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Enhanced expression of complement regulatory proteins on thyroid epithelial cells of Graves' disease.

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Abstract

Cytotoxic anti-thyroid microsomal autoantibodies are highly prevalent in sera of patients with Graves' disease, but in Graves' disease thyroid tissues rarely show destructive changes. We postulated that this might be due to membrane-associated complement regulatory proteins which protect target cells from injury by complement activation. We, therefore, investigated the expression of membrane attack complex inhibitory factor (MACIF) and decay accelerating factor (DAF) in the thyroid tissues from patients with Graves' disease, Hashimoto's thyroiditis, thyroid adenocarcinoma and normal human thyroid tissues. We found a high level of expression of MACIF and DAF in Graves' thyroid tissues. Using the membrane immunofluorescence and cell-ELISA techniques, we also investigated the factors which enhanced the MACIF and DAF expression in cultured thyroid cells. Thyroid stimulating hormone, phorbol 12, 13-dibutyrate and thyroid stimulating autoantibody enhanced the MACIF and DAF expression. These findings suggest that the membrane complement regulatory proteins increase in response to the thyroid stimulating factors such as thyroid stimulating autoantibody in Graves' disease and that this increase then protects the cells from damage due to complement activation by thyroid autoantibodies.

KEYWORDS: MACIF, DAF, TSH, phorbol 12, 13-dibutyrate, thyroid stimulating antibody

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Enhanced Expression of Complement Regulatory Proteins on Thyroid Epithelial Cells of Graves' Disease

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Cytotoxic anti-thyroid microsomal autoantibodies are highly prevalent in sera of patients with Graves' disease, but in Graves' disease thyroid tissues rarely show destructive changes. We postulated that this might be due to membrane-associated complement regulatory proteins which protect target cells from injury by complement activation. We, therefore, investigated the expression of membrane attack complex inhibitory factor (MACIF) and decay accelerating factor (DAF) in the thyroid tissues from patients with Graves' disease, Hashimoto's thyroiditis, thyroid adenocarcinoma and normal human thyroid tissues. We found a high level of expression of MACIF and DAF in Graves' thyroid tissues. Using the membrane immunofluorescence and cell-ELISA techniques, we also investigated the factors which enhanced the MACIF and DAF expression in cultured thyroid cells. Thyroid stimulating hormone, phorbol 12, 13-dibutyrate and thyroid stimulating autoantibody enhanced the MACIF and DAF expression. These findings suggest that the membrane complement regulatory proteins increase in response to the thyroid stimulating factors such as thyroid stimulating autoantibody in Graves' disease and that this increase then protects the cells from damage due to complement activation by thyroid autoantibodies.

Key words : MACIF, DAF, TSH, phorbol 12, 13-dibutyrate, thyroid stimulating antibody

There are several cytotoxic mechanisms postulated to have a role in autoimmune thyroid disease, including direct T cell cytotoxicity (1, 2), antibody-dependent cell-mediated cytotoxicty (3), and complement-dependent cytotoxicity (4). Complement-dependent cytotoxicity was first reported as the cytotoxic factor in the sera from patients with Hashimoto's thyroiditis which activated the complement (5). Further investigations showed that this factor was an anti-thyroid microsomal antibody (6) which was subsequently identified as an anti-thyroid peroxidase antibody (7). However, this anti-thyroid microsomal antibody is not only detected in the sera of patients with Hashimoto's thyroiditis, but is also frequently found in the sera of patients with Graves' disease which is associated with the thyroid cellular proliferation. Moreover, immunohistochemical observation of thyroid tissues has revealed the deposition of immunoglobulins and complements components on the follicular basement membrane in Graves' disease (8). However,

