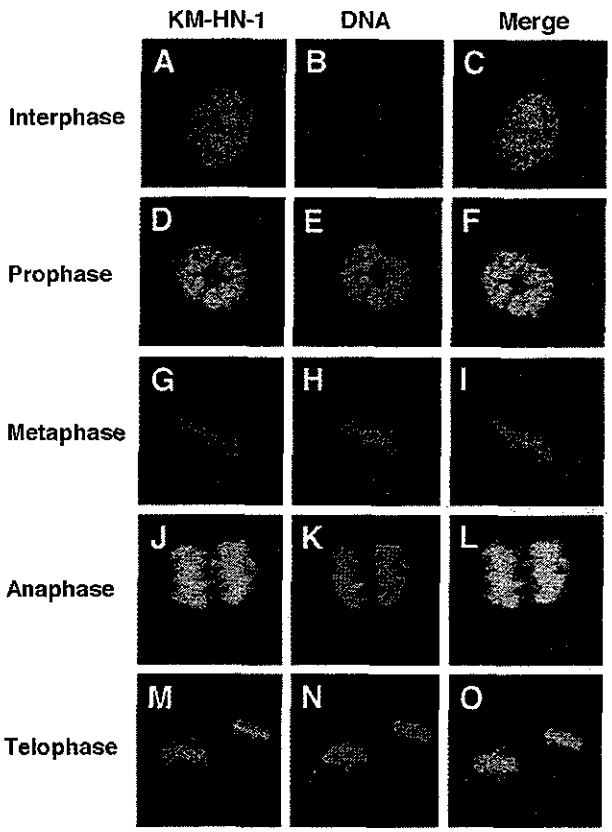

Fig. 3 Immunocytochemical analysis of KM-HN-1 protein with an esophageal cancer cell line, TE13, during mitosis: interphase (*A–C*), prophase (*D–F*), metaphase (*G–I*), anaphase (*J–L*), and telophase (*M–O*). TE13 cells were stained with anti-KM-HN-1 antibody (*green*). Chromosomes were visualized by propidium iodide staining (*red*).

against GST, ELISA reactivity was blocked by prior incubation of sera with an excess amount of purified GST-KM-HN-1 (data not shown). These results confirmed the specificity of IgG to KM-HN-1 in these patients and suggest that KM-HN-1 could elicit humoral immune responses in patients with a variety of tumors.

Association between *KM-HN-1* Expression in Cancer and Presence of KM-HN-1 Antibody in Patients' Sera. Both fresh-frozen tumor specimens and serum samples were available from 13 cancer patients (5 melanomas, 4 HCC, and 4 tongue cancer). Tumors were tested for *KM-HN-1* expression by quantitative RT-PCR, and sera were assayed for KM-HN-1 antibody by ELISA (Table 3). In this series of 13 patients, 5 had both KM-HN-1–positive tumors and KM-HN-1 antibodies. No KM-HN-1 antibody was detected in five patients with KM-HN-1 negative tumors. Three patients had KM-HN-1–positive tumors and no detectable KM-HN-1 antibody. Although the total numbers of samples were small, the association between *KM-HN-1* expression in cancer and presence of KM-HN-1 antibody in patients' sera was statistically significant ($P < 0.05$).

Establishment of CTL Lines Specific to KM-HN-1 Peptide. We attempted to generate KM-HN-1–specific CTLs from four *HLA-A*2402*–positive healthy individuals and four *HLA-A*2402*–positive SCCHN patients, by using KM-HN-1 peptide-pulsed dendritic cell. After three stimulations, CTL activity against peptide-pulsed C1R-A*2402 cells was examined by measuring IFN-γ production to assess peptide specificity. As shown in Fig. 6*A*, CTLs recognizing KM-HN-1 derived peptide were generated from all SCCHN patients and two of four healthy donors. The peptide-reactive CTL lines specific to HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$ were induced from two (SCCHN3 and HD1), three (SCCHN1, SCCHN3, and HD1), and four (SCCHN2, SCCHN4, HD1, and HD2) donors, respectively. The background levels of IFN-γ production in response to peptide unpulsed C1R-A*2402 cells were less than 20 pg/mL. These CTL lines did not produce IFN-γ when exposed to C1R-A*2402 cells pulsed with an HIV-1 nef-derived peptide that had a high binding affinity to HLA-A24, or when exposed to KM-HN-1 peptide-loaded parent cell line, C1R cells, negative for *HLA-A*2402* (data not shown). These data indicate that these CTL activities were KM-HN-1–derived peptide-specific and restricted by *HLA-A*2402* gene product.

