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Morphological properties of human thyroid tumor cells in collagen gel culture and metastatic or invasive ability

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Summary. Using normal human thyroid cells and tumor cells, the reconstruction of various diseased cells in collagen gel as well as the relationship between the morphology of colonies in collagen-embedded culture and the biological behavior (benignity, malignancy, metastasis, and invasion) of the original tumors were studied. In collagen gel culture, normal thyroid cells reorganized follicle-like constructions, and follicular adenoma cells showed *in vivo*-like constructions. However, two different types of colonies were observed in cultures of cells from papillary carcinomas. One was the branching type with many outgrowths projecting to three dimensions and the other was the spherical type without any outgrowths. These spherical colonies were observed in all cases of papillary carcinoma, but varied from one case to another. Metastasis and invasion were detected during pathological examination in cases with a high ratio of spherical colonies. Our results indicate that cells from highly metastatic and invasive thyroid cancer form spherical colonies in the collagen gel culture, and that this collagen culture is a useful method for studying the heterogeneity of tumor cells as well as the metastasis and invasive ability of tumor cells *in vitro*.

Key words: Thyroid cancer, Metastasis, Invasion, Collagen gel culture

Introduction

It is well known that histological and functional differentiation of cells, such as duct-like formation of mammary tumor cells (Yang et al., 1979, 1981; Lawler et al., 1983; Yang and Nandi, 1983) and reconstruction of blood vessels by endothelial cells (Mandri et al., 1983; Montesano et al., 1983), is induced by the three dimensional culture of these cells in collagen gel.

On the other hand, reconstruction of thyroid follicles has been reported in cases of rat and porcine thyroid cells (Chambard et al., 1981). As for human thyroid cells, only a few studies have been performed on reconstruction (Toda and Sugihara, 1990) and iodide uptake of human thyroid cells in collagen gel culture (Morvan et al., 1988). However, there is no information about the morphological characteristics of diseased cells of human thyroid cultured in collagen gel.

Yang et al. (1979, 1981), Yang and Nandi (1983) and Lawler et al. (1983) have demonstrated outgrowths of various morphologies in normal mammary gland cells and mammary tumor cells cultured in collagen gel. They suggested that these colonies of different morphologies may have derived from different types of cells. Lawler et al. (1983) reported that more outgrowths were produced by mammary gland tissues obtained from older mice, but the implications of these morphologically different colonies *in vivo* are yet to be clarified.

In this paper, we investigated the reconstruction of various diseased cells of human thyroid in collagen gel as well as the relationship between the ratio of morphologically different colonies in the collagen gel culture and diverse clinicopathological conditions, such as benignity, malignancy, metastasis, and invasive ability of the cells.

Materials and methods

Tissue preparation

Surgical specimens from patients with papillary carcinoma of thyroid (8 cases), follicular adenoma (3 cases) and Graves' disease (2 cases), as well as normal thyroid gland tissue (5 cases) were used for the experiments. The normal tissues were taken from apparently normal areas of thyroid glands excised from patients with adenomas and carcinomas. These tissues were histopathologically checked before culture. The surgical specimens were finely minced and treated with 0.1% collagenase (Type I, Sigma) + 0.2% dispase (Godo Shusei, Tokyo) for 30 to 120 min at 37 °C. The cells were collected by centrifugation at 90g for 3 min and

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washed several times with Hanks' balanced salt solution followed by centrifugation at 90g for 1 min. Cells were cultured according to two procedures.

Monolayer culture

The cells were cultured in plastic dishes in Dulbecco's modified Eagle medium (DME, Nissui Seiyaku, Tokyo) supplemented by 10% fetal bovine serum (FBS, Gibco) in a humidified incubator under 5% CO_2-95% air conditions.

Collagen gel culture

Eight volumes of Type I collagen solution (Cellmatrix, Type I-A, Nitta Gelatin Inc., Yao) were mixed with one volume each of x10 concentrated Ham's F12 (without $NaHCO_3$), reconstituted buffer (2.2g $NaHCO_3$ + 4.77g Hepes in 100 ml of 0.05N NaOH) and FBS and kept in ice. Aliquots (0.5 ml) of this reconstituted collagen solution were placed in the wells of Corning multiwell-plates (2 cm^2/well), and immediately warmed to 37 °C to allow gel formation. This base layer was overlaid with a reconstituted collagen gel (0.5 ml) containing the cells ($1x10^5$ cells/ml). Finally, the collagen gels were overlaid with 1 ml of DME supplemented with 10% FBS or 10% FBS + Thyroid stimulating hormone (TSH, 1 mU/ml).

Overlaid medium was changed every other day.

Morphological studies

These cultured cells were fixed in 10% buffered formalin, followed by the preparation of paraffin sections. Some sections were stained with hematoxylin and eosin (HE), others were reacted with anti-thyroglobulin antibodies (1:400, Medac diagnostika) or anti-epidermal growth factor receptor (EGF-R) antibodies (1:400, Cambridge). Then the avidin-biotin-peroxidase complex method (Hsu et al., 1981) was used to visualize the binding of anti-thyroglobulin and EGF-R antibodies. As a control, normal rabbit serum was employed.