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histological evidence of cytotoxic activity is rare in thyroids with Graves' disease, although an increased cytotoxic activity is shown in sera of patients with Graves' disease. We postulated that this discrepancy between the actual findings in Graves' disease and the *in vitro* findings might be due to the involvement of membrane-associated complement regulatory proteins which protect the follicular epithelial cells from complement mediated cytotoxicity. Based on this hypothesis, we investigated the expression of the membrane attack complex inhibitory factor (MACIF, CD59) (9-13) and the decay accelerating factor (DAF, CD55) (14-18) on the follicular epithelial cells of normal human thyroid glands and thyroid glands affected by various diseases. In addition, the factors enhancing the expression of MACIF, and DAF on the cultured human thyroid cells were investigated using the membrane immunofluorescence technique and cell-enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Reagents. Fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG, C3, and C1q and peroxidase-labeled goat anti-mouse IgG were purchased from Medical & Biological Laboratories (Nagoya, Japan). FITC-labeled goat anti-mouse IgG was purchased from E-Y Laboratories, Inc. (San Mateo, CA, USA). The monoclonal murine anti-MACIF antibody was a gift of Dr. Y. Sugita (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan), and the monoclonal murine anti-DAF antibody IA10 was provided by Prof. T. Kinoshita (Osaka University, Osaka, Japan). Monoclonal murine anti-thyroid microsomal antibody was prepared in our laboratory (19, 20). Thyroid stimulating antibody (TSAb)-positive serum was obtained from a patient with Graves' disease. The titer of TSAb was assayed at Otsuka laboratory (Tokushima, Japan) by a method previously reported (21). RPMI1640 medium and fetal calf serum (FCS) were purchased from Gibco (New York, USA). Collagenase and o-phenylendiamine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Thyroid stimulating hormone (TSH) and phorbol 12, 13-dibutyrate were purchased from Sigma (St. Louis, MO, USA), and 96-well tissue culture plates came from Becton Dikinson (New Jersey, USA).

Immunohistochemical studies. Frozen sections (5 μm) were prepared using the tissues obtained during surgery from 12 patients with Graves' disease, 5 with thyroid cancer, and 2 with Hashimoto's disease. The normal control group of tissues was comprised of the uninvolved thyroid tissues which were obtained during surgery of 9 patients with thyroid neoplasias. IgG, C3, and C1q reactivity was investigated in unfixed sections using FITC-labeled antibodies according to the standard direct fluorescence antibody technique. Sections were fixed in acetone for 15 min for the detection of MACIF and DAF. Sections were washed in phosphate-buffered saline (PBS) and then subjected to the standard indirect fluorescence antibody technique. Sections were incubated at room temperature for 30 min with monoclonal anti-MACIF or anti-DAF antibodies and then, following washing in PBS, treated with FITC-labeled goat anti-mouse IgG for an additional 30 min. After a final wash in PBS, the sections were observed under a fluorescence microscope (Olympus BF2-RFCA, Tokyo, Japan). The intensity of fluorescence was estimated arbitrarily and expressed as $(-)\sim(+++)$ in intensity. (See Fig. 1)

Cell culture studies. Follicular epithelial cells were isolated from the thyroid tissues of patients with Graves' disease and from normal thyroid tissues using collagenase (1 mg/ml in RPMI1640). Isolated cells were suspended at a final concentration of 1×10^6/ml in RPMI1640 supplemented with 10 % FCS and were treated as follows. The cells from Graves' disease thyroid were incubated in the above medium without additives or in the medium supplemented with TSH (10^{-3} U/ml) or 100 nM phorbol 12, 13-dibutyrate. The cells isolated from normal thyroid tissues were also incubated in the medium containing the same additives described above as well as in the medium containing the serum from a patient with high TSAb titer (equivalent to 3×10^{-4} U/ml TSH) or containing the same amount of normal human serum (NHS).

Immunofluorescence. Cells were cultured at 37°C for 5 days in 5 % CO_2 containing air. The monolayers which grew on the glass plates were then incubated on ice at 4°C for 30 min with the monoclonal anti-MACIF or anti-DAF antibodies, washed in RPMI1640, and incubated with FITC-labeled goat anti-mouse IgG for a further 30 min. Fluorescence microscopy was then performed after a final wash in RPMI1640. The membrane

immunofluorescence of follicular epithelial cells was also assessed immediately after their isolation.

ELISA. To investigate the effects of the above-mentioned additives on the expression of MACIF and microsomal antigen, a cell-ELISA study was performed. Normal thyroid cells were adjusted to 1×10^6/ml as mentioned above, and 100 μl aliquots were added to each well of a 96-well culture plate. The cells were then cultured in a CO_2 incubator for 5 days under the conditions mentioned above. Monolayers were fixed with methanol for 15 min before they were stripped from the culture plate. After washing in PBS, the monoclonal anti-MACIF or anti-thyroid microsomal antibodies were allowed to react with the plate for 60 min. Then, after further washing in PBS, peroxidase-labeled goat anti-mouse IgG was allowed to react for 60 min. After a final wash in PBS, the absorbance was measured at 492 nm following the addition of o-phenylendiamine with H_2O_2. All assays were performed in triplicate and results were shown as the mean ± SD. The differences between groups were analyzed by Student's t-test, and $p < 0.05$ was taken to indicate a significant difference.