To investigate whether 3 KM-HN-1–derived peptides, recognized by CTL lines, are naturally processed and presented by *HLA-A*2402*–encoded molecules, SCCHN cell line HSC-4 (*HLA-A*2402* positive, KM-HN-1 negative) and HSC-4-HN-1, the HSC-4 cells transfected with full-length *KM-HN-1* cDNA, were used as target cells. As shown in Fig. 6*B*, CTL lines induced by HN-$1_{196-204}$, HN-$1_{499-508}$ produced substantial amounts of IFN-γ when exposed to HSC-4-HN-1, but CTL lines induced by HN-$1_{770-778}$ did not do so. The background levels of IFN-γ production by the cells in response to HSC-4 were less than 30 pg/mL in Fig. 6*B*.

Reactivity and Cytotoxicity of CTL Lines against Cancer Cell Lines. HLA-A24 expression in the cancer cell lines was examined by staining with anti-HLA-A24 monoclonal antibody (One Lambda, Inc., Canoga Park, CA) followed by flow cytometry and genotyping, and *KM-HN-1* expression was examined by Northern blot analyses. Among the seven cell lines examined, TE9 were negative for HLA-A24, and HSC-4 were negative for KM-HN-1. Because the total amount of blood obtained from cancer patients was limited, we could use CTL lines from cancer patients only for limited experiments. The CTL reactivity was detected when CTLs were exposed to cancer cell lines positive for both HLA-A24 and KM-HN-1. Representative data are shown in Fig. 7*A*, *B*, and *C*. CTL lines induced by HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$ produced substantial amounts of IFN-γ in response to all cancer cell lines positive for both HLA-A24 and KM-HN-1. Cytotoxic activity of these CTL lines was also examined. These CTL lines exhibited cytotoxicity only against TE3 and TE11, which were positive for both HLA-A24 and KM-HN-1, but not against TE9 and HSC-4 (Fig. 8*A–G*).

To further confirm that the cytotoxicity of these CTL lines against cancer cells was mediated by specific recognition of endogenously processed KM-HN-1, we did a cold target inhibition assay as shown in Fig. 8*J*, *K*, and *L*. In the presence of cold target C1R-A*2402 loaded with HN-$1_{196-204}$, HN-$1_{499-508}$, or HN-$1_{770-778}$ peptides, cytotoxic activity of these CTL lines against PC-9 (Fig. 8*J* and *K*) or IFN-γ treated TE13 (Fig. 8 *L*) was markedly inhibited, whereas the addition of HIV-1 nef-derived peptide-loaded or -unloaded C1R-A*2402 had no effect on cytotoxicity. These findings clearly indicate