A part of collagen gel containing these cultured cells was fixed in 2% glutaraldehyde-2% paraformaldehyde-cacodylate buffer, post-fixed in 1% osmic tetroxide at 4 °C for 1 hour and embedded in Epon resin.

Results

In the monolayer culture of cells obtained from normal thyroid or tumor tissues, polygonal cells with projections proliferated, forming not colonies or follicles, but sheets (Fig. 1). In the collagen gel culture, however, many colonies could be observed. These colonies had a 3-dimensional structure. Immunohistochemical staining with anti-thyroglobulin antibody revealed positive staining of colonies grown in collagen gel (Fig. 2). These results suggested that these colonies had derived from thyroid epithelial cells.

In collagen gel culture of normal thyroid cells, the colonies had many outgrowths projecting in three dimensions (Fig. 3A). HE staining of these colonies revealed follicle-like constructions (Fig. 3B). In follicular adenoma cells, a characteristic branching type of colony was observed (Fig. 3C). These colonies were organized in small follicles similar to those observed in the original tumors (Fig. 3D).

In the case of papillary carcinoma, two different kinds of colonies were observed: the branching type, in which colonies had many outgrowths projecting in three dimensions; and the spherical type, without any outgrowth (Figs. 3E,F). HE staining revealed follicle-like formations in both types of colonies derived from papillary carcinoma cells (Figs. 3G,H). As shown in Table 1, the ratio of the two kinds of colonies varied.

Table 1 shows the percentage of spherical colonies in the collagen gel culture, and the presence of pathological metastasis in the regional lymph nodes as well as the presence of invasion beyond thyroid capsule in each case. The spherical colonies in culture predominated in cases 1-5 showing, clinically, a high degree of metastasis in lymph node and invasion beyond thyroid capsule. In

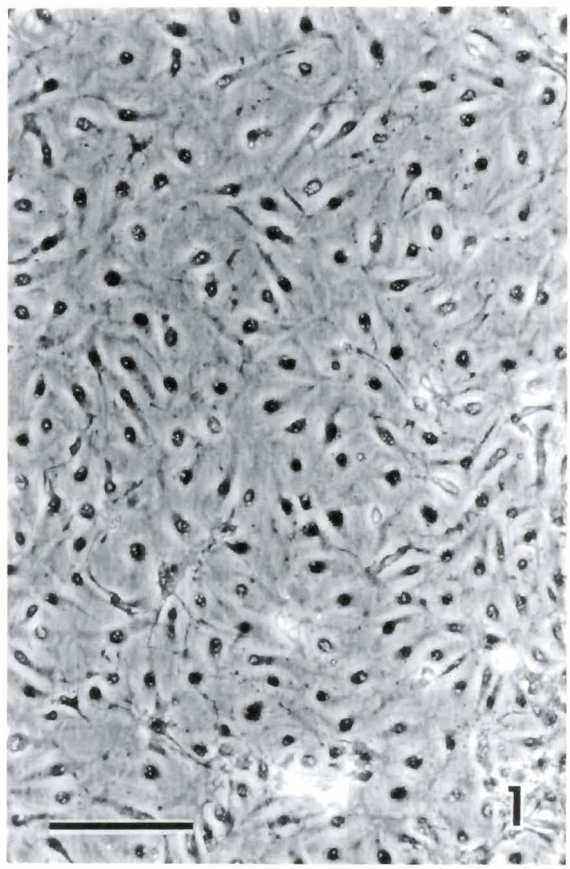


Fig. 1. This photograph shows monolayer culture of normal thyroid cells. Polygonal cells with projections proliferate like a pavement, and do not form follicles. Bar = 50 μm

Table 1. Percentage of spherical colony of various thyroid diseases in collagen gel culture.

DIAGNOSIS	CASE No.	SEX	AGE	PERCENTAGE OF SPHERICAL COLONIES	pTNM	INVASION BEYOND THE CAPSULE	METASTASIS OF REGIONAL LYMPH NODE
PC	1	F	41	90.2	$pT_4N_1M_0$	+	+
	2	F	59	90.0	$pT_4N_1M_0$	+	+
	3	F	53	85.3	$pT_4N_1M_0$	+	+
	4	F	58	84.8	$pT_4N_1M_0$	+	+
	5	F	70	78.5	$pT_4N_1M_0$	+	+
	6	F	44	19.7	$pT_2N_0M_0$	-	-
	7	F	42	12.2	$pT_2N_0M_0$	-	-
	8	F	47	5.6	$pT_2N_0M_0$	-	-
FA	9	F	60	0	---	-	-
	10	F	48	0	---	-	-
	11	F	32	0	---	-	-
GD	12	F	24	0	---	-	-
	13	F	27	0	---	-	-
Nor	14	F	60	0	---	-	-
	15	F	50	0	---	-	-
	16	F	48	0	---	-	-
	17	F	47	0	---	-	-
	18	F	41	0	---	-	-

PC: papillary carcinoma; FA: follicular adenoma; GD: Graves' disease; Nor: normal thyroid tissue; pTNM: post surgical histopathological classification of UICC.