Results

Immunohistochemical Studies

As shown in Table 1, IgG and C3 were often present in granular deposits on the follicular basement membrane in Graves' disease, but were not present in any of the tissues of the normal thyroids and in only some of the adenocarcinoma tissues. A small amount of granular deposits was present in the tissues with Hashimoto's disease. MACIF was strongly expressed in all of the thyroids with Graves' disease, and was largely localized at the luminal surfaces of the follicular epithelial cells (Fig. 1 a). The MACIF expression varied from negative to weakly positive in normal thyroid tissues (Fig. 1 b) and was negative in all adenocarcinoma patients except for one with strong positivity. In Hashimoto's disease, some of the remaining follicular epithelial cells were positive for MACIF (Fig. 1 c). DAF was detected in the follicular epithelial cells of 11/12 patients with Graves' disease. It was chiefly localized at the luminal surfaces as was the case with MACIF (Fig. 1 d). DAF was not detected in either the normal thyroid tissues or in those of Hashimoto's disease (Fig. 1 e, f), and was found in only one of adenocarcinoma cases.

Table 1 Expression of MACIF, DAF, IgG, C3 and C1q in various thyroid tissues

Source of tissues	Case	MACIF	DAF	Immune deposits			MCA (×100)
				IgG	C3	C1q	
Graves' disease	Y.T.	+++	+	+	+	±	2^6
	E.E.	+++	++	++	+	±	2^{12}
	K.O.	++	−	−+	++	±	2^8
	C.K.	++	+	++	+++	±	2^8
	M.O.	++	±	−	+	−	<1
	A.T.	++	±	+	+	−	2^3
	K.N.	++	±	−	±	−	2^8
	N.T.	++	±	+	+	−	2^5
	C.I.	++	+	−	−	−	<1
	S.H.	++	+	−	−	−	2^{18}
	K.S.	++	+	±	+	−	2^2
	K.N.	++	+	++	++	−	2^6
Normal	M.S.	+	−	−	−	−	<1
	A.K.	−	−	−	−	−	<1
	K.S.	−	−	−	−	−	<1
	K.M.	+	−	−	−	−	<1
	Z.K.	++~−	−	−	−	−	<1
	S.N.	±	−	−	−	−	<1
	H.H.	+	−	−	−	−	<1
	M.U.	+	N.D.	−	−	−	<1
	Y.U.	+	N.D.	−	−	−	<1
Adeno-carcinoma	M.S.	++	+	+	+	+	<1
	A.K.	−	−	±	−	−	<1
	K.S.	−	−	−	+	−	<1
	Z.K.	−	−	−	−	−	<1
	H.H.	−	−	+	+	−	<1
Hashimoto's disease	Y.K.	+~−	−	±	+	−	2^{12}
	S.O.	+~−	N.D.	±	+	−	2^{12}

Analysis was done by the imunofluorescence technique on tissuses from 12 cases of Graves' disease, 9 normal individuals, 5 cases of thyroid adenocarcinoma and 2 cases of Hashimoto's disease. The degree of fluorescence is shown as (+++), very strongly positive; (++), strongly positive; (+), positive; (±), weakly positive; and (−), negative. (+)~(−) indicates the coexistence of positive and negative cells.

MACIF; membrane attack complex inhibitory factor; DAF; decay accelerating factor; MCA; microsome aggulutination test; N.D.; Not done.