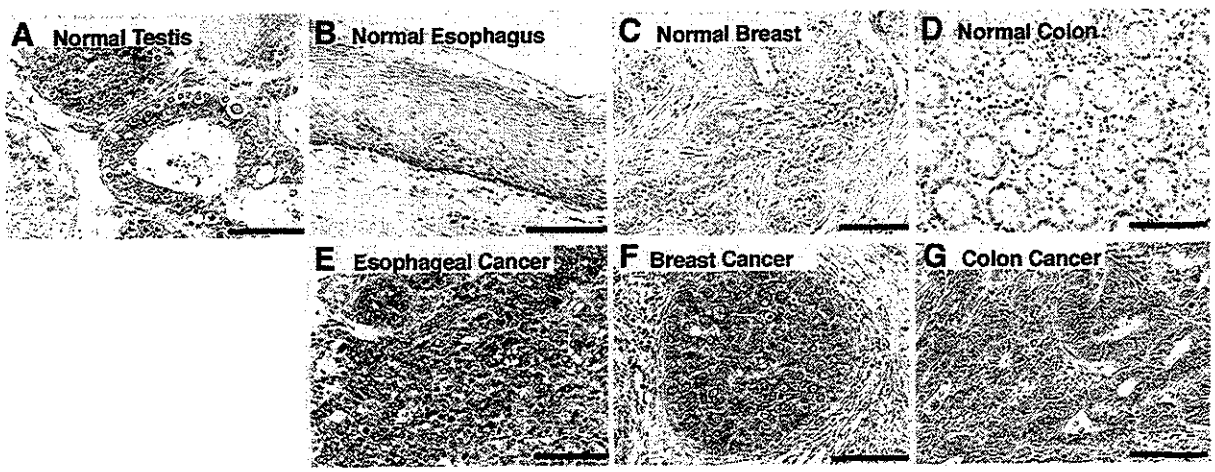

Fig. 4 Immunohistochemical staining of KM-HN-1 antigen in normal and cancer tissues by using avidin-biotin complex method and 3,3′-diaminobenzidine chromogen. *A*. in testis, seminiferous tubules were positively stained with strong intratubular staining of mostly spermatogenic cells, whereas no reactivity with spermatids or with Sertoli cell or interstitial tissue was observed. *B*, *C*, and *D*. KM-HN-1 staining was not detected in the normal esophagus, breast, or colon. *E*. Heterogeneous KM-HN-1 staining was observed in esophageal cancer. Homogeneous KM-HN-1 staining was observed in both breast cancer (*F*) and colon cancer (*G*). ×200. *Scale bar*, 100 μm.

that HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$ peptides are naturally processed from KM-HN-1 protein in cancer cells, expressed in the context of HLA-A24, and recognized by KM-HN-1–specific CTL lines to induce cytotoxicity against cancer cells.

Effects of IFN-γ on Susceptibility of Cancer Cells to CTL-Mediated Cytotoxity. Until now, several antigenic peptides that were processed exclusively by immunoproteasome but not standard proteasome, were reported (31, 32). CTL lines specific to HN-$1_{770-778}$ peptide derived from four different donors were reactive against HN-$1_{770-778}$ peptide-pulsed C1R-A*2402 cells but not against HSC-4-HN-1 (Fig. 6*A* and *B*). We thought that this peptide might be processed by immunoproteasome. Most nonlymphoid cells, be they normal or tumoral, constitutively express standard proteasomes and switch to immunoproteasomes when exposed to

