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PRODUCTION AND PROPERTIES OF NOVEL HUMAN THYROID CANCER SPECIFIC MONOCLONAL ANTIBODIES*

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Monoclonal antibodies (TCM-7, -9 and -12) against human thyroid differentiated cancers were established by screening with human thyroid cancers, normal and benign thyroid tissue, and normal human serum protein. A monoclonal antibody (TCM-9) with strong specificity for human thyroid cancer but not for Graves' disease, adenoma or normal thyroid, was shown to recognize a 300 K protein but not to bind to native or mature human thyroglobulin. When TCM-9 was used in immunohistochemical staining tests on more than 30 types of non-thyroid lesions, no reactivity of TCM-9 was observed except with skin immature teratoma, lip squamous carcinoma and stomach adenocarcinoma, which revealed weak reactivities. TCM-9 also showed strong reactivity with two undifferentiated thyroid cancer cell lines and one tissue specimen. Thus TCM-9 is a novel monoclonal antibody against the thyroid cancer.

Current diagnosis of differentiated thyroid cancer relies on palpation, various imaging techniques (ultrasonography, computed X-ray tomography, radioisotope scintigraphy and/or magnetic resonance imaging) and detection by needle or aspiration biopsy. Although these methods have greatly improved the accuracy of diagnosis, it is still quite difficult to distinguish follicular cancer from benign follicular adenoma. After the development of hybridoma technology, a variety of monoclonal antibodies (MoAbs) are established and found to have wide applications including the diagnosis of various cancers (1-3). Our aim in this study was to produce MoAbs that were capable of distinguishing human thyroid cancer (especially follicular cancer) from other benign thyroid nodules.

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MATERIALS AND METHODS

1. Membrane preparation

Various thyroid tissues were obtained at surgery, homogenized in 0.25M sucrose, 10 mM Tris-HCl, pH 7.6, 4 mM $MgCl_2$, then gauze filtered, and centrifuged at 180 x g for 5 min at 4℃. The supernatant was recentrifuged at 15,000 x g for 30 min and the precipitant was suspended in phosphate buffered saline and stored at -80℃ until use.

2. Cells

Mouse myeloma cells X63-Ag8.653 and human embryonal lung fibroblast cells (HEL) were provided by Prof. Tsuyoshi Uchida (Institute for Molecular and Cellular Biology, Osaka University). The human undifferentiated thyroid cancer cell line (HTC/C3) was isolated in our laboratory (4). Other human cancer cells of gastric cancer (AZ-251), human undifferentiated thyroid cancer (5-C-O), renal cell cancer (Thomas-Wirts), and fibrosarcoma (HT1080) were provided by the Japanese Cancer Research Resources Bank.

3. Thyroglobulin preparation

Human thyroglobulin (hTg) was prepared according to the method described by Bilstad et al. (5) with minor modifications. Human thyroid tissue was homogenized, and then centrifuged at 15,000 x g for 30 min. The supernatant was precipitated with 1.4-1.8 M ammonium sulfate and the precipitant was dissolved in 2 x condensed PBS (2 X PBS) and dialyzed. Then the sample was applied to HPLC G3000SW column (Tosoh, Japan), and the void fraction was collected and stored at -80℃ until use.

4. Production of hybridomas

Spleen cells were prepared from Balb/c mice immunized with membrane fractions of the differentiated thyroid cancer, and fused with X63-Ag8.653 as described previously (6). ELISA plates were coated with purified membranes' preparations from 25 cancers (17 papillary cancer and 8 follicular cancer), 6 adenoma, 6 Graves' tissues and 5 normal human thyroids; guinea pig fat cell membrane, normal human serum protein and hTg preparations and used for screening. The class and subclass of each MoAb were identified by an isotyping kit for mouse monoclonal antibodies (Serotec, Oxford. U.K).

5. Immunohistochemical staining

Immunohistochemical analysis was performed following the methods of De Micco et al.(7). Tissue sections were incubated with MoAb at a 1:100 dilution of ascites with normal mouse serum used as a control, and ABC-alkaline phosphatase staining (Vector laboratories) was performed.