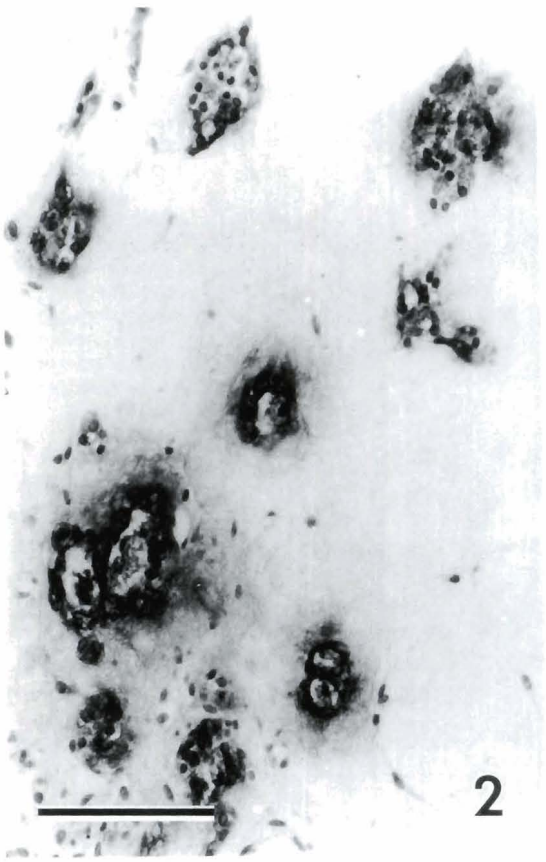


Fig. 2. This photograph shows immunohistochemical study with anti-thyroglobulin antibody. Positive reactions are observed on cells of colonies in collgen gel. Immunoreactivity is visualized by the ABC method. Bar = 100 μm

cases 6-8, most of the colonies in culture were the branching type. Clinically, no metastasis in the lymph node was observed, and invasion took place only inside the capsule. All colonies were the branching type in follicular adenoma, Graves' disease and normal thyroid gland tissue.

In the colonies derived from normal, Graves' diseased and follicular adenoma cells, there was an increase in the number of outgrowths and they were bigger after adding TSH to the medium in collagen gel culture (Fig. 4A). There was, however, no morphological change of the spherical colonies derived from papillary carcinoma cells (Fig. 4B).

In the immunohistochemical study with anti EGF-R antibody, normal and Graves' diseased cells showed negative or very weak reactions. Follicular adenoma cells showed weak or moderate intensity. But immunohistochemical intensity in spherical colonies was strong, and was stronger than in branching colonies (Figs. 5A,B).

Ultrastructural analysis of cells from normal and adenoma tissues was performed after 10 days of culture. On electron microscopy, the colonies exhibited morphological polarity. Mitochondria, rough endoplasmic reticulum, and the Golgi apparatuses were located around the nucleus. Numerous microvilli were observed at the apical surface. Lysosomes, colloid droplets, junction complexes and colloid-filled luminal space were also seen (Fig. 6A). Electron microscopic examination of colonies derived from thyroid carcinoma revealed many microvilli at the basal surface of the cells in the spherical colonies. However, in branching colonies, few microvilli were observed (Figs. 6B, C).

