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A histopathological, immunohistochemical and ultrastructural study of intranuclear cytoplasmic inclusions in thyroid papillary carcinoma

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Summary. Intranuclear cytoplasmic inclusions (ICI) in 38 cases of thyroid papillary carcinoma were studied histopathologically, immunohistochemically, and ultrastructurally in order to examine the frequency of ICI and their relationship to both the histological structure and cytological findings in thyroid papillary carcinoma. The fine-structure and biochemical state of ICI were also studied. ICI occurred in all 38 cases. ICI occurrence ranged from one in several microscopic fields to more than ten per field. The number of ICI divided by the number of nuclei on the microscopic photographs ranged from 0.013 to 0.116. The frequency of ICI was strongly influenced by the state of nuclear chromatin and pleomorphism, but was not influenced by a pattern of papillary or follicular tumour growth. Immunohistochemically, 10–30% of ICI revealed strong thyroglobulin (Tg), which was ascertained by immunoelectron microscopy. Neither T3 nor T4 was detected in ICI (with some exceptions). Some ICI showed keratin and vimentin. PAS-positive ICI were observed. Ultrastructurally, enlarged r-ER, many Golgi vesicles and small vesicles (diameter of 300–500 nm) and sacs were observed in ICI. These findings suggested increased protein synthesis and/or protein accumulation. Abundant secondary lysozomes, showing degradation of ICI, and bundles of condensed intermediate filaments were also detected. The character and genesis of ICI are discussed.

Key words: Papillary carcinoma of the thyroid – Intranuclear cytoplasmic inclusions – Immunohistochemistry – Thyroglobulin

Introduction

Intranuclear cytoplasmic inclusions (ICI) are weakly eosinophilic and spherical inclusions in the nuclei observed by light microscopy. Cytoplasmic inclusions containing cell organelles surrounded by a nuclear envelope are observed by electron microscopy (Doniach 1970; Carcangiu et al. 1985).

ICI appear to be the "poorly stained giant nucleoli" reported by Söderström in 1966 as a finding strongly suggesting malignancy in fine-needle aspiration biopsy of the thyroid. The incidence of ICI is still a valuable sign of malignancy in aspiration biopsy of the thyroid gland. It is one of the diagnostic findings for papillary thyroid carcinoma since ICI occur in 70~90% of papillary carcinomas (Christ et al. 1979; Hanawa et al. 1979; Toriya et al. 1984). ICI are now also known to be appear in medullary carcinoma (Kakudo et al. 1978) and follicular carcinoma (Michael et al. 1984) of the thyroid.

Ultrastructurally, Gray et al. (1968) found that ICI were not nucleoli but intranuclear cytoplasmic inclusions. They are thought to be caused by invagination of the cytoplasm. Söderström et al. (1973) reported moderately electron-dense spherical bodies (M-bodies) 0.5–1.0 μ in diameter in ICI, and Carneiro et al. (1980) reported abundant microfilaments. The presence of r-ER in addition to these findings suggests sequential decomposition processes in ICI.

The purpose of the present study has been to investigate the relation between the frequency of ICI and histological and cytological findings of papillary carcinoma. I have also examined the fine structure and the biochemical state inside ICI using immunohistochemistry and ultrastructural studies in surgical material from papillary thyroid carcinoma.

Materials and methods

38 cases of thyroid papillary carcinoma were investigated at the second Surgical Department of the Gunma University School of Medicine between February 1985 and February 1987. The diagnosis of papillary thyroid carcinoma was based upon

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the WHO classification (Hedinger et al. 1974). The tumours possessed papillae composed of a fibro-vascular core covered with carcinoma cells. Some were mainly composed of papillary structures, but others included various amounts of follicular growth. Small blocks of 1 cm^3 on the border of normal thyroid tissue were examined by light microscopy and immunohistochemistry. The tissue blocks were fixed in 10% phosphate-buffered formalin for 24–48 h and embedded in paraffin. The paraffin sections were then stained with haematoxylin and eosin (H.E.) and periodic acid Schiff (PAS).

For immunohistochemical investigations, the same paraffin material was used. Light microscopic examinations were made by observing sections stained with H.E.

The frequency of ICI in papillary thyroid carcinoma was examined by two methods. In the first the numbers of ICI of three fields which had a relatively high incidence (observed by light microscopy at 400 magnification) were counted and the average, per visual field, was calculated. The tumours were then grouped into five types according to the average finding, + + + (more than ten per field), + + (3–9 per field), + (1–2) per field), ± (one per several fields), − (ICI not detected). In the second the numbers of ICI in 300–400 nuclei in three microscopic fields with a magnification of 700 were counted in cases which had a relatively high frequency of ICI. The number of ICI divided by the numbers of nuclei (ICI/nuclei) was calculated and the incidence of ICI per nucleus was then expressed in decimal fractions.

The classification of histological structure of papillary thyroid carcinoma was made according to the ratio of papillary and follicular structures. The tumours were grouped into four types, the papillary where papillary structure was predominant, a predominantly papillary type where papillary structures predominated but follicular areas existed, a mixed papillary and follicular type where papillary and follicular structure occurred in almost the same ratio, and a predominantly follicular type where follicular structure predominated (including the so-called follicular variant).

The state of nuclear chromatin and nuclear pleomorphism were divided into a clear type where nuclei with peripherally located chromatin and sharp nuclear membrane were present. Most of this type have scattered coarse-granular chromatin. A fine-granular type was also seen where the distribution of chromatin was uniform, although some of this type had coarse chromatin as found in the clear type. Finally a condensed type with nuclei with uniform condensed chromatin was present.

According to the degree of nuclear pleomorphism, the tumours were grouped into three classes as follows. (+) – round or oval uniform nuclei, (+) – nuclei with moderate irregularity in shape, including nuclear clefts, and moderate variety in size, (+ +) – nuclei with severe irregularity in shape and severe variety in size. Large round or bizarre nuclei were occasionally seen.

Histochemical observations were made with PAS staining and immunohistochemical observations in 15 of the 38 cases (No. 4, 6, 8, 9, 14, 17, 22, 23, 24, 25, 29, 31, 33, 34, 38) with a high frequency of ICI and sufficient material using the avidin-biotin peroxidase complex method. The paraffin sections were stained for thyroglobulin (Tg), triiodothyronine (T3), thyroxine (T4), keratin, vimentin and actin. The antisera for Tg, keratin (wide spectrum), and vimentin were obtained from Dako corporation (California, USA) and used at a dilution of 1:500 for thyroglobulin and keratin, and at 1:200 for vimentin. The antisera for T3, T4, and actin were obtained from Bio-Yeda (Kiryat Weizmann, Israel) and used at a dilution of 1:200 for T3 and T4, and at 1:100 for actin. The antisera for anti-rabbit IgG or anti-mouse IgG and abidin-biotin peroxidase complex were obtained from Vector Laboratories. Inc. (California, USA) as VECTASTAIN ABC KIT and used according to the procedure.

Conventional electron microscopic observation was made in 10 of 38 cases (No. 2, 4, 22, 23, 24, 27, 29, 33, 34, 36), having a high frequency of ICI and well-fixed material. Small blocks were immediately fixed in 2.5% glutaraldehyde solution for 3–4 h, post-fixed in 1% osmium tetroxide, dehydrated and embedded in epoxy resin (Epon 812) as usual. Ultrathin sections were made on a LKB 2088 ultrotome V and stained with uranyl acetate and lead citrate.

Immunoelectron microscopy for thyroglobulin was carried out by the pre-embedding immunoenzyme method (Kawaoi 1984). Small blocks from 3 cases (No. 33, 34, 36) with a volume of 0.5 cm^3 were fixed in a PLP solution for 3–4 h at 4° C, then successively immersed through 10, 20 and 20% sucrose with 5% glycerin solutions in PBS, each for 6–12 h at 4° C with gentle agitation. They were then quickly frozen in embedding matrix. The indirect immunoperoxidase method was applied to the frozen sections. The sections, with a thickness of 8–10 μ, were incubated in the same anti-thyroglobulin antiserum as used for light microscopy at 4° C for 24–48 h. The material was then washed in PBS and incubated with horseradish peroxidase-labelled anti-rabbit IgG, goat IgG Fab'fragment solution, obtained from Biosys, SA (Compiègne France), at 4° C for 24–48 h. The peroxidase activity was visualized by incubating in a hydrogen peroxidase-free solution of 3,3′-diaminobenzidine (DAB) in Tris-HCl buffer for 30 min at room temperature and subsequently in DAB solution containing 0.005% hydrogen peroxidase for 5–10 min at room temperature. The sections were then post fixed in 1% osumium tetroxide for 1 h at room temperature, dehydrated and embedded in epoxy resin (Epon 812) using upside-down application of gelatin capsules. Ultrathin sections were made on LKB 2088 V ultrotome and observed without counterstaining.