6.Thyroglobulin specificity of MoAbs

Specificity of MoAb against thyroglobulin was evaluated by: 1) anti-thyroglobulin and anti-thyroid microsomal antibody measurement using a passive agglutination kit (Fujirebio, Tokyo, Japan), 2) Immunoprecipitation with ^{125}I-labelled human thyroglobulin (Midorijuji, Osaka, Japan) by Affigel Protein A (Bio-Rad), and 3) absorption of standard thyroglobulin preparations in an IRMA kit (Midorijuji, Chiba, Japan). In the IRMA kit, various concentrations of standard hTg were incubated with or without monoclonal antibodies or controls in anti-hTg MoAb coated tube. Then the solutions were removed and ^{125}I-MoAb directed against another hTg epitope was added, then the bound radioactivities were measured.

RESULTS

1. Immunohistochemical staining of thyroid gland

Extensive cloning was performed using membranes from 25 thyroid cancers, 6 adenoma, 6 Graves' tissues and 5 normal thyroids, and finally three clones (TCM-7, -9 and -12) that produced MoAbs with reactivity against

thyroid cancer membrane (but not adenoma and human serum protein) were selected from the 58 clones screened. All 3 MoAbs were identified to be IgG2b. Further immunohistochemical staining was performed as described (Table 1a). The MoAbs TCM-7, -9 and -12 were found to react strongly with human thyroid follicular cancer tissue, papillary cancer, but did not react with Graves' or adenoma tissue. The staining was exclusively in cytoplasma, not in colloid nor interstitial tissues (Fig.1). The highest level of binding was consistently seen in tests with TCM-9. The antibody TCM-9 reacted strongly with the undifferentiated cancer cell lines HTC/C3 and 5-C-O; TCM-7 reacted weakly with HTC/C3; and TCM-12 bound to

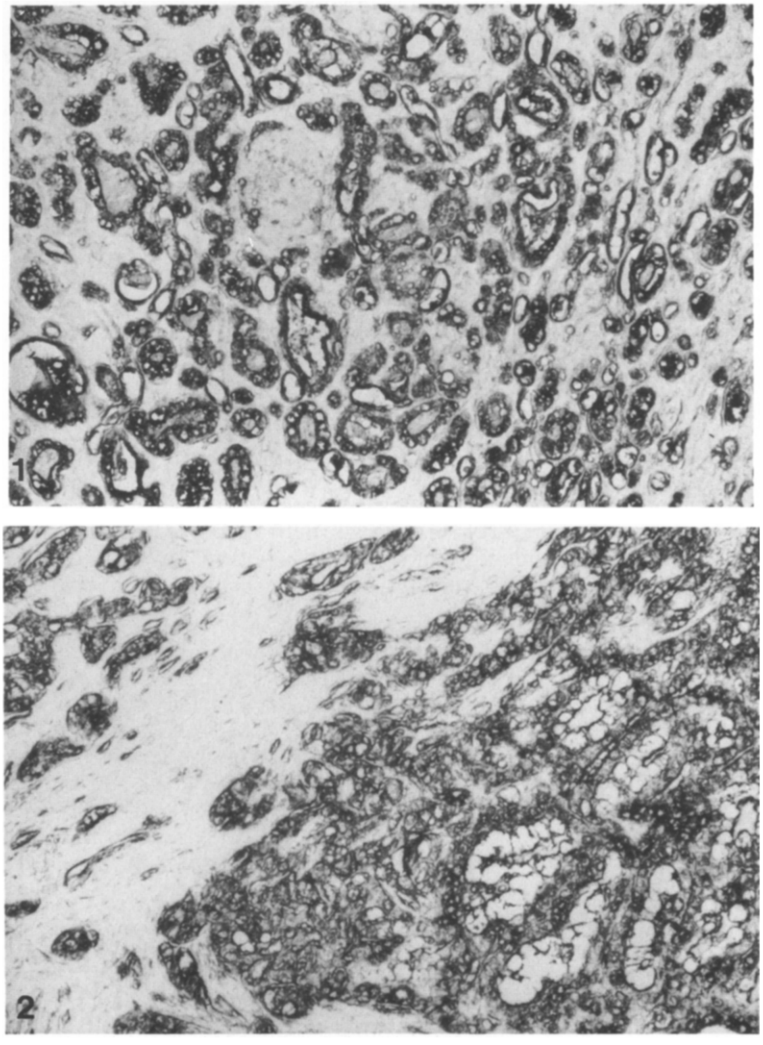

Fig. 1.
Immunohistochemical staining of thyroid tissue sections by TCM-9. 1, follicular cancer; 2, papillary cancer; 3, Graves' disease; 4, adenoma; and 5, undifferentiated thyroid cancer cell line HTC/C3.

