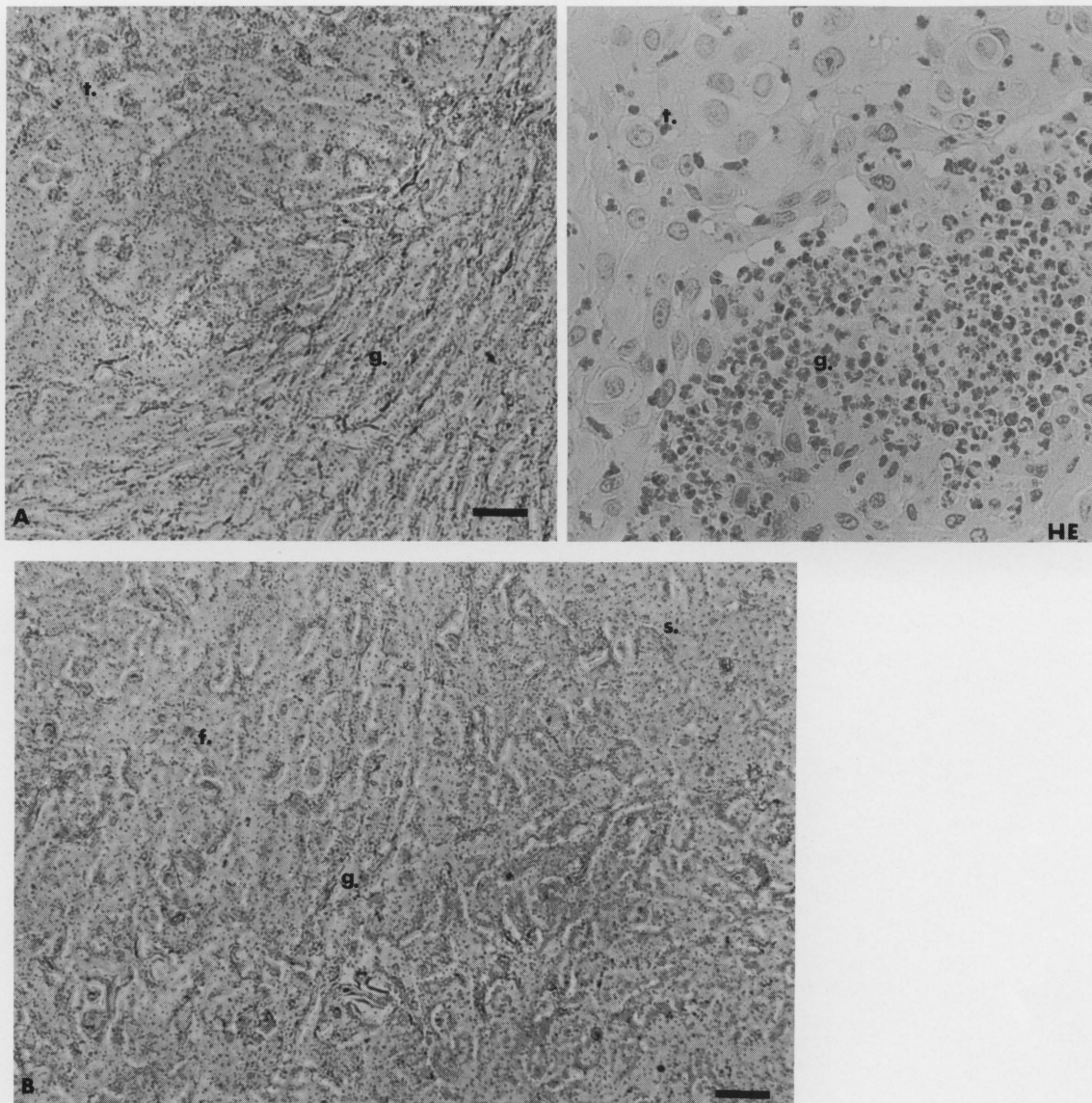


Fig. 7 Microautoradiograms of the tissue section. (A): Microautogram (left) and photomicrograph (right) show that a higher grain level within the granulation tissue than the viable tumor cells is observed. This region is the marginal areas surrounding necrosis near the center of tumor (Fig. 5D) (B): The grains are localized diffusely on viable tumor cells independent of the histologic features of the tumor tissues. (g: granulation tissue, t: tumor cell, f: follicle forming tumor cell, s: trabecularly growing cell); Scale bar = 5.5 μ m.

reflect proliferative activity and implicit aggressiveness of a tumor.

However, we could not find a correlation between the amount of S-phase cells and the histologic features of the tumor.

Kubota and associates¹⁷ reported that in an intratumoral [¹⁸F]FDG distribution study of a C3H/He mouse FM3A tumor model, [¹⁸F]FDG accumulation was relatively higher in macrophages and young granulation tissue than in the tumor cells. Our macroautoradiographic examinations also revealed that the areas with granulation tissues and/

or the margin of necrotic tissues showed higher accumulation of [¹⁴C]DG than tumor cells of trabecularly and follicularly growing tissues of thyroid cancer AC2 xenografts. No accumulation of [¹⁴C]DG was observed in microscopically necrotic tissue.

Our findings therefore indicate that the degree of deoxyglucose uptake may reflect not only tumor cell viability and proliferation but also inflammatory reaction and degenerative reaction to tumors.

Further studies are under way to evaluate the correlation between the uptake of other metabolic substrates and

malignant thyroid tumor grade, using our animal model which will be helpful in understanding the role of PET in thyroid cancer research.

ACKNOWLEDGMENTS

A part of this research was supported by a Grant-in-Aid from the Cancer Research Fund provided by the Ministry of Education (Group leader; Yukio Tatenno) of Japan.

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