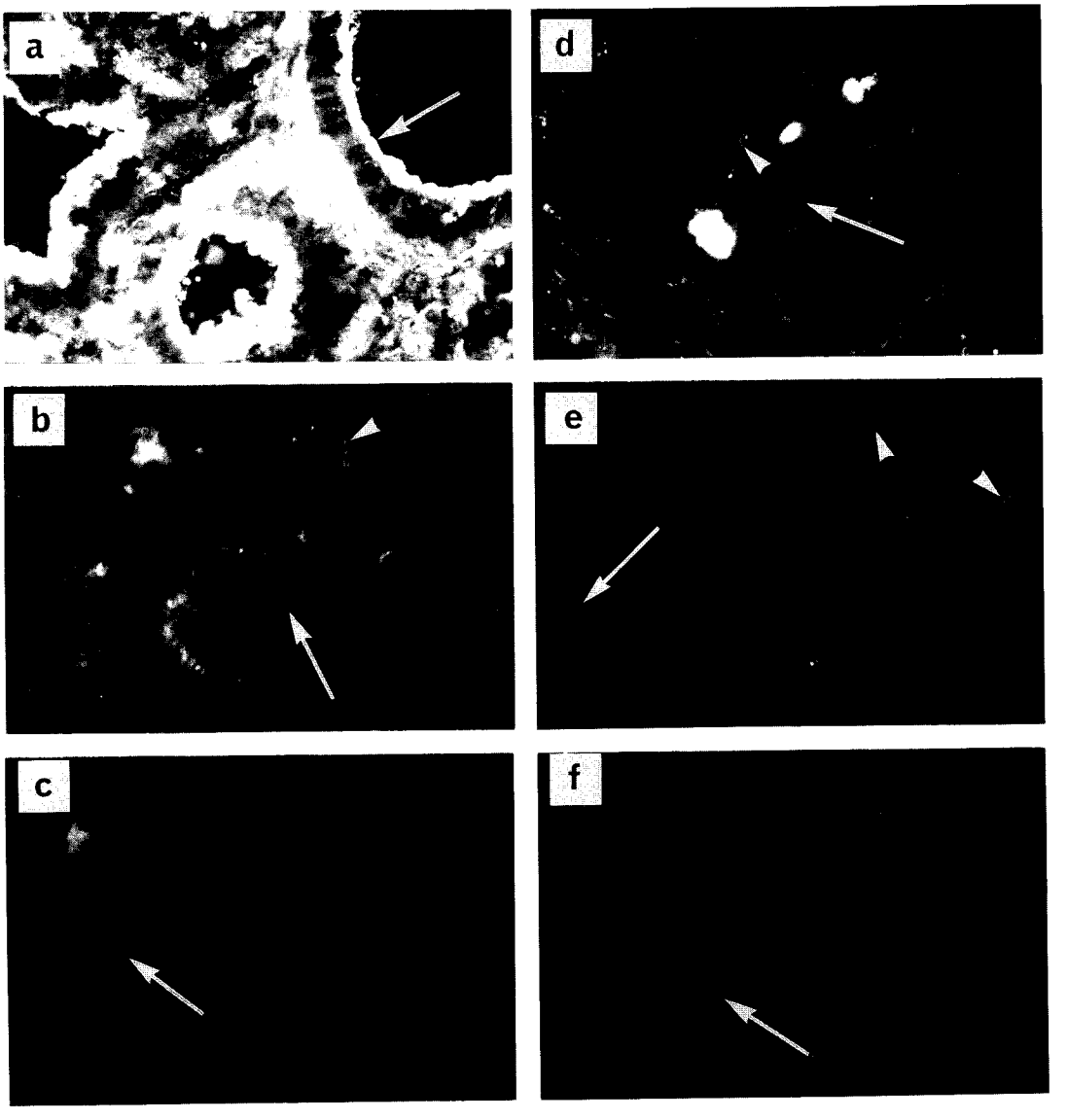

Fig. 1 Expression of membrane attack complex inhibitory factor (MACIF) and decay accelerating factor (DAF) in normal thyroids, Graves' disease thyroids and Hashimoto's disease thyroids, as shown by the indirect fluorescence antibody technique. Magnification × 200.

a) Staining of MACIF in Graves' disease thyroid tissue. MACIF was expressed in all the follicular cells, more strongly at the luminal surfaces of the cells. The intensity of fluorescence was estimated as (+++).

b) Staining of normal thyroid tissue for MACIF. Expression by some of the follicular cells was observed, but it was weaker than in Graves' disease. The intensity of fluorescence was estimated as (+).

c) Staining of MACIF in Hashimoto's disease thyroid tissue. Expression by some of the remaining follicular epithelial cells was observed. The intensity of fluorescence was estimated as (+).

d) DAF staining in Graves' disease tissue. The fluorescence of DAF was weaker than that of MACIF, but showed a similar pattern to that for MACIF. The intensity of fluorescence was estimated as (++).

e) DAF staining appeared to be almost absent in normal thyroid tissue. The intensity of fluorescence was estimated as (−).

f) DAF staining was also largely negative in Hashimoto's disease tissue. The intensity of fluorescence was estimated as (−).

The arrows indicate the apical surface of the follicular cells. The arrow heads indicate autofluorescence by lipofuscin granules.