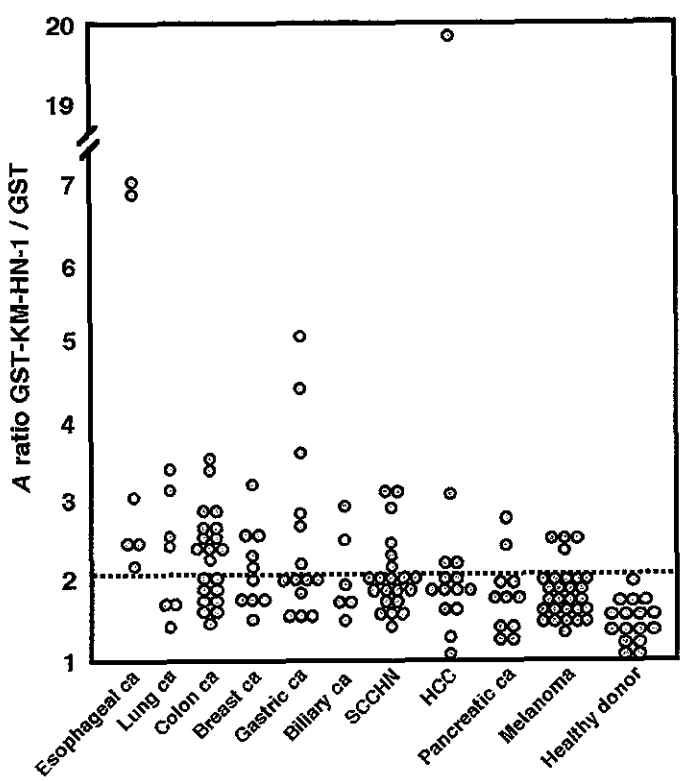

Fig. 5 Quantitation of KM-HN-1– specific IgG measured by ELISA in sera from age-matched healthy donors and patients with various types of cancer. Serum samples were diluted at 1:100 and were analyzed by ELISA to detect antibodies reactive to GST-KM-HN-1 fusion protein and GST alone. The ratio of the absorbance (*A*) for GST-KM-HN-1 to the absorbance for GST was calculated to express the degree of specific reactivity above background. *Dashed line*, the upper normal value (two SDs above the mean value in healthy donors).

Table 3 Correlation between *KM-HN-1* mRNA expression in cancer and the presence of anti-KM-HN-1 antibody in patients' sera

	mRNA*		IgG†	
Patients		Relative ratio		Absorbance ratio
Melanoma				
1	−	ND	−	1.68
2	+	0.1	+	2.36
3	+	0.5	−	1.71
4	+	0.7	+	2.49
5	−	ND	−	1.87
HCC				
1	−	ND	−	1.99
2	−	ND	−	1.39
3	+	4.0	+	3.46
4	−	ND	−	1.64
Tongue cancer				
1	+	0.5	−	1.39
2	+	1.0	−	1.74
3	+	5.0	+	3.14
4	+	1.0	+	2.56

Abbreviation: ND, not determined.

* The relative expression ratio of the *KM-HN-1* mRNA was calculated using the following formula: ratio = $(E_{\text{KM-HN-1}})^{\Delta CP_{\text{KM-HN-1}}(\text{testis-sample})} / (E_{\beta\text{-actin}})^{\Delta CP_{\beta\text{-actin}}(\text{testis-sample})} \times 100$.

† Antibody titer was determined using indirect ELISA with KM-HN-1-GST fusion protein. The cutoff value (2.15) is the mean plus two *SD for healthy donors' sera*; positive values are underlined.

IFN-γ (33). Therefore, we investigated effects of IFN-γ on the susceptibility of cancer cells to CTL-mediated cytotoxicity. Target cell lines (HSC-4 and HSC-4-HN-1) were pretreated with IFN-γ according to the protocol described in Materials and Methods. CTL lines specific to HN-$1_{770-778}$ peptide produced IFN-γ in response to IFN-γ treated target cells (Fig. 6*C*). Furthermore, HN-$1_{196-204}$, and HN-$1_{499-508}$ peptide-specific CTL lines also showed increased IFN-γ production in response to IFN-γ-treated target cells. All of CTL lines that were reactive to peptide-pulsed C1R-A*2402 cells produced substantial amounts of IFN-γ in response to HSC-4-HN-1 treated with IFN-γ, but not in response to HSC-4 treated with IFN-γ (data not shown). The background levels of IFN-γ production by the cells in response to HSC-4 were under 50 pg/mL in Fig. 6C. Next, we investigated effects of IFN-γ on the presentation of naturally processed KM-HN-1 peptide in cancer cell lines. Cytotoxic activities of all of these CTL lines tested were also increased when TE13 was pretreated with IFN-γ (Fig. 8*H* and *I*), but not when TE9 and HSC-4 were pretreated with IFN-γ (data not shown).