Negative controls: The sections were incubated with non-immune rabbit serum at the same dilution as thyroglobulin antiserum in place of the first antibody, and were processed in the same way as the sections treated with the antiserum.

Electron and immunoelectron microscopic observations were made using JEM 7A, 100C and 200CX.

Results

Thirty seven females and one male ranging in age from 18 years to 60 years (mean 44 years) were studied. The primary region of the carcinoma was in the left lobe in 17 cases, the right lobe in 18 cases and the isthmus in 3 cases. At the time of operation, the carcinoma was localized to the thyroid gland in 21 cases. In 17 cases (45%), the carcinoma showed invasion or adhesion to the surrounding tissues such as the anterior cervical muscles, trachea, oesophagus, internal jugular vein, common cartid artery and recurrent laryngeal nerve. In 11 of these cases, invasion was confirmed histologically. Regional lymph-node metastasis was shown in 27 cases (71%). Distant metastasis was not shown. As of March 1988, all patients are alive.

ICI was apparent in all cases. The frequency of ICI according to the 38 cases, more than ten per field in 3 cases (8%), 3–9 per field in 11 cases (29%), 1–2 per field in 12 cases (32%), and one

Table 1. Surgical findings and frequency of ICI

Case			Tumor		Lymph. meta.	Extra. invasion[a]	Frequency of ICI[b]	
No.	Age	Sex	Lobe	Size (cm)			per field	ICI/nuclei
1	35	♀	isth.	1.2 × 0.8	7/15	−	+ +	0.055
2	40	♀	left	0.8 × 0.8	0/19	−	+ +	0.059
3	34	♀	left	2.0 × 2.0	5/26	−	+	0.025
4	40	♀	right	2.4 × 2.2	5/33	−	+ +	0.077
5	32	♀	left	2.1 × 1.8	10/21	+	+	0.025
6	59	♀	right	2.5 × 2.3	7/19	+	+ +	0.067
7	37	♀	left	0.8 × 0.7	0/26	−	+	0.031
8	44	♀	isth.	0.8 × 0.7	3/46	−	+ + +	0.059
9	63	♀	right	0.8 × 0.7	2/30	−	+ +	0.050
10	45	♀	right	1.4 × 1.0	3/30	±	±	0.021
11	35	♀	left	2.4 × 1.5	10/18	+	±	0.013
12	36	♀	right	0.5 × 0.5	0/14	−	+ +	0.067
13	40	♀	right	1.6 × 1.5	11/33	+	±	0.027
14	57	♀	right	1.2 × 0.9	5/47	−	+ +	0.034
15	56	♀	right	0.6 × 0.5	0/19	−	±	0.033
16	30	♀	right	0.2 × 0.2	0/27	−	+	0.026
17	60	♀	left	4.0 × 3.4	7/28	+	+	0.029
18	45	♀	right	1.6 × 1.2	2/41	±	+	0.034
19	40	♀	left	1.7 × 1.5	1/33	±	+ +	0.048
20	36	♀	right	3.0 × 2.5	5/64	±	±	0.032
21	67	♀	right	6.8 × 4.8	23/38	+	±	0.022
22	62	♀	left	1.0 × 1.0	11/50	−	+ + +	0.116
23	51	♀	left	1.5 × 3.8	0/33	−	+ +	0.061
24	60	♀	left	6.0 × 3.5	14/74	+	+ +	0.056
25	46	♀	isth.	1.0 × 0.8	0/33	−	+	0.045
26	45	♀	right	1.8 × 1.6	2/24	−	±	0.017
27	32	♀	left	2.3 × 2.5	0/50	−	+	0.029
28	38	♀	left	2.4 × 1.8	6/21	±	±	0.018
29	50	♀	left	4.0 × 2.8	8/38	±	+	0.027
30	18	♀	left	2.5 × 1.9	4/34	−	±	0.022
31	40	♀	right	2.0 × 2.4	10/40	+	+	0.026
32	59	♂	right	5.0 × 4.8	9/16	+	±	0.022
33	51	♀	left	3.3 × 3.2	13/17	−	+ +	0.055
34	48	♀	right	3.0 × 2.2	21/33	±	+ + +	0.038
35	38	♀	right	1.2 × 1.2	0/34	−	±	0.037
36	41	♀	left	3.5 × 2.5	0/35	−	+	0.019
37	39	♀	left	3.7 × 2.5	0/43	+	±	0.021
38	24	♀	right	2.5 × 2.2	13/33	−	+	0.032

[a] Carcinoma showing invasion or adhesion to the surrounding tissues such as anterior cervical muscles, trachea, esophagus, internal jugular vein, common cartid artery etc. is expressed by ±, and in these, carcinoma in which invasion was certained histologically, is expressed +.

[b] The incidence of ICI was examined by two methods: (1) The number of ICI per visual field of light microscope with a magnification of ×400. (2) The number of ICI divided by the number of nuclei

per several fields in 12 cases (32%) (see Table 1). The number of ICI divided by nuclei (ICI/nuclei) ranged from 0.013 to 0.116 (mean value: 0.039).

The frequency of ICI in 5 cases with large tumour size (more than 4 cm), regional lymph-node metastasis and extraglandular invasion to the surrounding tissues (No. 17, 21, 24, 29, 32) was 3–9 in one case, 1–2 in two cases, and one per several fields in two cases and the mean value of ICI/nuclei was 0.039. However, the frequency of ICI in 7 cases with small tumour size (less than 2 cm), no lymph-node metastasis and no invasion (No. 2, 7, 12, 15, 16, 25, 35) was 3–9 in two cases, 1–2 in three cases, and one per several fields in two cases and the mean value of ICI/nuclei was 0.043. The mean of the major axis and minor axis in regard to tumour size showed no significant difference in the frequency of ICI between the group (18 cases) with larger than average tumour size (2.07 cm), and the group (20 cases) with smaller tumour size. There was also no significant difference in the frequency of ICI between the group with regional lymph-node metastasis (27 cases) and the group without metastasis (11 cases). The frequency of ICI in the

Table 2. Papillary (P), follicular (F) structure and frequency of ICI

Histological[a] structure	Frequency of ICI				
	The number of ICI per field				ICI/nuclei (average)
	+++	++	+	±	
P (8 case)	0	2	3	3	0.035
P>F (19 case)	2	4	6	7	0.039
P=F (4 case)	1	1	1	1	0.040
P<F (7 case)	0	3	3	1	0.041

[a] P: Almost papillary structure
P>F: Predominantly papillary structure
P=F: Papillary and follicular structure in the same ratio
P<F: Predominantly follicular structure

Table 3. The state of nuclear chromatin and frequency of ICI

Chromatin[a]	Frequency of ICI				
	The number of ICI per field				ICI/nuclei (average)
	+++	++	+	±	
Cd (5 case)	2	2	1	0	0.059*
Fg (20 case)	1	7	7	5	0.040*
Cl (13 case)	0	2	4	7	0.030*

[a] Nuclei were grouped into three classes; clear type, fine-granular type, condensed type and the tumors then grouped into three classes; Cd, Fg, Cl according to the quantities of these three types-nuclei

* Statical examination of the differences in the groups by Kruskal-Wallis test ($P=0.13$)

group without invasion (21 cases), however, was higher than in the group invasion (17 cases)*.

The tumors were grouped into 4 types; papillary, predominantly papillary, mixed papillary and follicular and follicular type according to the ratio of papillary and follicular structures (Table 2). They were then compared with regard to the frequency of ICI. In each type of tumour, the frequency of ICI detected by light microscopy ($\times 400$) was distributed almost equally between one per several fields and more than ten per field. The mean values of ICI/nuclei of 4 types ranged from 0.035 to 0.041 and there was no significant difference (Kruskal-Wallis test, $P=0.73$). The frequency of ICI varied locally in one specimen of papillary carcinoma but was not influenced by the pattern of papillary or follicular tumour growth.