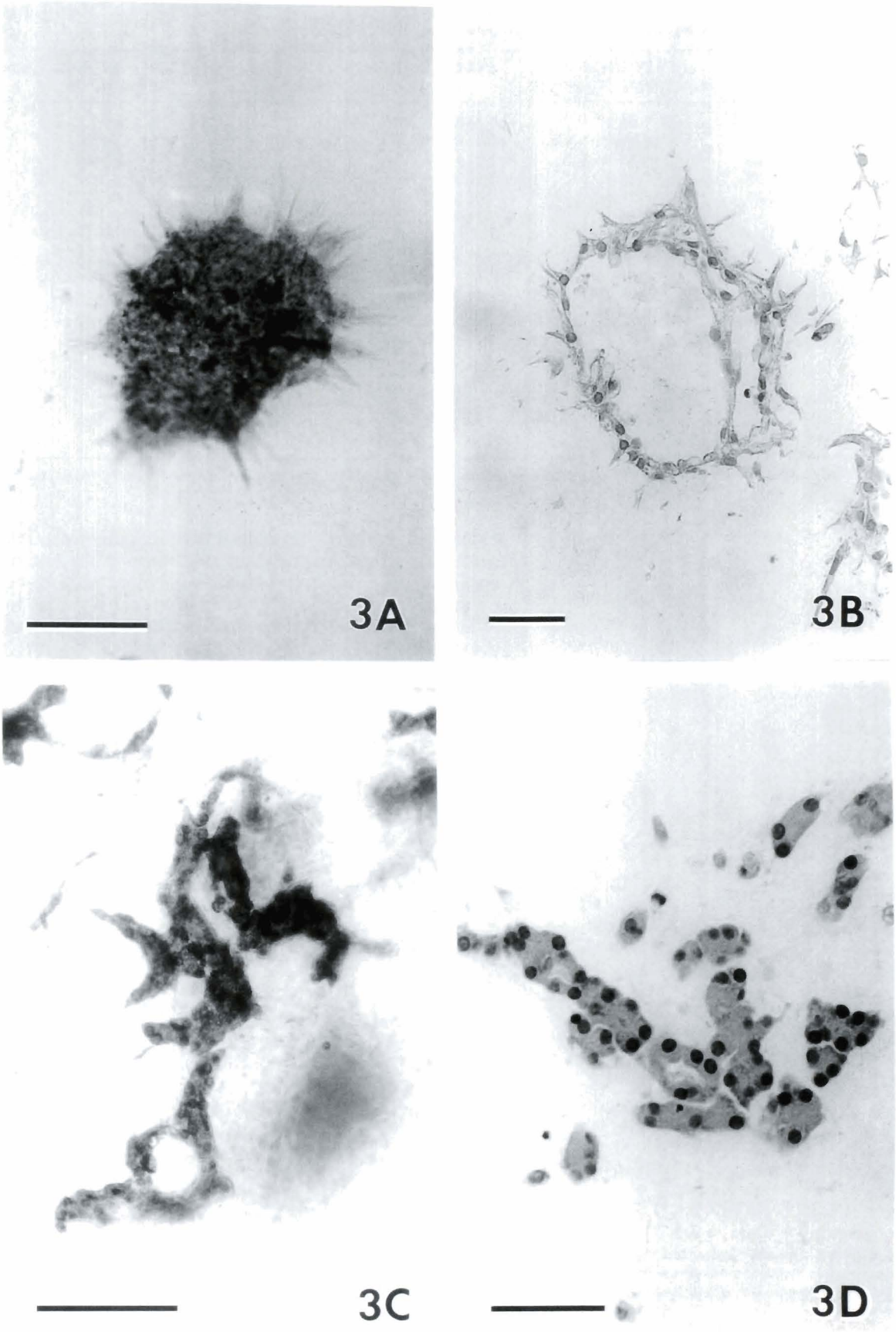
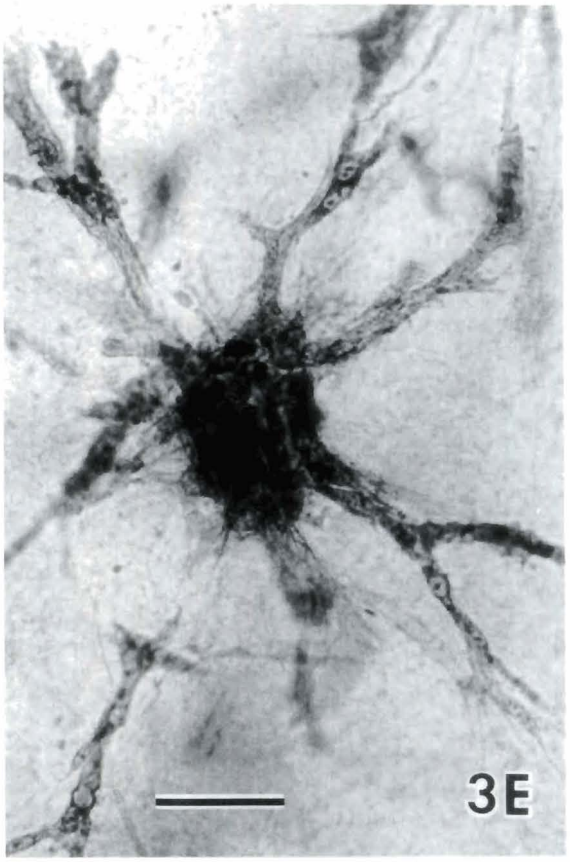