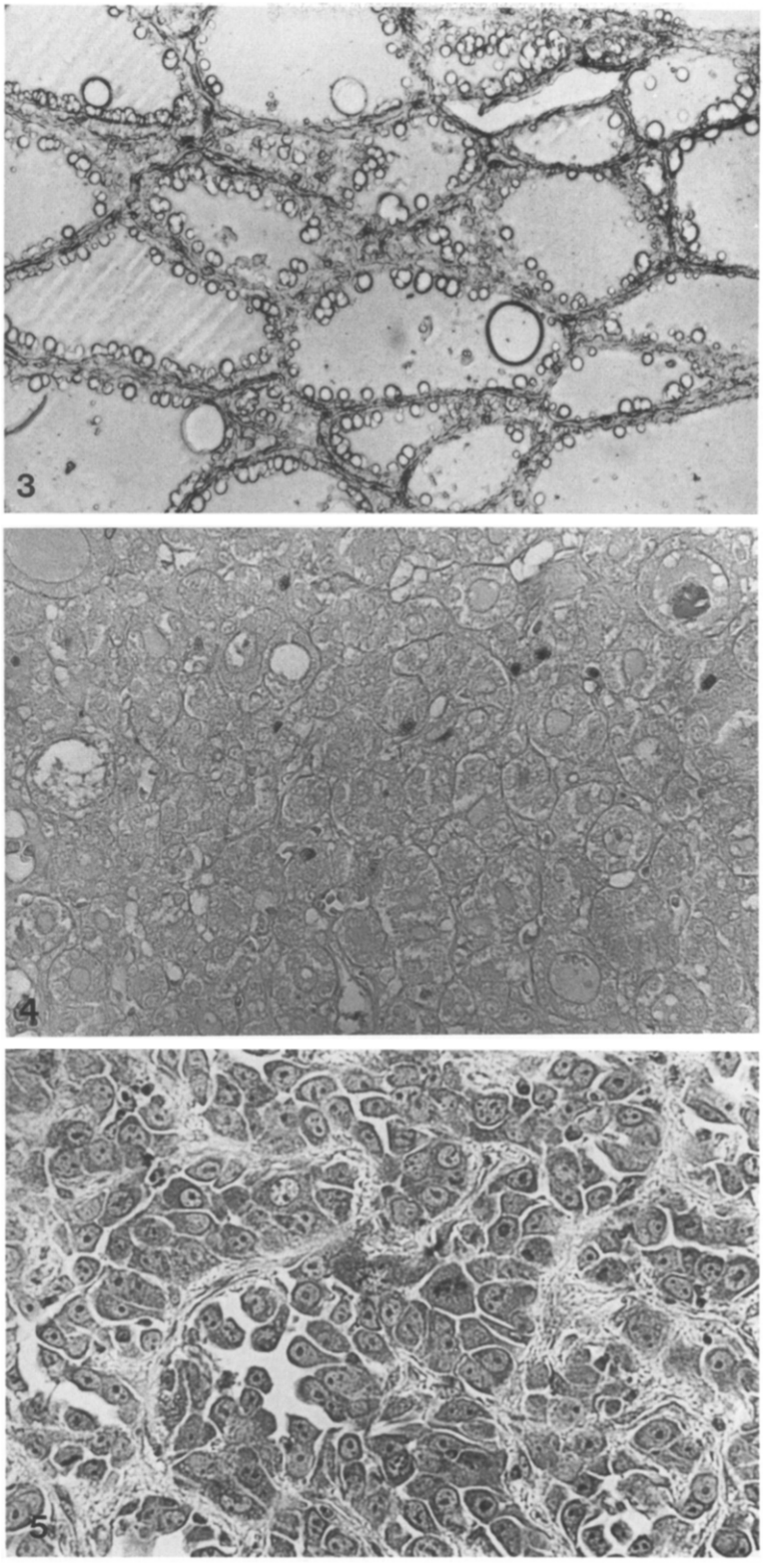

Fig. 1 - Continued

Table 1. Immunohistochemical staining

a) To thyroid lesions*

Thyroid disease	Number of positive staining	Reactivity TCM-7	TCM-9	TCM-12	NMS
Follicular cancer	2	+ +	+++	+	-
Papillary cancer	1	+	+ +	±	-
Graves' disease	0	-	-	-	-
Adenoma	0	-	-	-	-

b) To other carcinoma cell lines**

cell line	origin	TCM-7	TCM-9	TCM-12	NMS
AZ521	gastric	-	-	-	-
5-C-0	thyroid	-	+ +	-	-
Thomas-Wirts	renal	±	±	±	±
HTC/C3(11)	thyroid	±	+ +	-	-
OG-90	lung	-	-	-	-