The tumours were grouped according to the state of nuclear chromatin and nuclear pleomorphism, and the groups were compared as to the frequency of ICI (Tables 3, 4). The Cl group (Fig. 1) was mainly composed of cases with a low frequency of ICI (one per several fields or 1–2 per field). The Fg group (Fig. 2) was composed of cases with varying frequency of ICI. The Cd group (Fig. 3) was composed of cases with a high frequency of ICI (more than 10 or 1–2 per field). The average of ICI/nuclei also increased from 0.030 to 0.059 in accordance with the change of the state of nuclear chromatin (Kruskal-Wallis test, $P=0.13$).

The low nuclear pleomorphism group (±) (Fig. 1) was composed of cases with a low frequency of ICI (one in several fields or 1–2 per field). The moderate nuclear pleomorphism group (+)

* Statical examination by U-test. $P=0.49$ (size), 0.62 (metastasis), 0.04 (invasion)

Table 4. Nuclear pleomorphism and frequency of ICI

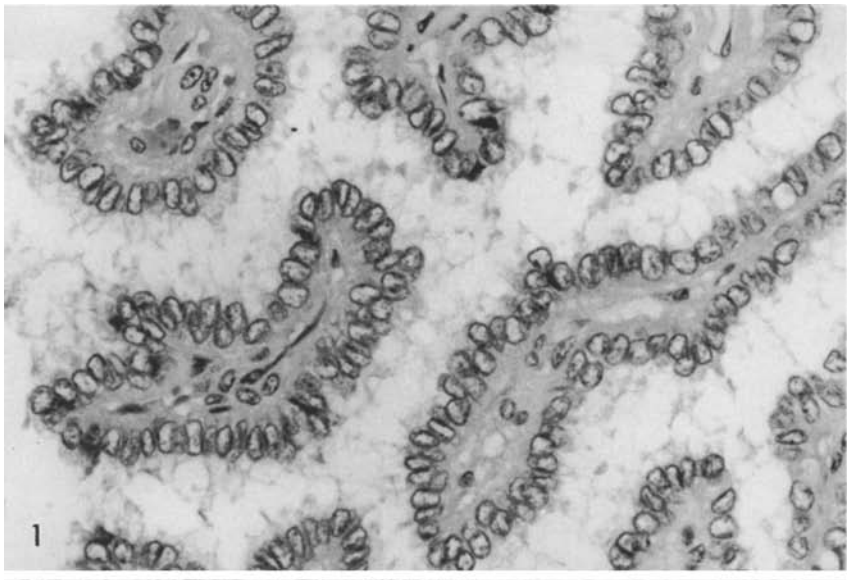
Pleomorphism[a]	Frequency of ICI				
	The number of ICI per field				ICI/nuclei (average)
	+++	++	+	±	
++ (7 case)	2	4	1	0	0.062*
+ (23 case)	1	6	9	7	0.037*
± (8 case)	0	0	3	5	0.022*

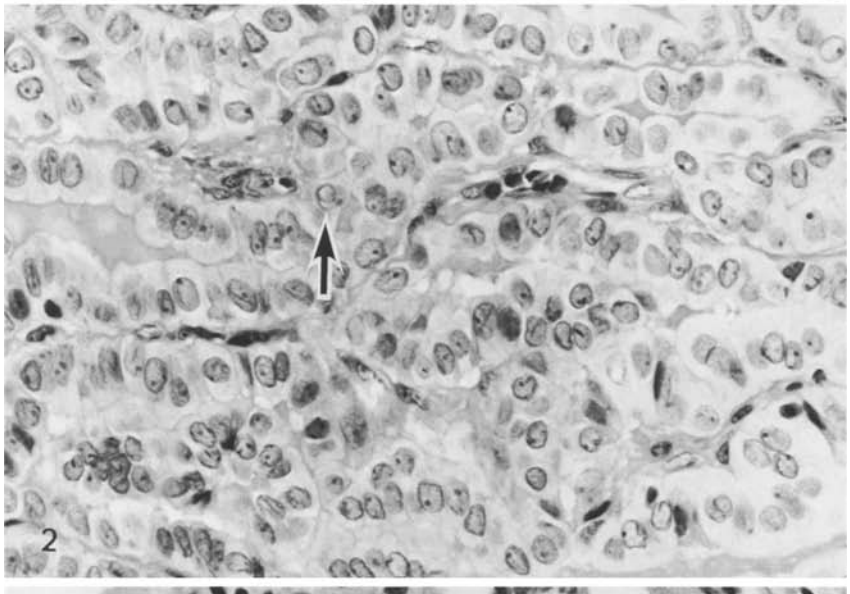
[a] ++: Nuclei with severe irregularity in shape and severe variety in size +: Nuclei with moderate irregularity in shape and moderate variety in size ±: Round or oval uniform nuclei

* Statical examination of the differences in the groups by Kruskal-Wallis test ($P=0.007$)

showed a range of incidence of ICI. The severe nuclear pleomorphism group (Fig. 3) was composed of cases with more than ten or 3–9 ICI per field. The average of ICI/nuclei also increased from 0.026 to 0.065 in accordance with nuclear pleomorphism (Kruskal-Wallis test, $P=0.007$). This same tendency applies to similar specimens. Most nuclei with ICI showed fine-granular or condensed chromatin and were large and irregular with invagination of a nuclear envelope.

Immunohistochemically, diffuse or mosaic-like staining for thyroglobulin was detected in ICI (Fig. 4) and ICI stained blackish brown were regarded as "positive". Thyroglobulin was positive in ICI in all cases. More than 30% of ICI were stained in one case, 10–30% of ICI in 12 cases, and less than 10% of ICI in 2 cases. A T3-positive cytoplasmic reaction was observed in 5 out of 15 cases and T4 in 7 out of 15 cases but these reactions occurred in only a small part of the tumour. Only 2–3 ICI showed T3-positive globularity in 2 cases





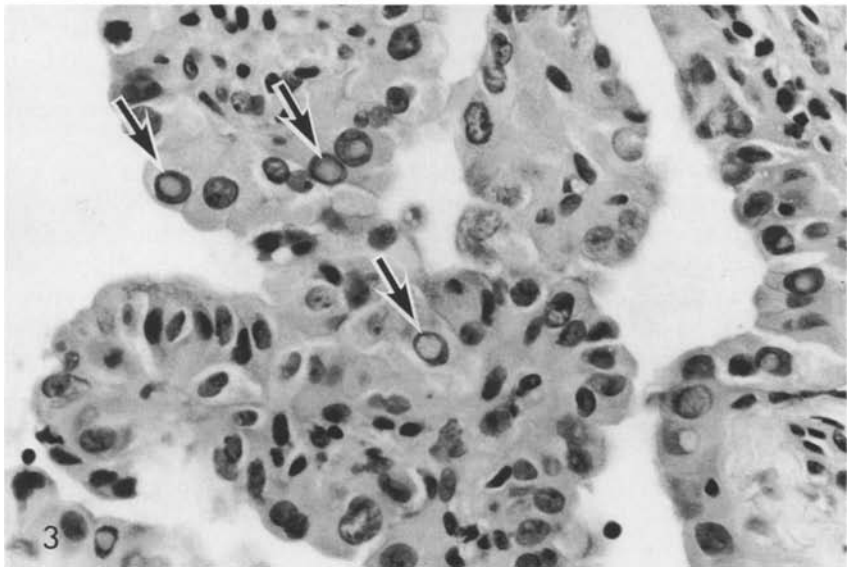


Fig. 1. Papillary carcinoma of the thyroid (Case No. 15, 56 years, female). The nuclei are round or oval and uniform in size. The contents of the nuclei are clear (clear type). ICI is not detected. × 500. Stained with H.E

Fig. 2. Papillary carcinoma of the thyroid (Case No. 5, 32 years, female). The nuclei have a moderate variety of size and of irregularity in shape. The nuclear chromatin is fine-granular (fine-granular type). One ICI (*arrow*) is detected. × 500. Stained with H.E