DISCUSSION

A prerequisite for a broader application of antigen-specific immunotherapy for cancer is the molecular definition of antigens that are specifically expressed in commonly occurring neoplasms, *e.g.*, breast, lung, prostate, or colorectal carcinoma. The recognition that members of the *MAGE, BAGE, GAGE, HOM-MEL-40,* and *NY-ESO-1* gene families form a class of tumor antigens with restricted expression confined to cancer and testis has let to the designation of *CT* antigens. This characteristic expression profile suggested that testicular tissue is a prime candidate source for the identification of additional CT antigens by SEREX. To further increase the yield of unidentified CT antigens, we applied SEREX with serum from a SCCHN patient and a testis cDNA expression library to identify KM-HN-1.

Although KM-HN-1 contains no Pfam motif (http://pham.wustl.edu/), it does consistently exhibit a low level (<20%) of substantial homology with CENP-F (centromere protein F) and

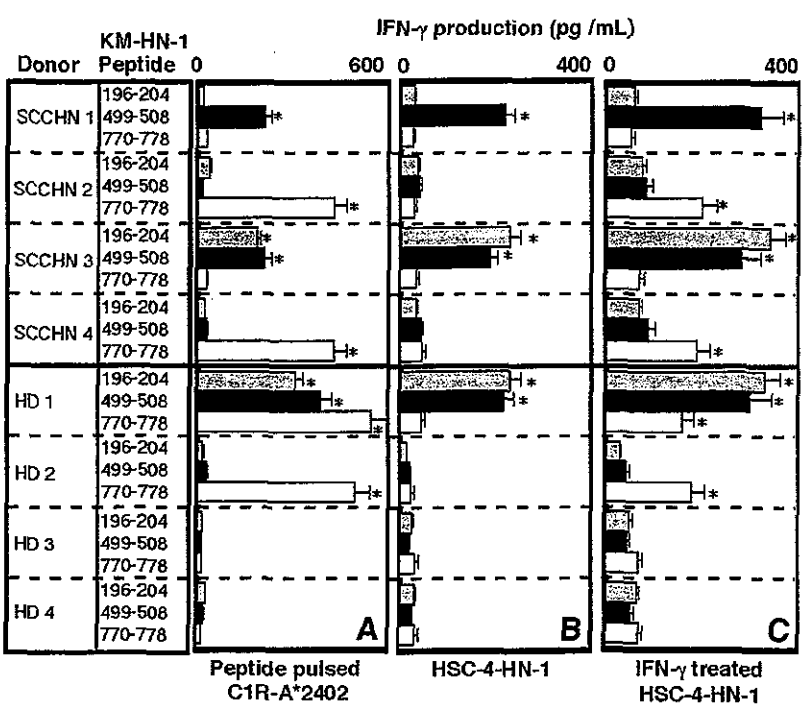

Fig. 6 Induction of CTLs by the KM-HN-1—derived peptides. CTL lines specific to three peptides, HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$, could be generated from HLA-A24–positive healthy donors and cancer patients. CTL responses to the peptide-loaded C1R-A*2402 cells (*A*), HSC-4 cells transfected with *KM-HN-1* gene (expression vector), HSC-4-HN-1 (*B*), and HSC-4-HN-1 pretreated with IFN-γ (*C*) were examined. CTL lines were cultured with each cell for 16 hours, and the culture supernatant was harvested for the measurement of IFN-γ production. *Values*, the means of duplicate assays. Two-tailed Student's *t* test was used for the statistical analysis of difference between IFN-γ production by the cells in response to the corresponding peptide-loaded C1R-A*2402 cells, HSC-4-HN-1, or IFN-γ–treated HSC-4-HN-1, and that in response to unloaded C1R-A*2402 cells, HSC-4, or IFN-γ–pretreated HSC-4, respectively. *, $P < 0.05$. Effector to target ratio was 10. (*HD*, healthy donor.)