Normal mouse serum (NMS) was simultaneously used as a control.
*: Two human thyroid follicular cancers, one papillary cancer, one Graves' tissue and one adenoma were tested.
**: TCM-9 shows cross-reactivity with undifferentiated thyroid cancer, but not with gastric and lung cancer cells.

neither(Table 1b). The reactivity of MoAbs with thyroid cancer was not reduced by 100% methanol for 30 min or 50mM periodate treatment at room temperature for 30 min(data not shown). Neither normal mouse serum nor a mouse MoAb against human alpha atrial natriuretic peptide (α-ANP)(6) gave a positive result with the thyroid cancer cells tested.

2. Binding of MoAbs with other cancer cell lines and non-thyroidal tissues.

All the 3 MoAbs (TCM-7,-9,-12) and the normal mouse serum control reacted weakly with a renal cancer cell line (Thomas-Wirts). They did not react with gastric or lung cancer cell lines. As shown in Table 2, most of the non-thyroidal tissues did not react with TCM-9, regardless of whether they were benign or malignant. Some cases of stomach cancer, immature teratoma and skin squamous cell carcinoma did, however, exhibit weak reactivity with TCM-9.

3. Reactivity with Tg and thyroid microsomal antigen

In SDS-PAGE and electroblotting assays, follicular cancer membrane reacted with TCM-9 and gave a single band with relative molecular weight of approximately 300 K under reducing conditions (Fig.2). The reactivity of TCM-9 was stronger than that of TCM-7, which showed a similar band at 300 K (data not shown). The lack of positive immunostaining of the colloid,

Table 2. Reaction of TCM-9 with various benign and malignant lesions

Malignant lesions		Benign lesions	
Uterine cervix			
Squamous cell ca. case1	-	cervical polyp	-
case2	-		
Ovary			
serous cyst adenocarcinoma	-		
adenocarcinoma	±		
Liver			
hepatocellular ca. case1	-	cirrhosis	-
case2	-		
Soft tissue			
synovial sarcoma	-	neurofibroma case1	-
immature teratoma		case2	-
epidermis	+	neurinoma case1	-
serous epithelium	+	case2	-
sweat gland	+	case3	-
		granular cell tumor	-
		angiolipoma	-
		hemangioma	-
		pyogenic granuloma	-
		leiomyoma	-
		dermatofibroma	-
		desmoid tumor	-
Bone			
		osteochondroma	-
		fibrous dysplasia	+?
Stomach			
adenocarcinoma case1	+	ATP	-
case2	±	inflammatory polyp	-
case3	-		
Large intestine			
adenocarcinoma case1	±	adenoma case1	-
case2	-	case2	-
Pituitary gland			
		adenoma	-
Skin			
squamous cell ca.(lip)	+		
malignant melanoma	-		
Prostate			
adenocarcinoma	-		
Lymph node			
non Hodgkin's lymphoma	-	normal lymph node	-
Kidney			
renal cell ca.	-		
Nasopharynx			
nasopharyngeal ca. case1	-		
case2	-		

The bone fibrous dysplasia specimen contained abundant calcification.

membrane preparation of Graves' tissue, and purified hTg indicated that these MoAbs likely do not bind to hTg, or at least to its native form. This was further confirmed by several methods. Antibodies TCM-7 and 9 did not aggregate latex beads coated with hTg or thyroid microsomal antigen(data not shown). When ^{125}I-labeled hTg was incubated with TCM-7 or