Fig. 3. Papillary carcinoma of the thyroid (Case No. 22, 62 years, female). The nuclei have marked variety of size; large round or irregular-shaped nuclei are observed. The nuclear chromatin is mostly condensed (condensed type). Various ICI (*arrowheads*) can be seen. × 500. Stained with H.E

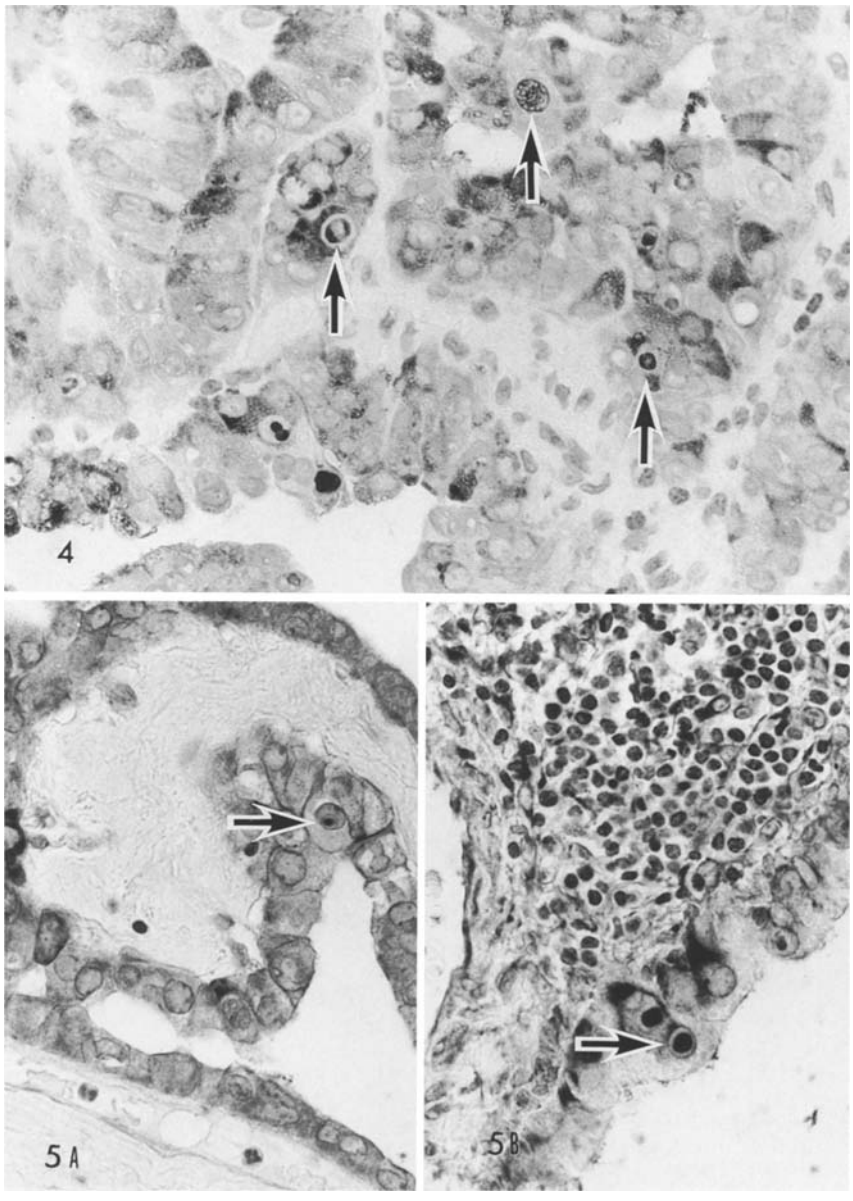


Fig. 4. Immunohistochemical staining for thyroglobulin of papillary carcinoma (Case no No. 22, 62 years, female). Some ICI show diffuse or mosaic staining (*arrows*) for thyroglobulin. × 500. ABC method.

Fig. 5. (**a**) Immunohistochemical staining for keratin in papillary carcinoma (Case No. 4, 40 years, female). One ICI shows globular staining (*arrow*) for keratin. Keratin is weakly and diffusely positive in the greater part of cytoplasm. × 500. ABC method.
(**b**) Immunohistochemical staining for vimentin in papillary carcinoma (Case No. 4, 40 years, female). One ICI shows globular staining (*arrow*) for vimentin. Vimentin is strongly positive in the basal portion of cytoplasm. × 500. ABC method

having a high frequency of ICI and T3 positivity in the cytoplasm. T4 was not detected in ICI. PAS-positive fine-granularity, or occasional globularity, was detected in ICI in 7 out of 15 cases. Positivity was mostly present in about 10% of the ICI in each of these 7 cases.

Keratin or vimentin was detected in some ICI in a globular or lumped shape (Figs. 5a, b). Keratin was observed in ICI in 14 cases, and vimentin in 13 out of 15 cases. Each of them was observed once or twice per several fields. Through observation of the serial sections, the coexistence of both keratin and vimentin in the same ICI was ascertained, although it was rare. Most papillary carcinoma cells contained actin, but the location could not be determined as some parts of the cytoplasm and nucleus were stained diffusely.

On electronmicroscopy the nuclei of papillary carcinoma with a high frequency of ICI showed severe irregularity of the nuclear envelope and the continuity of small irregular-shaped multiple ICI in the same nucleus was occasionally ascertained through observation of serial sections. Cytoplasmic invaginations, with cell organelles in the cytoplasm were occasionally observed. The membrane of ICI was composed of the inner leaflet of the nuclear envelope and heterochromatin on the outside and the outer leaflet of the nuclear envelope on the inside (Figs. 6, 7, 8, 10, 11). Cell organelles in the surrounding cytoplasm, especially in the cy-

Table 5. Immunohistological and histochemical results in ICI

No.	Tg[a]	PAS[b]	Thyroid hormone		Cytoskelton[d]	
			T_3	T_4	keratin	Vimentin
4	+	+	−	−	+	+
6	+	±	−	−	+	+
8	±	−	−	−	−	−
9	+	+	−	−	+	+
14	+	±	−	−	+	+
17	+	−	−	−	+	+
22	+	+	±[c]	−	+	+
23	+	−	−	−	+	+
24	+	±	−	−	+	−
25	±	−	−	−	+	+
29	+	−	−	−	+	+
31	+	−	−	−	+	+
33	++	+	±[c]	−	+	+
34	+	−	−	−	+	+
38	+	−	−	−	+	+

[a] Diffuse or mosaic-like blackish brown immunoreaction for thyroglobulin was regarded as positive. ++: More than 30% of ICI stained +: 10–30% of ICI stained, ±: Less than 10% of ICI stained −: no positive staining

[b] PAS-positive fine-granularity, or occassional globularity, was regarded as positive. The classification of the number of PAS-positive ICI is the same for thyroglobulin

[c] T_3-positive globularity was demonstrated in only 2–3 ICI

[d] Globular or lumped shape-immunoreaction for keratin, vimentin was regarded as positive(+) regardless of the number of positive ICI

toplasm near the nucleus, were observed in ICI. They resembled local organelles but varied considerably. ICI containing a large number of mitochondria were rare although mitochondria were frequently present in large quantities in the cytoplasm of papillary carcinoma. 5 to 20 mitochondria with other cell organelles were found in some ICI (Fig. 6). They were round or oval and the cristae usually appeared perpendicular to the long axis and well extended across the width. Fragments of mitochondria or crumpled membranes were occasionally observed in ICI, showing variable stages of degradation. R-ER were frequently present in ICI. Two types of r-ER were observed. One consisted of several laminae of flattened cisternae without content, while the other consisted of enlarged cisternae, globular or ellipsoid in shape, containing low or moderately electron-dense fine-granular material throughout the ICI. Small vesicles with a diameter of 300–500 nm (Figs. 8, 9, 10) and sacs with a minor axis of 200–300 nm (Fig. 10) were observed in ICI in close relationship to the r-ER. Small vesicles with a diameter of 300–500 nm sometimes occupied the whole ICI. Their shapes varied from almost complete spheres to enlarged irregular or saccules shapes and their contents varied from relatively dense material (Fig. 8) to fine-granular material (Fig. 9). The small vesicles were frequently coated with ribosomes (Fig. 9). Spherical swelling of a part of r-ER was observed; these closely resembled the small vesicles. Sacs with a minor axis of 200–300 nm were frequently close to the small vesicles and had similar contents (Fig. 10), although the laminar structure of gathered sacs occasionally occupied the entire ICI. Membrane-limited structures without ribosomal coating were present in moderate numbers in ICI but it was difficult to distinguish s-ER from the r-ER, small vesicles, and sacs. Golgi complexes were frequently observed. ICI with abundant small vesicles having a diameter of 50–100 nm and containing moderately electron dense material, seeming to be Golgi vesicles, were occasionally observed (Fig. 7) and larger vesicles with relatively electron-dense contents resembling dense bodies, sometimes accompanied the small vesicles. Free ribosomes were present in large numbers in the cytoplasm of most papillary thyroid carcinoma and were also abundant in ICI. Dense bodies were few in ICI and varied from spheres with a diameter of 100 nm with high electron dense contents to ellipsoids with a major axis of 300 nm and moderately electron dense contents.