Fig. 3. Reconstructed normal and diseased thyroid cells in collagen gel. **A.** normal thyroid cells. **B.** HE staining of normal thyroid colony. Follicle-like structure is observed. **C.** Follicular adenoma cells. **D.** HE staining of follicular adenoma colony. In vivo-like structure is shown. E-H: These photomicrographs show two different kinds of colonies in collagen gel in the case of papillary carcinoma. **E.** Branching type colony: long and thick outgrowths from spherical mass are observed. These outgrowths are thicker and longer than of colonies produced by normal thyroid cells (see Fig. 3A). **F.** Spherical type colony; This colony do not have any outgrowths. **G.** HE staining of branching type colony. **H.** HE staining of spherical colony. Bar = 50 μm.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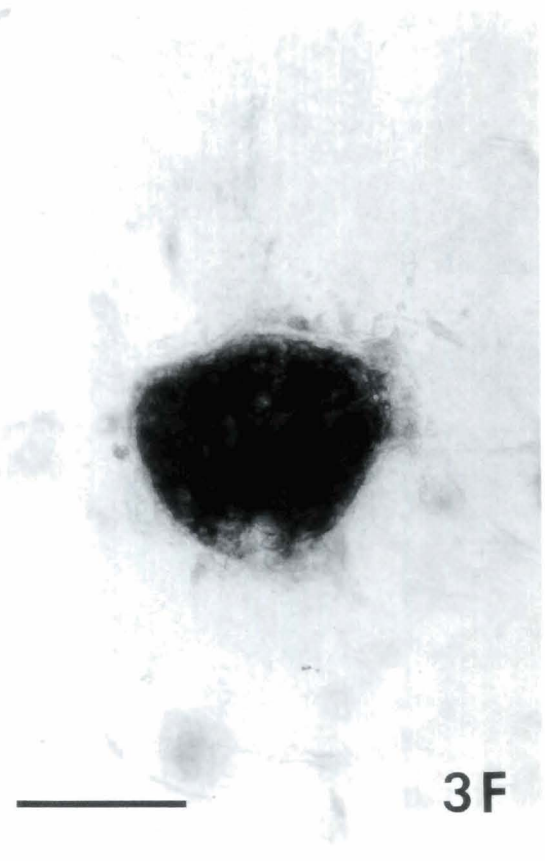
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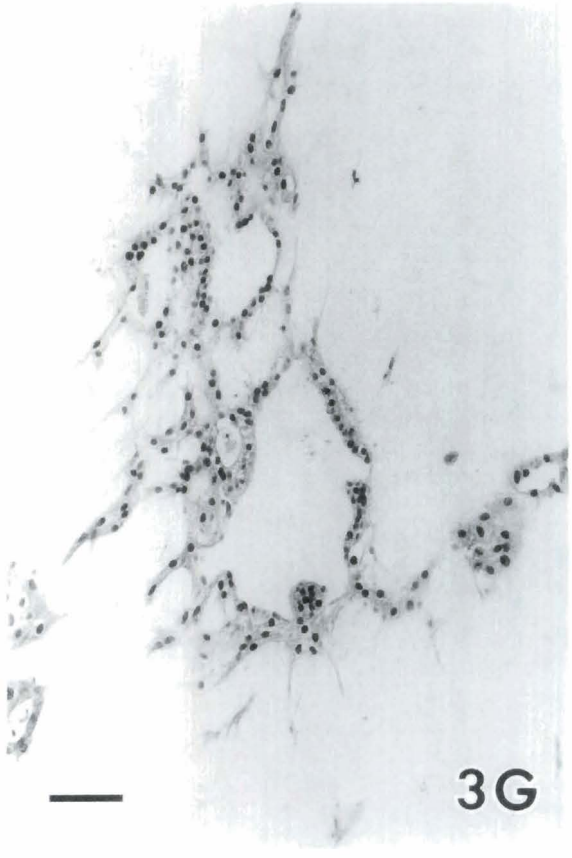
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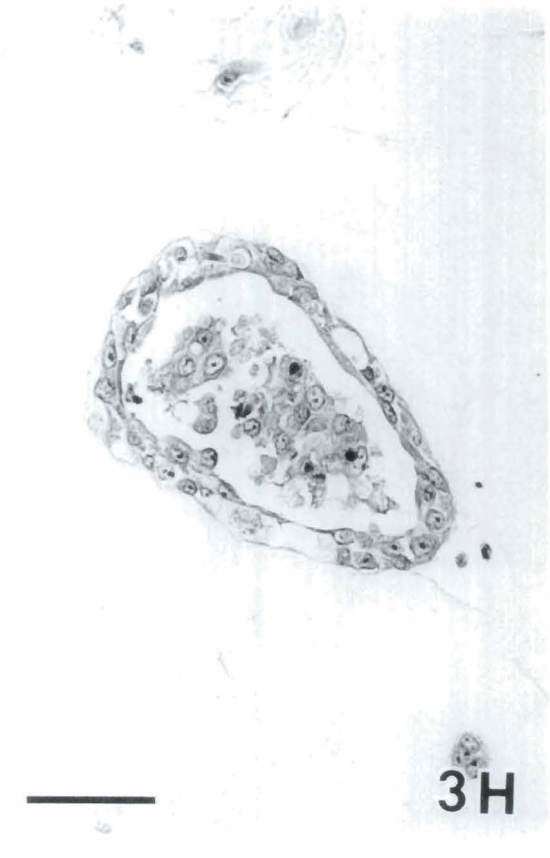
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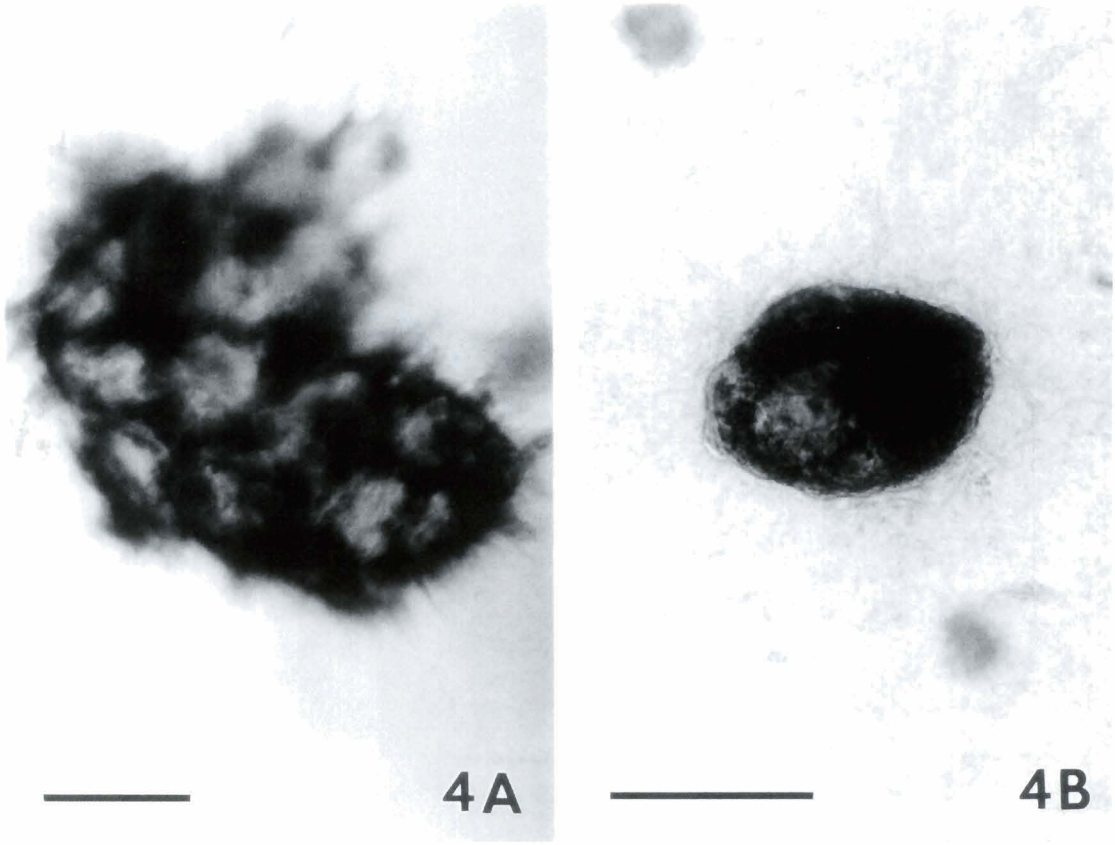