Table 2 Expression of MACIF and DAF on thyroid cells after culture for 5 days with various additives

	Case	No additives	TSH	Phorbol ester
Graves' disease (n = 5)	N.T. MACIF	++	+++	N.D.
	DAF	+	++	N.D.
	T.U. MACIF	+	+++	+++
	DAF	+	++	+
	K.Y. MACIF	++	+++	+++
	DAF	+	++	++
	T.O. MACIF	++	+++	+++
	DAF	±	+	+
	T.N. MACIF	++	++	+++
	DAF	+	+	+
Normal (n = 3)	Z.K. MACIF	+	++	N.D.
	DAF	±	++	N.D.
	M.C. MACIF	+	+++	+++
	DAF	−	++	++
	H.T. MACIF	+	++	+++
	DAF	±	+	+

The membrane immunofluorescence technique was used to examine cells from 5 Graves' disease thyroids and 3 normal thyroids. The degree of fluorescence is shown as (+++): very strongly positive, (++): strongly positive, (+): positive, (+−): weakly positive and (−): negative. Thyroid-stimulating antibody-positive Graves' disease serum and normal human serum were also added to cells obtained from a normal thyroid gland (See Fig. 2). TSH: thyroid stimulating hormone; phorbol ester: phorbol 12, 13 dibutyrate. Other abbreviations: See Table 1.

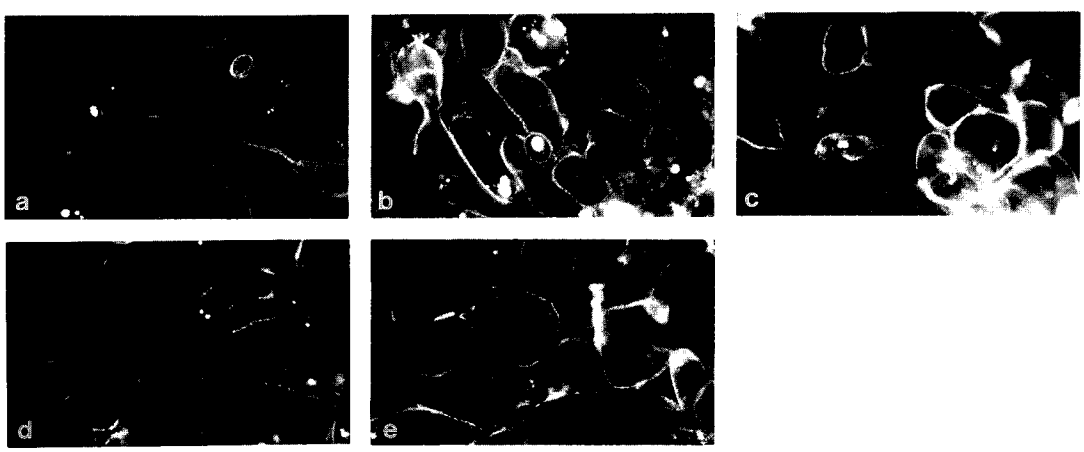

Fig. 2 Effects of thyroid stimulating hormone (TSH) phorbol 12, 13-dibutyrate, and thyroid-stimulating antibody (TSAb)-positive serum on the membrane attack complex mhibitory factor (MACIF) expression on normal thyroid cells after 5 days of culture. TSH, phorbol 12, 13-dibutyrate, TSAb-positive serum, or normal human serum (NHS) were added to cells isolated from normal thyroid tissue. After incubation for 5 days, the monolayers growing on the glass plate were analyzed by the membrane immunofluorescence antibody technique. Magnification × 100.

a) No additives, b) Addition of TSH, c) Addition of phorbol 12, 13-dibutyrate, d) Addition of NHS, e) Addition of TSAb-positive serum.

Relatively high levels of MACIF expression were observed on cells after the addition of phorbol 12, 13-dibutyrate, THS and TSAb-positive serum. Low levels of expression were seen in cells cultured with no additives or NHS.