Most ICI contained secondary lysozomes and residual bodies in various numbers (Fig. 8), sometimes occupied ICI throughout. Autophagosomes containing crumpled membranes or fine-granular material were also observed. Microfilaments were frequently seen. They were mostly intermediate filaments; some filaments lay parallel to one another in loose aggregates similar to the surrounding cytoplasm, others lay in condensed circular or U-shaped bundles (Figs. 6, 8). The tubular bodies clustered irregularly and were surrounded by a circular bundle of intermediate filaments (Fig. 11). R-ER or small vesicles with a diameter of 300–500 nm were observed near the tubular bodies.

On immunoelectron microscopy some ICI showed thyroglobulin. Immunoreactive Tg was demonstrated in r-ER of the cytoplasm (Figs. 12, 13) and perinuclear space of ICI and in globular, saccular and lamellar shape in ICI (Fig. 12). Secondary lysozomes were negative for thyroglobulin (Fig. 13), but the fine-structure of Tg could not be clearly determined by immunoelectron microscopy.

No positive staining was detected in ICI incubated with non-immune rabbit serum in place of the antisera for thyroglobulin.

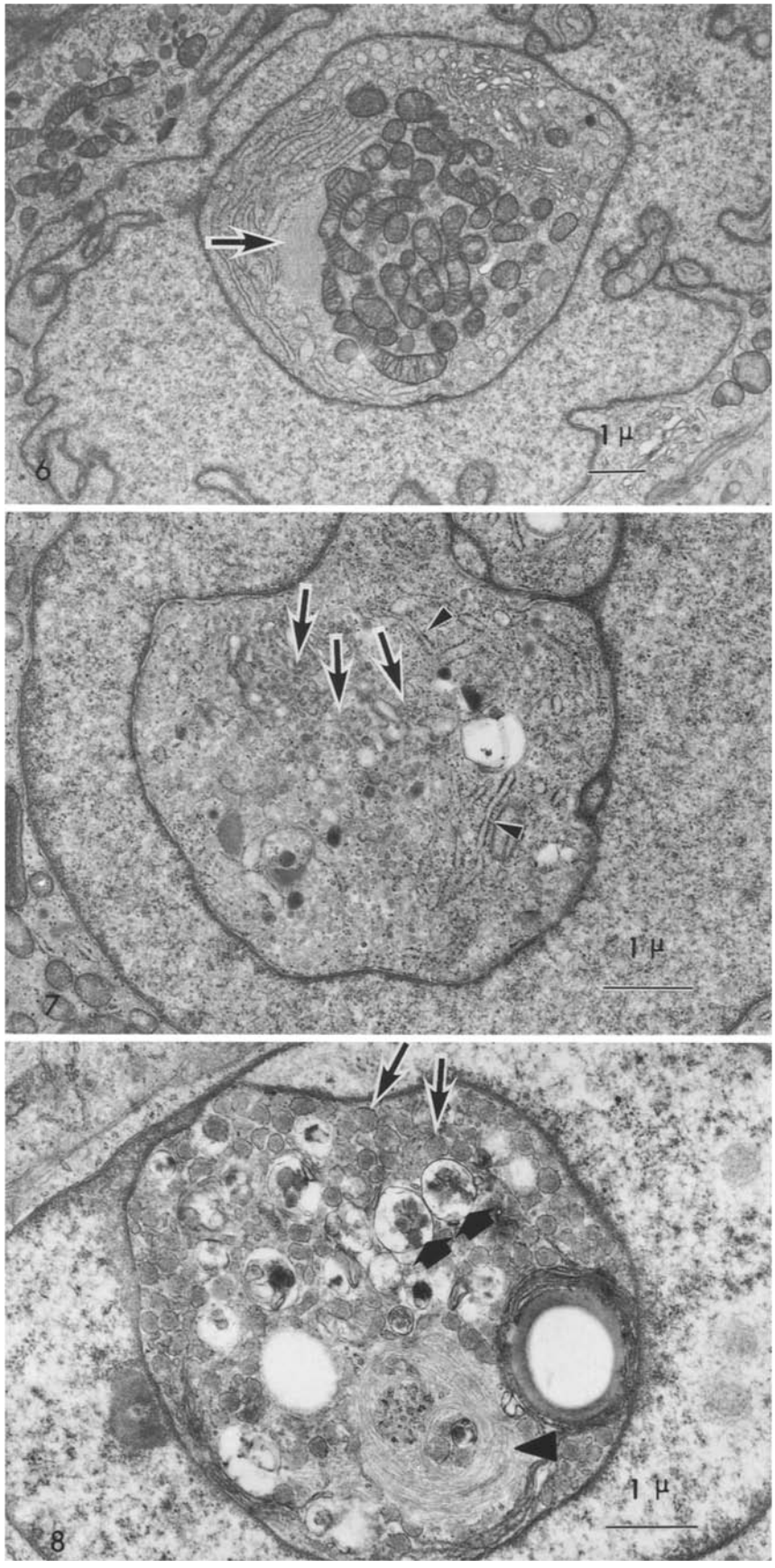
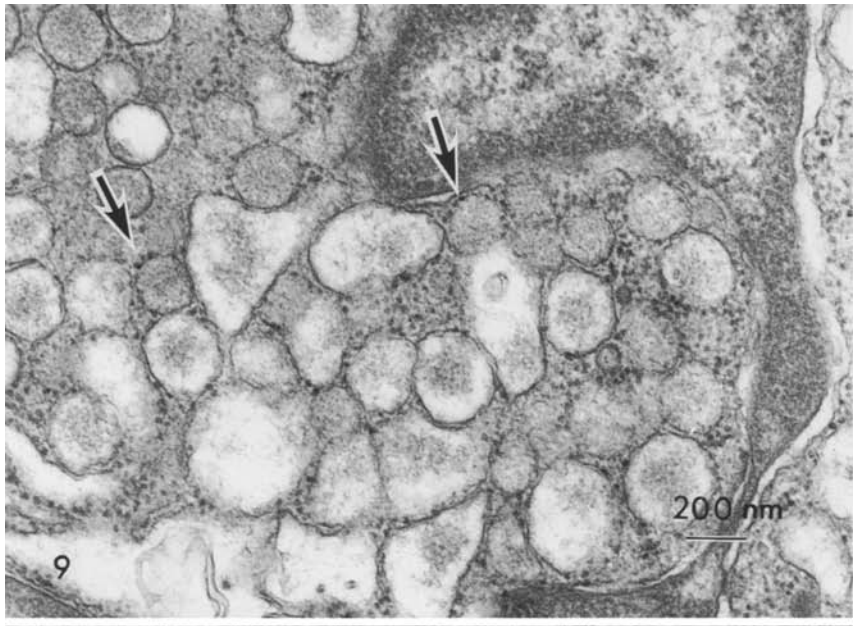


Fig. 6. Electron micrograph of ICI (Case No. 22, 62 years, female). Mitochondria, r-ER and Golgi complex are observed in ICI. They are slightly crowded but similar to the cytoplasmic organelles in the surrounding cytoplasm. A bundle of intermediate filaments (*arrow*) is observed

Fig. 7. Electron micrograph of ICI (Case No. 22, 62 years, female). R-ER (*arrowheads*) and many Golgi vesicles (*arrows*) are observed in ICI