Fig. 4. Effects of the addition of TSH on the morphological change of colonies in collagen gel culture. **A.** Culture of normal human thyroid cells in the addition of TSH (1 mU/ml). The colony increase in size and is bigger than normal thyroid colony cultured with DME + 10% FBS (see Fig. 3A). Bar = 50 μm. **B.** Culture of spherical colonies produced by papillary carcinoma cells in the addition of TSH (1mU/ml). No morphological change is observed. Bar=50μm.

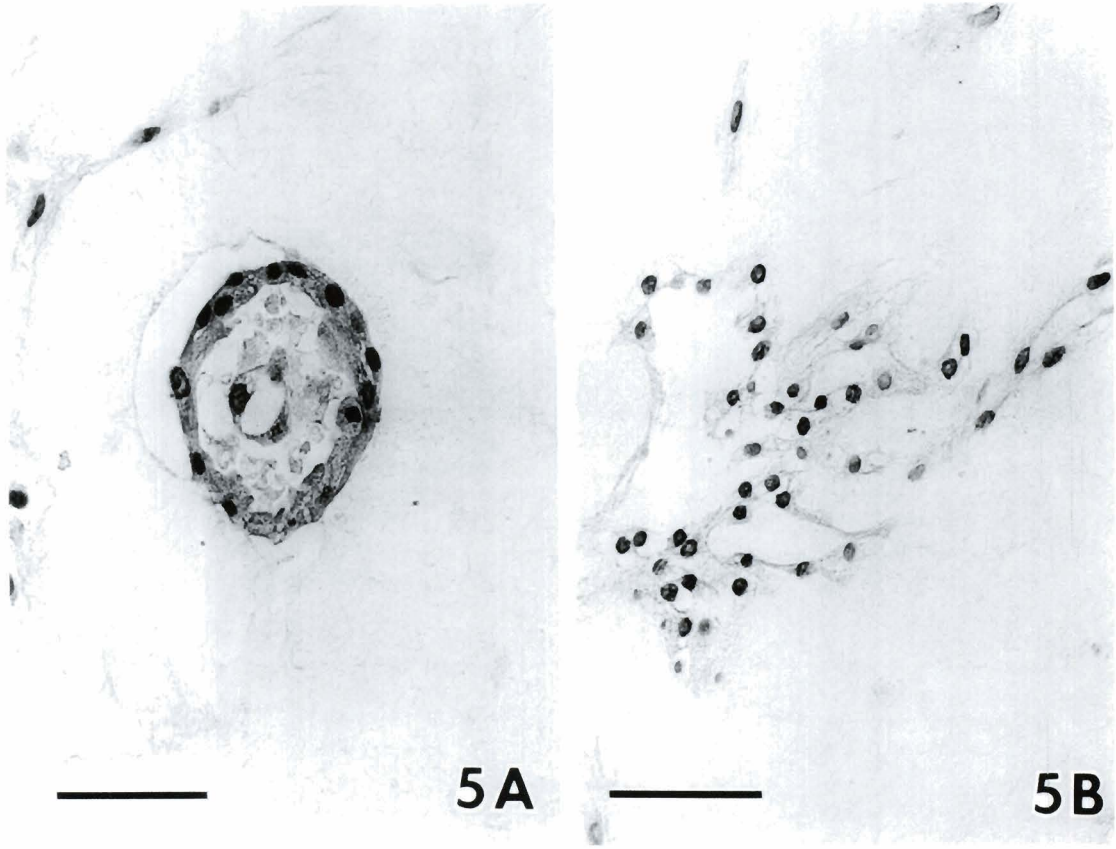
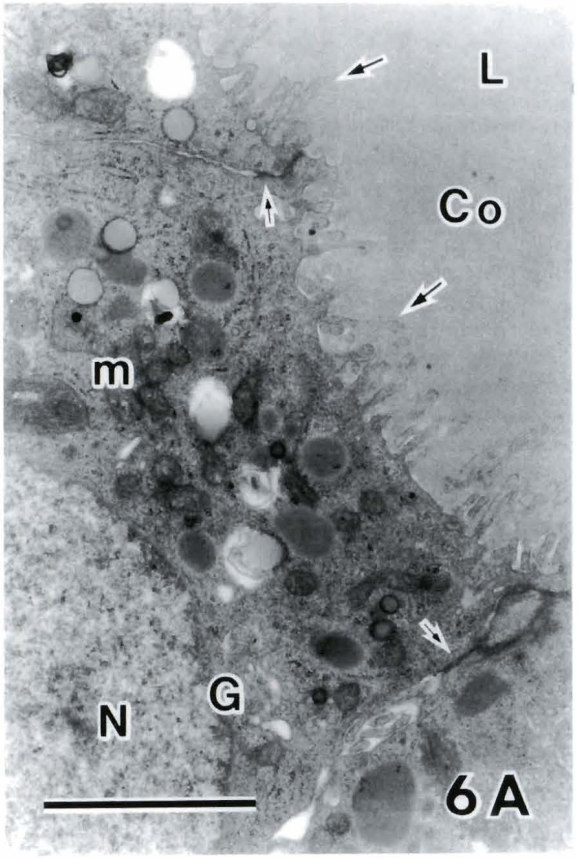


Fig. 5. Immunohisto-chemical observations of EGF-R. **A.** Spherical colony derived from papillary carcinoma. Strong positive reaction is observed. **B.** Branching colony derived from papillary carcinoma. Weak reaction is observed. (Counter staining with haematoxylin). Bar=50 μm.