Table 3 Effect of various additives on MACIF and microsomal antigen (Mic) expression in cultured thyroid cells

		TSH	Phorbol ester	TSAb-positive serum	NHS	No additives
Experiment 1	MACIF	595.0 ± 80.0*	739.0 ± 50.0***	511.0 ± 3.0**	424.7 ± 3.5	410.7 ± 24.5
	Mic	127.0 ± 6.0**	50.0 ± 7.0	131.7 ± 50.5	103.7 ± 7.5*	85.7 ± 6.5
Experiment 2	MACIF	261.5 ± 21.9*	239.5 ± 6.4	182.0 ± 45.3	93.0 ± 26.9	158.0 ± 5.7
	Mic	84.5 ± 3.5*	48.0 ± 1.4*	59.5 ± 3.5*	38.0 ± 2.8	43.0 ± 1.4

Cell ELISA was performed on cells isolated from normal thyroid tissues obtained from two patients with thyroid adenoma at surgery. The absorbance at 492 nm was measured and the net values are indicated. Values are given as the mean ± SD of three determinations. Expression of MACIF and microsomal antigen in the cells cultured with various additives was compared with those cultured without any additive. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$)

MACIF expression was increased by the addition of TSH, phorbol 12, 13-dibutyrate and thyroid-stimulating antibody (TSAb)-positive Graves' disease serum. Microsomal antigen was increased by the addition of TSH and TSAb-positive serum. Normal human serum (NHS) did not alter the expression of MACIF or microsomal antigen. Other abbreviations: See Table 1.

Cell Culture Studies

Immunofluorescence. Immediately after isolation, thyroid epithelial cells from Graves' disease showed circumferential DAF positivity and strong MACIF positivity. Normal cells showed weak circumferential DAF posotivity, and some of them showed weak to strong MACIF positivity.

Increase in the expression of DAF and MACIF occurred on the 5th incubation day after the addition of TSH or phorbol 12, 13-dibutyrate in the cultures of Graves' disease and normal thyroid cells (Table 2). DAF and MACIF expression also increased after the addition of TSAb-positive serum from a patient with Graves' disease, but not after the addition of NHS in the cultures of normal thyroid cells (Fig. 2).

ELISA. An increase of MACIF staining was observed after the addition of TSH, phorbol 12, 13-dibutyrate, and TSAb-positive Graves' disease serum, but not after the addition of NHS. Microsomal antigen was increased by the addition of TSH and TSAb-positive serum from a patient with Graves' disease, but not by the addition of phorbol 12, 13-dibutyrate or NHS (Table 3).

Discussion

The anti-thyroid microsomal antibody is frequently detected in Graves' disease and is known to cause *in vitro* cytotoxicity *via* complement activation. The reason why it does not induce tissue injury in the thyroid gland with Graves' disease has not yet been clarified. DAF and MACIF are membrane-associated complement regulatory proteins that protect cells from self complement attack. DAF is a phosphatidyl inositol (PI)-anchored membrane protein with a molecular weight of 70kDa that protects cells by inhibiting the formation of C3 convertase and C5 convertase (16, 17). MACIF is another PI-anchored membrane protein with a molecular weight of 20kDa that blocks formation of the membrane attack complex (MAC) by binding to C8, C9 or both (9, 13). It is known that the lack of these proteins induces paroxysmal nocturnal hemoglobinuria (12, 18). We have proposed that these membrane-associated complement regulatory proteins have a role in protecting thyroid cells with Graves' disease from injury by complement activation mediated *via* anti-thyroid microsomal antibody. This study showed that the expression of MACIF and DAF was greater on follicular epithelial cells in thyroid tissues of patients with Graves' disease than in similar tissues with Hashimoto's disease and normal tissues.

The culture of thyroid follicular epithelial cells revealed that the expression of both MACIF and DAF was increased by the addition of TSH,

phorbol 12, 13-dibutyrate, a protein kinase C activation factor that is known to increase DAF in various cells (22, 23), and TSAb-positive Graves' disease serum which increases c-AMP production by stimulating thyroid cells as does TSH (24-26). TSH and TSAb-positive Graves' disease serum also increased the expression of microsomal antigen on the thyroid epithelial cells in culture. These findings indicate that in Graves' disease thyroid follicular epithelial cells show an increase of autoantigen expression in response to thyroid stimulating factors such as TSAb. At the same time, the expression of membrane-associated complement regulatory proteins is also increased. In addition, sera from patients with Hashimoto's disease showed a small degree of cytotoxic effect on thyroid epithelial cells isolated from patients with Graves' disease. However, the same sera showed a significantly enhanced cytotoxic effect on the cells in the presence of anti-MACIF antibody (umpublished data). These results imply that these complement regulatory proteins might have a protective role against the thyroid cell damage due to complement activation by autoantibodies, especially anti-thyroid microsomal antibody.

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