Fig. 8. Electron micrograph of ICI (Case No. 34, 48 years, female). Small vesicles (*narrow arrows*), are observed measuring 300–500 nm in diameter and containing moderately electron-dense material. Secondary lysozomes (*wide arrows*) containing highly electron-dense irregular material, are also seen. A whirl of intermediate filaments (*arrowhead*) are present



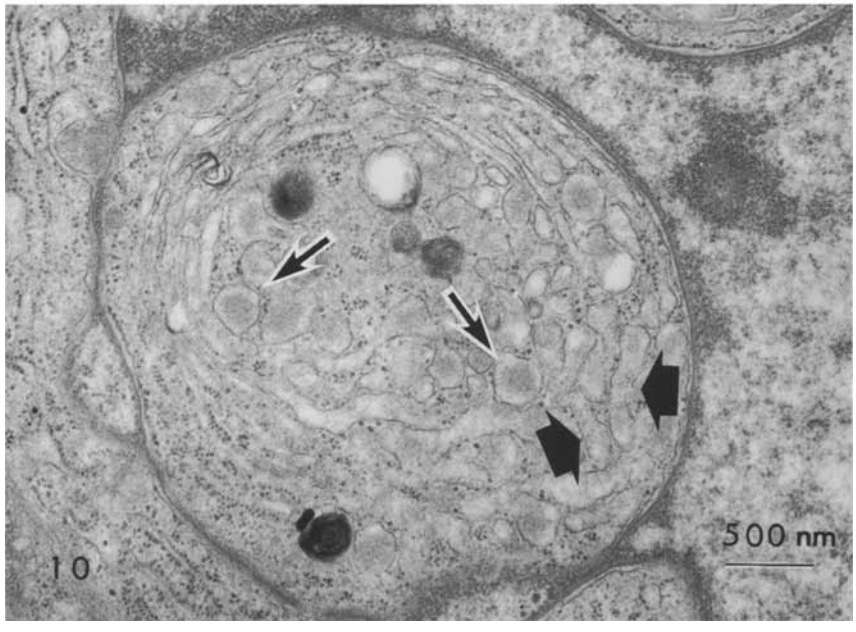


Fig. 9. Electron micrograph of ICI (Case No. 34, 48 years, female). ICI are filled with small vesicles, measuring 300–500 nm in diameter and containing fine-granular material, and some of them are enlarged. Ribosomes are attached around the smaller vesicles (*arrows*)

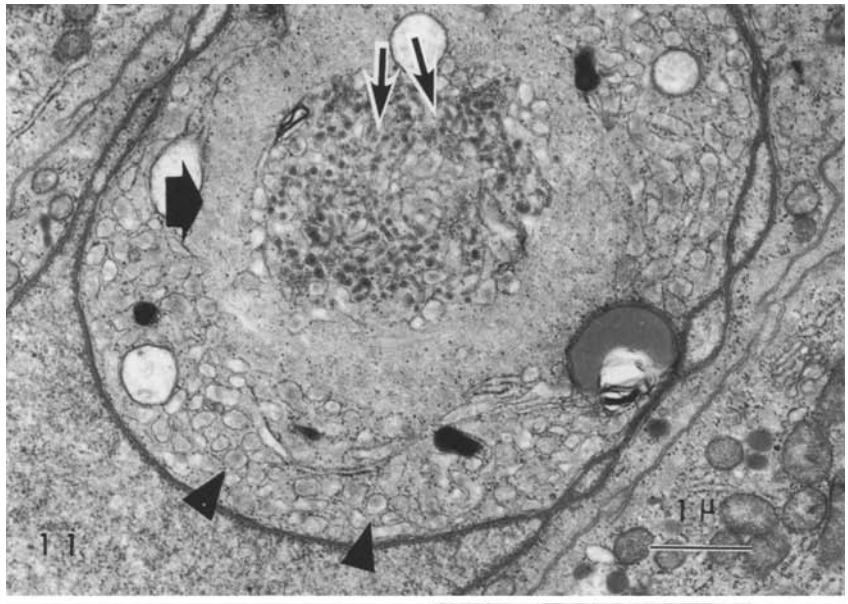
Fig. 10. Electron micrograph of ICI (Case No. 22, 62 years, female). Small vesicles (*narrow arrows*), measuring 300–500 nm in diameter and containing moderately electron-dense material. Sacs with a minor axis of 200–300 nm (*wide arrows*), containing the same material, are also observed in ICI

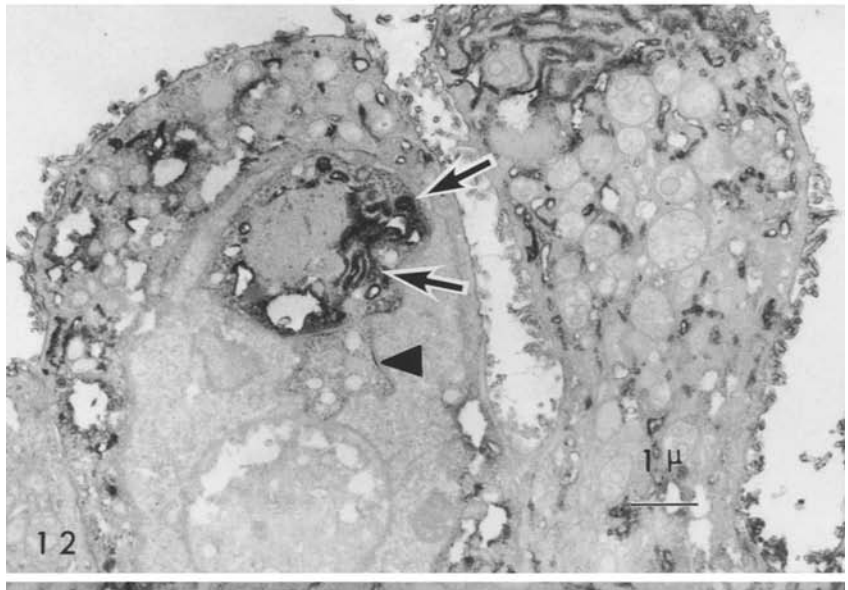
Discussion

Intranuclear cytoplasmic inclusions (ICI) in the thyroid gland are weakly eosinophilic spherical inclusions in the nuclei observed by light microscopy. On electron microscopy they are cytoplasmic inclusions containing cell organelles surrounded by a nuclear envelope (Doniach 1970; Carcangiu et al. 1985). ICI are identical to the nuclear inclusions in various organs that have been shown to contain cytoplasm as determined by histochemical studies or electron microscopy (Sobel et al. 1969).

ICI in the thyroid gland where previously reported by Söderström in 1966 as "poorly stained giant nucleoli". He considered them a sign strongly suggesting malignancy in fine-needle aspiration biopsy. Later, Gray et al. (1968) confirmed that ICI were cytoplasmic inclusions by electron microscopy. They are still useful in interpreting aspiration biopsies of the thyroid gland and are one of the diagnostic findings for papillary carcinoma because of their high frequency (70–90% – Christ et al. 1979; Hanawa et al. 1979; Toriya et al. 1984). However, they are now known to appear in medullary carcinoma (Kakudo et al. 1978) and follicular carcinoma (Michael et al. 1985) of the thyroid.