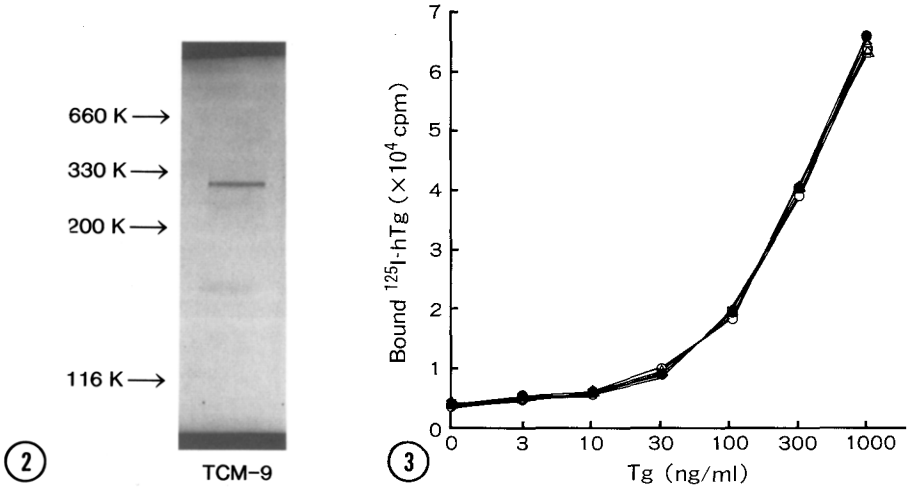

Fig. 2.
Western blotting with TCM-9. SDS-PAGE of a follicular carcinoma membrane reacted with TCM-9 showing a single band (300 K). Lines indicate the position of standard molecular markers; 19S thyroglobulin (660 K), reduced thyroglobulin (330 K), myosin (200 K) and E. coli β-galactosidase (116 K).

Fig. 3.
Effect of TCM-9 on the binding of hTg with anti-hTg antibodies. Addition of TCM-7 (open circle) or TCM-9 (closed circle) did not influence the standard binding curve when compared to the addition of control MoAbs (anti-human ANP antibody) (closed triangle) or buffer (open triangle).

-9 and precipitated by Affi-Gel Protein A, no significant increase in the precipitated radioactivity was observed when compared to anti-human α-ANP MoAb (a negative control), or polyclonal hTg antibody (a positive control, data not shown). Further, incubation of standard hTg preparations with TCM-7, -9 and control MoAb did not affect the standard curves of hTg IRMA (Fig. 3). The IRMA system is composed of two anti-hTg MoAbs, which recognize different epitopes on the hTg molecule. If TCM-7 or TCM-9 bound to the same or proximal site as the IRMA anti-hTg MoAbs, the standard curve would shift. The absence of this result indicates that no inhibition of the binding of hTg with the provided monoclonal anti-hTg antibody occurred.

The undifferentiated thyroid cancer cell lines (HTC/C3 and 5-C-O) were not immunostained with anti human Tg (data not shown). As described above, TCM-9 reacted with the undifferentiated thyroid cancer (two cell lines and one tissue lesion). Thus, TCM-9 recognizes a novel thyroid cancer specific epitope not a molecule of thyroglobulin.

DISCUSSION

Immunohistochemical studies of various thyroid tissues with these MoAbs revealed that all three stained differentiated cancer cells.

Recently, there have been many reports indicating that cancer cells are unique in that they have various abnormalities in the carbohydrate moiety of the structure of proteins that can be detected by MoAb (8). Periodate and methanol treatments of the membrane preparation did not affect the binding of TCM-9. These observations indicate that TCM-9 recognizes the peptide portion but not the carbohydrate nor lipid component of the antigen molecule. Many MoAbs produced by immunization with purified hTg have been reported (9-14), and a certain amount of structural heterogeneity of Tg in cancer tissues or sera of patients have been indicated by these MoAbs. These previous reported MoAbs likely react with native hTg, whereas our results indicate that TCM-7, -9 and -12 do not react with native hTg from benign thyroid tissues. All the Tg specific monoclonal antibodies reported previously recognizes the peptide portion of the molecule predominantly (15) or a conformational structure of Tg (11). The monoclonal antibodies in these studies were selected to recognize native Tg, although some showed different reactivities against serum Tg in thyroid cancer patients and patients with non-malignant thyroid diseases (9). The monoclonal antibody TCM-9 was selected not to recognize native Tg. Furthermore, to our knowledge, there is no report about the monoclonal antibody against human undifferentiated thyroid cancer. Thus, TCM-9 is a novel MoAb against thyroid cancer specific antigen. It will potentially have application in future studies.

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