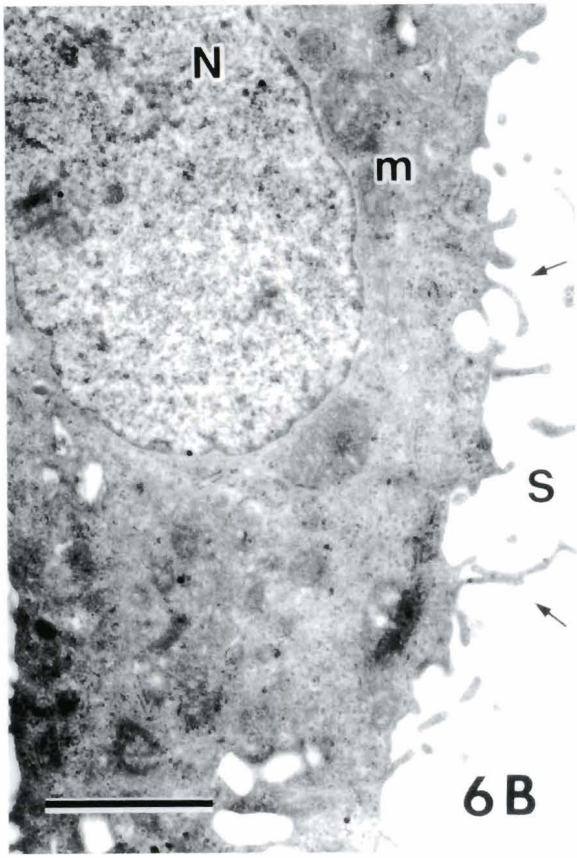


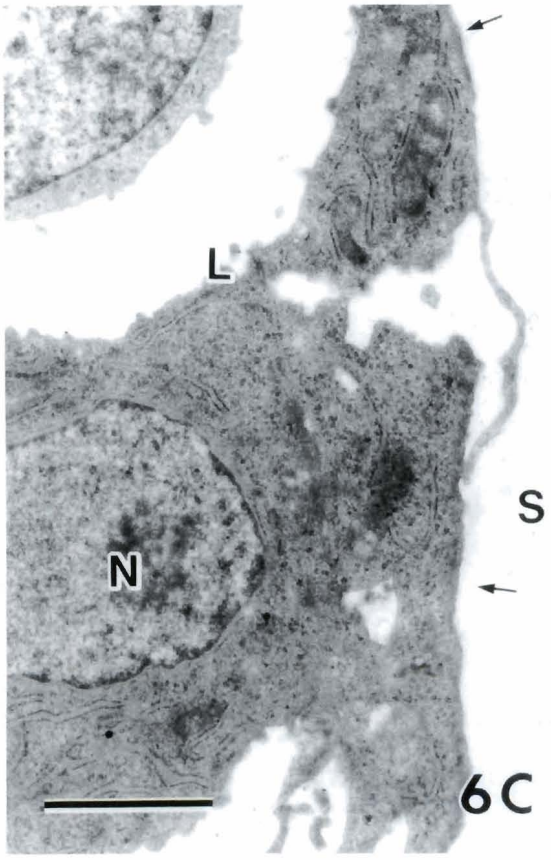
Discussion

Yang et al. (1979, 1981), and Yang and Nandi (1983) cultured normal mammary epithelial cells in collagen gel and observed three kinds of colonies with different morphologies. They suggested that these three kinds of colonies might consist of different types of cells. Lawler et al. (1983) studied whether the type of outgrowth was indicative of the neoplastic state and reported that more outgrowth was produced by mammary gland tissues obtained from older mice. In our experiment using thyroid epithelial cells, we were also able to observe colonies of different morphologies in collagen gel culture.

In this study, we concentrated our attention on spherical colonies derived from thyroid carcinoma. The colonies we defined as spherical did not develop any

Fig. 6. Electron microscopic observation of normal and carcinoma cells in collagen gel. **A.** Normal thyroid cells; The cells are polarized with microvilli and Golgi apparatus (G) apical to the nucleus (N). Many microvilli (large arrow) are seen at the apical surface of its follicle lumen (L). Cells have lysosomes, mitochondria (m), and rough endoplasmic reticulum. Junctional complexes (small arrows) and colloid-filled luminal space (Co) are observed at the apical side. **B and C.** Electron microscopic examination of two different kinds of colonies derived from papillary carcinoma cells in collagen gel. B: Spherical colony; Many microvilli (arrows) are observed at the basal surface (S) of cells. C: Branching colony; Smooth cell surface (arrows) is observed at the basal surface (S) of cells. L; Lumen. N; Nucleus. m; Mitochondria. Bar = 2 μm.





projections even after 14 days of culture or longer. These spherical colonies were observed in all cases of papillary carcinoma but the ratio varied among individual cases. Clinically, metastasis and invasion of the tumor tended to occur more frequently as the percentage of spherical colonies increased. This suggests that highly metastatic or invasive thyroid cells may form spherical colonies in collagen gel culture. On the other hand, all the colonies derived from cells obtained from normal thyroid gland and Graves' disease were the branching type, suggesting that the spherical colony formation is associated with tumorigenesis of thyroid epithelial cells. In only a few cases, we also obtained the same results as human colon and lung cancer cells in highly metastatic cases which showed spherical-type colonies in collagen gel (unpublished data).

The TSH receptors which induce the differentiation of thyroid cells are heterogeneous (Field et al., 1978; Abe et al., 1981). They decrease with the advance of the thyroid carcinoma stage (Siperstein et al., 1989). Moreover, high affinity TSH receptors are reported as undetectable in some papillary carcinomas (Abe et al., 1981; Chang et al., 1988; Siperstein et al., 1989) and in most undifferentiated carcinomas (Abe et al., 1981). In our present experiment, the colonies derived from normal thyroid cells grew further after the addition of TSH to the collagen gel culture, but spherical colonies derived from cells from papillary carcinoma showed no change in shape.

Moreover, in the case of colonies derived from carcinoma cells, the spherical colonies showed a stronger positive immunohistochemical reaction of EGF-R than the branching colonies. And on electron microscopic examination, cells in the spherical colonies displayed many microvilli on their surface, whereas those in the branching colonies had a smooth surface with few microvilli. Many researchers have demonstrated increased immunohistochemical expression of EGF-R (Masuda et al., 1988; Lemoine et al., 1991) and increased binding of radiolabelled EGF (Makinen et al., 1988; Kanamori et al., 1989) in thyroid cancers compared with adenomas or normal gland tissues. Ren et al. (1990a,b) found that microvilli were more abundant on the surface of highly metastatic clone cells. They also showed that the c-neu oncogene product, which is closely related to the EGF-R, is positively stained in microvilli of tumor cells with high growth potential and high metastatic ability, whereas tumor cells with low growth potential and weak metastatic ability are not stained. Their results are in agreement with ours and support our hypothesis that the spherical colonies produced by thyroid carcinoma in collagen gel culture have higher metastatic and growth potential than branching colonies.

The results presented above indicate that collagen gel culture may be a useful method to differentiate subpopulations in a single tumor which have different biological characteristics, such as the morphology of colonies. Moreover they suggest that a single thyroid carcinoma may consist of at least two different types of cells.

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