The formation of ICI is thought to be caused





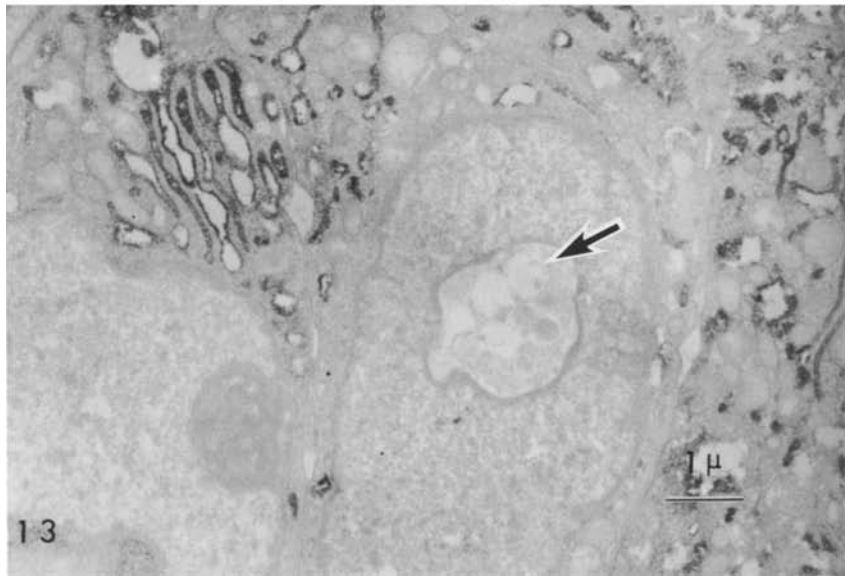


Fig. 11. Electron micrograph of ICI (Case No. 22, 62 years, female). An aggregate of tubles (*narrow arrows*), measuring 30–50 nm in width and containing highly electron-dense material, is present. It is surrounded by intermediate filaments (*wide arrow*). Small vesicles are observed outside the filaments (*arrowheads*)

Fig. 12. Immunoelectron micrograph for thyroglobulin of ICI (Case No. 33, 51 years, female). Thyroglobulin is demonstrated in the perinuclear space (*arrowhead*) and globular, saccular and lamellar structures (*arrows*) of ICI

Fig. 13. Immunoelectron micrograph for thyroglobulin of ICI (Case No. 33, 51 years, female). Secondary lysozomes and mitochondria are negative for thyroglobulin (*arrow*). R-ER in the cytoplasm is positive for thyroglobulin

by invagination of the cytoplasm. According to some authors (Sobel et al. 1969) cytoplasmic invagination is due to the expansion of cytoplasm into a nucleous. Others think invagination is caused by the increase of irregularity of the nuclear membranes as the result of the enlargement of the nuclear surface area (Noor Sunba et al. 1980). Another opinion is that ICI are formed by the trapping of cytoplasm after frequent or abnormal mitosis (Bernhard et al. 1963). Carneiro et al. (1980) noticed how the appearance of ICI differed from the surrounding cytoplasm and guessed that they might play a role in compensating for the disturbance of nucleo-cytoplasmic transfer of ribonucleoproteins in nuclei of papillary thyroid carcinoma.

ICI were termed "cytoplasmic pseudo-inclusions" (Gray et al. 1968) or "cytoplasmic invaginations" (Sobel et al. 1969) from the point of view that ICI were connected with the surrounding cytoplasm. It was thought that not all ICI were connected with the surrounding cytoplasm and some of them were true inclusions without connection. Such inclusions were termed "intranuclear bags" (Söderström et al. 1973). The term "intranuclear cytoplasmic inclusions (ICI)" has been used in this article because of its wide usage for similar lesions in various organs.

ICI occurred in all our 38 cases. Söderström et al. (1973) found it difficult to find any inclusions in routine histological specimens. Recently, however, the incidence of ICI has been reported to be 46% (Chan et al. 1986) or 89% (Katoh 1988) in papillary carcinoma. In aspiration biopsies, the incidence of ICI is 70–90% (Christ et al. 1979; Hanawa et al. 1979). The incidence of ICI on smears is thought to be higher than in histological sections but ICI were found in all cases following careful observation in the present study and the incidence of ICI in both histological sections and smears seems not to be different.

The number of ICI identified by light microscopy with a magnification $\times 400$ ranged from one per several fields to more than ten per field and the number of ICI divided by the number of nuclei (ICI/nuclei) in microscopic photographs with a magnification of $\times 700$ ranged from 0.013 to 0.116 (mean values: 0.039). Both values for the frequency of ICI were almost correlative, but occasionally inverse. For example, the number of ICI observed by light microscopy in case No. 35 ($\pm$) was smaller than for No. 36 (+), but ICI/nuclei of case No. 35 (0.037) was larger than for No. 36 (0.019). The number of ICI/nuclei counted in microscopic photographs represents the frequency of ICI according to the cases more correctly than the number of ICI per visual field as determined by light microscopy. The former is not influenced by the difference of nuclear density of papillary carcinoma as is the latter. But ICI can be detected by field focusing when counting the latter and the frequency of ICI can best be reported by a comparison of both.

Although systematical studies of the frequency of ICI in histological specimens have been sparsely reported, the frequency varies from 0.48–4.01% in wet-fixed smears and 0.29–3.87% in air-dried smears by Hanawa et al. (1979) on the aspiration biopsies. ICI in the present study occurred in 1.3–11.6% of the nuclei in the histological sections and so more frequently than in the report described above. One reason for this difference seems to be that the author identified small nuclear inclusions (irregular or slender in shape) as ICI on histological sections. However, only expansive round inclusions are detected as ICI on smears. Another reason seems to be that the average number of ICI/nuclei was calculated using three photographs of the lesions with a relatively high incidence of ICI in the present study.

As to the frequency of ICI, papillary carcinoma have been grouped into two types; one type with an exceptionally high frequency of ICI, such as No. 4, 21, and another major type with a low frequency, in which ICI occurred in less than 5% of the nuclei.

The frequency of ICI was not influenced by the papillary or follicular growth of the papillary carcinoma. These findings applied to individual cases, and ICI appeared diffusely, regardless of the papillary or follicular structure in those cases with a high incidence of ICI. According to the WHO classification, papillary carcinoma of the thyroid may include various amounts of follicular structure. The incidence of ICI in both follicular structure and papillary structure of papillary thyroid carcinoma showed similar characteristics.

The frequency of ICI increased in accordance with nuclear pleomorphism and changes in the state of nuclear chromatin from the clear type to the condensed type. In the present study, the close relation between the frequency of ICI and nuclear atypia on smears (reported by Hanawa et al. 1979) was demonstrated on histological sections. Noor Sunba et al. (1980) supposed that there might be an increase in the effective nuclear surface as to the genesis of ICI in malignant melanoma. The relation between the frequency of ICI and the high irregularity of the nuclear membranes in the present study supports the idea.

As to the state of nuclear chromatin, "ground-glass nuclei" are widely thought to exist on histological sections stained with H.E., although they do not exist on smears. Many authors regard these nuclei as one of the diagnostic findings of papillary thyroid carcinoma, together with papillae (Lindsay 1960; Franssila 1973). They were considered to contain peripherally located chromatin with thickened nuclear membranes. In this study, typical ground-glass nuclei (included in clear type) were oval and uniform in size and ICI occurred frequently (Fig. 1). Ground-glass nuclei seem to occur in inverse proportion to ICI in histological specimens of papillary thyroid carcinoma.

There were no case of metastasis to organs. All patients were operated on within the last three years and they are presently alive. Comparing the frequency of ICI with tumour size, extraglandular extension, and cervical lymph-node metastasis, showed no significant differences. However, ICI appeared less frequently in cases with extraglandular extension than in cases without extraglandular extension. The frequency of ICI seems not to be influenced by the stage of the tumour.

Thyroglobulin (Tg) was occasionally detected in ICI by immunostaining. Immunoproducts varied from yellowish brown, as in most of the surrounding cytoplasm, to a mosaiced or diffuse, blackish brown reaction (Fig. 4). This type of reaction was observed in 10–30% of all ICI. Because a small amount of ICI containing PAS-positivity was detected, polysaccharides incorporated into Tg may somtimes be contained in ICI. Neither T3 nor T4 was regularly observed in ICI although T3 was demonstrated in 2–3 ICI in 2 cases (No. 22 and 33). As T3 was exceptionally positive in some ICI in cells, possessing T3-positive cytoplasm and frequent ICI, T3 and T4 negativity in ICI seems to be due to the inability of the carcinoma cells to produce T3 and T4 rather than as a result of a different behavior of ICI from the surrounding cytoplasm.

By electron microscopy, mitochondria, r-ER, Golgi complex, free ribosomes, dense bodies and intermediate filaments etc., which seem to reflect cell organelles in the surrounding cytoplasm, were observed in ICI, but they were occasionally modified in quality or in quantity. R-ER in papillary carcinoma are not developed as well as in normal thyroid tissue. Most of them were fragmented into small vesicles or composed of flattened cisternes. However, globular or sacular r-ER with cisternal contents or large amounts of small vesicles with a diameter of 50–100 nm were detected in ICI (Fig. 7) and appear to be Golgi vesicles (Fig. 7).

Small vesicles with a diameter of 300–500 nm (Figs. 8, 9, 10) are the same as what Söderström et al. (1973) termed "M-bodies". These sacs with a minor axis of 200–300 nm (Fig. 10) were frequently observed close to the small vesicles with a diameter of 300–500 nm. The sacs contained moderately electron-dense contents similar to the small vesicles (Fig. 10). Perpendicular sections of a part of lamellar sacs might look like the small vesicles.

Carneiro et al. (1980) thought that these M-bodies originated from r-ER. As the small vesicles and sacs were frequently coated with ribosomes (Fig. 9) in the present study, and since the lamellar structure of gathered sacs as r-ER was occasionally observed, the small vesicles and sacs are regarded as figures made from r-ER with cisternal storage.

If ICI are true inclusions and not connected to the surrounding cytoplasm, it is reasonable to assume that storage suggests increased synthesis, as storage or condensation of products could occur naturally in ICI. But it seems probable that ICI are initially formed by invagination of the cytoplasm into the nucleus and remain connected to the cytoplasm. So the accumulation in ICI may be caused by increased protein (material) synthesis. This suggestion is supported by Noor Sunba et al. (1980) and Carneiro et al. (1980).

Another characteristic of electron microscopy are findings of cell degradation in many ICI (Fig. 8), suggesting that ICI are destined to become autophagolytic. Carneiro et al. (1980) supposed that lytic enzymes were stored in M-bodies, but found no acid phosphatase activity and detected thyroglobulin in the globular or sacular shapes by immunoelectron microscopy. The difficulties of the nature of the contents of various types of membrane-limited structures have yet to be resolved.

Tubular bodies with a minor axis of 30–50 nm with high electron-dense contents were rarely observed in ICI (Fig. 11). Endoplasmic reticulum may be the source of these bodies as they contain highly electron-dense material and are surrounded by intermediate filaments as if they had been confined. Small vesicles with a diameter of 300–500 nm can be observed near-by (Fig. 11).

Bundles of circular intermediate filaments were observed in ICI (Figs. 6, 8), and these findings seem to correspond with the keratin or vimentin globular positivity determined by immunostaining. Keratin and vimentin are seldom observed in the same ICI through observation of serial sections. Papillary carcinoma of the thyroid gland is known to show both keratin and vimentin (Miettinen et al. 1984), and some authors have reported that anap-

lastic transformation accompanied the loss of keratin in papillary carcinoma (Kawahara et al. 1984). So the positivity of both keratin and vimentin in ICI indicates that the character of the surrounding cytoplasm is reflected in them. But intermediate circular or "U" shaped filaments are not usually observed in the cytoplasm of papillary carcinoma. They may be caused by "twist" of the cytoplasm at invagination or may be formed as a result of abnormal protein synthesis in ICI. These filaments also might play a role in the formation and maintenance of the ICI.

Because thyroglobulin (Tg) was demonstrated in globular, saccular and lamellar shape in ICI (Figs. 11, 12) by immunoelectron microscopy, it is certain that Tg exist in some of r-ER, the r-ER-related vesicles or sacs, or the Golgi apparatus. But fine structures were not clarified in detail by the present method. From the normal function of follicular epithelial cells, it can be concluded that lysozomes and peroxidase may also be produced and stored.

The nuclei of papillary carcinoma with a high frequency of ICI showed severe irregularity of the nuclear envelope. The inter-connection of small and irregular-shaped multiple ICI in the same nuclei was ascertained by observation of ultra-thin serial sections. It is thus certain that ICI are formed by the invagination of the cytoplasm to the nucleus. The irregularity of nuclear membranes seems to result from an increase of the nuclear surface area, possibly in relation to activated and abnormal metabolism because of the presence of morphological evidence of hyperactivity of the nuclei in papillary carcinoma (prominent nucleoli, complex nuclear bodies). This hyperactivity may indicate an increase in protein synthesis in ICI.

The author has some doubt as to whether all ICI finally connect with the surrounding cytoplasm because of the presence of large, spherical ICI throughout the nucleus. This problem must be solved by careful observation of serial sections.

Most tumours with frequent appearance of ICI possess high malignancy and show severe atypia, for example, malignant melanoma and glioblastoma multiforme. However, ICI is known to appear in benign lesions like menigioma and naevi and in normal tissues (in the liver and adrenal glands). It is not possible to conclude that ICI should be regarded as one character of the nucleus of a malignant cell. ICI have been known to appear in non-papillary carcinoma of the thyroid. But the high frequency of ICI in papillary carcinoma when considered together with other findings (the existence of papillary frons and grooved nuclei) are of value in diagnosis.

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References

Bernhard W, Granboulan N (1963) The fine structure of the cancer cell nucleus. Exp Cell Res, Suppl 9:19–53

Carcangiu ML, Zampi G, Rossai J (1985) Papillary thyroid carcinoma: A study of its many morphologic expressions and clinical correlates. Pathol Annu 20:1–44

Carneiro F, Gonçalves V, Sobrinho-Simões MA (1980) Intranuclear cytoplasmic inclusions in thyroid papillary carcinomas. J Submicros Cytol 12:137–143

Chan JKC, Saw D (1986) The grooved nucleus. Am J Surg Pathol 10:672–679

Christ M, Hara J (1979) Intranuclear cytoplasmic inclusions in thyroid aspirations. Acta Cytol 23:327–331

Doniach I (1978) The thyroid gland. In: Symmers WSC (ed) Systemic pathology, ed 2, vol 14. Churchill Livingstone, Edinburgh, pp 1975–2037

Franssila KO (1973) Is the differentiation between papillary and follicular thyroid carcinoma valid? Cancer 32:853–864

Gray A, Doniach I (1968) Morphology of the nuclei of papillary carcinoma of the thyroid. Br J Cancer 23:49–51

Hanawa T, Ihara S, Horinaka E (1979) Cytologic study of internuclear cytoplasmic inclusions of the thyroid gland. J Jpn Soc Clin Cytol 18(3):302–311

Hedinger Chr, Sobin LH (1974) Histological typing of thyroid tumors. In: International histological classification of tumours No. 11. World Health Organization, Geneva

Kakudo K, Miyauchi A, Ogihara T, Takai S, Kitamura H, Kosaki G, Kumahara Y (1978) Medullary carcinoma of the thyroid. Arch Pathol Lab Med 102:445–447

Katoh R (1988) Papillary carcinoma-histologic architectures and cytologic features. Pathology and Clinical Medicine 6:746–755

Kawahara E, Ooi A, Oda Y, Katsuda S, Terahata S, Michigishi T (1986) Papillary carcinoma of the thyroid gland with anaplastic transformation in the metastatic foci. Acta Pathol Jpn 36:921–927

Kawaoi A (1984) Immunoenzyme method. In: The Japanese Society of Histochemistry and Cystochemstry (eds) Histochemistry and Cytochemistry. Gakusaikikaku, Tokyo, pp 89–110

Lindsay S (1960) Carcinoma of the thyroid gland. A clinical and pathologic study of 293 patients at the University of California Hospital. Charles C Thomas, Springfield, Illinois

Michael DG, Eric KB, Diane DD (1984) Intranuclear cytoplasmic inclusions in aspirates of follicular neoplasms of the thyroid. Acta Cytol 28:576–580

Miettinen M, Franssila K, Lehto VP, Paasivou R, Virtanen I (1984) Expression of intermediate filament proteins in thyroid gland and thyroid tumors. Lab Invest 50:264–270

Noor Sunba M, Rahi AHS, Morgan G (1980) Tumors of the

anterior uvea. Intranuclear cytoplasmic inclusions in malignant melanoma of the iris. Br J Ophtalmol 64:453–456
Sobel H, Schwarz R, Marquest E (1969) Nonviral nuclear inclusions. Arch Pathol 87:179–192
Söderström N (1966) Fine-needle aspiration biopsy. Grune and Stratton, New York, pp 96–108
Söderström N, Biörklund A (1973) Intranuclear cytoplasmic inclusions in some types of thyroid cancer. Acta Cytol 17:191–197
Toriya S, Sengoku K (1984) The thyroid gland. In: Yamada T, Kakihana M (eds) Atlas of aspiration biopsy cytology. Bunkodo, Tokyo, pp 72–93

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