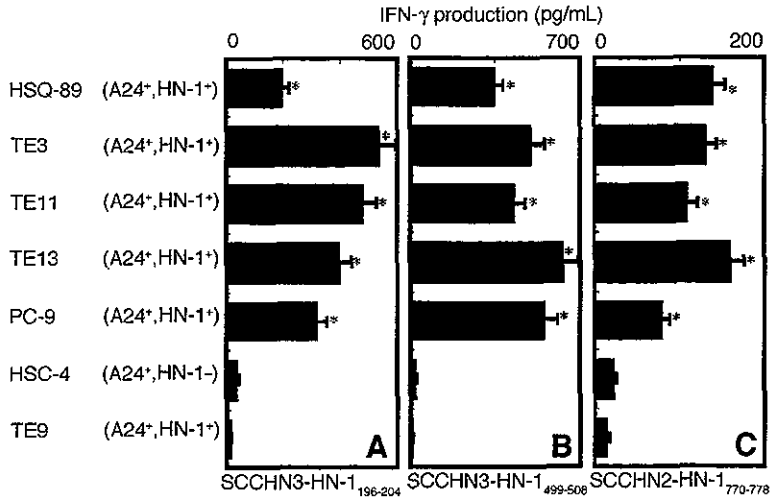

Fig. 7 CTL responses to cancer cell lines. Representative results of at least two reproducible experiments are shown: *A*, HN-$1_{196-204}$ peptide-specific CTL line generated from the patient SCCHN3; *B*, HN-$1_{499-508}$ peptide-specific CTL line generated from the patient SCCHN3; C, HN-$1_{770-778}$ peptide-specific CTL line generated from the patient SCCHN2. CTL lines were cultured with each cancer cell, and the culture supernatant was harvested for the measurement of IFN-γ production. Values represent the means of duplicate assays. Two-tailed Student's *t* test was used for the statistical analysis of difference in IFN-γ production between cancer cell line-stimulated CTLs and HSC-4–stimulated CTLs. *, $P < 0.05$.

SCP-1 (sterol carrier protein-2). CENP-F protein localizes to the spindle midzone and the intracellular bridge in late anaphase and telophase, respectively. Localization of this protein suggests that it may play a role in chromosome segregation during mitosis (34, 35). Furthermore, autoantibodies against CENP-F protein were found in patients with cancer (36). SCP-1 had been described as a major component of the synaptonemal complex (37). The synaptonemal complex is a tripartite macromolecular assembly formed between homologous chromosomes during the meiotic prophase (38). SCP-1 had also been described as one of the CT antigens, recognized by both humoral and cellular immune systems in cancer patients (39). In this study, KM-HN-1 is colocalized with chromosome during mitosis (Fig. 3), and an antibody against KM-HN-1 is observed in cancer patients (Fig. 5 and Table 1), which suggests a functional similarity between KM-HN-1 and these proteins.

Our expression analysis demonstrates the aberrant expression of the *KM-HN-1* gene in a broad spectrum of human neoplasms and characterizes KM-HN-1 as a CT antigen. KM-HN-1 has several similarities to known CT antigens. First, there is the mRNA expression profile; *KM-HN-1* was expressed only in testis among normal tissues but was expressed in various types of cancer, *e.g.*, SCCHN, esophageal cancers, and so forth. Second, there is the protein expression pattern in testis tissues: in the testis, CT antigens expression was detected exclusively in spermatogonia (40). As shown in Fig. 4, our data were similar to the observations in previous studies. Third, KM-HN-1 is immunogenic in cancer patients: KM-HN-1 elicited humoral (Fig. 5) and cellular (Figs. 6, 7, and 8) immune response in cancer patients. On the other hand, KM-HN-1 differs from known CT antigens by chromosomal localization. Although nearly all of the hitherto known genes encoding CT antigens have been mapped to the X chromosome (41), mapping of the human *KM-HN-1* gene has been assigned to chromosome 4. Furthermore, some CT antigens, *e.g.*, MAGE, GAGE, and NY-ESO-1, were shown to be members of multigene families (42, 43), but the family gene of KM-HN-1 was not found.

Because previous studies showed that some of SEREX-defined antigens elicited humoral immune responses in many types of cancer patients (44, 45), we looked for the KM-HN-1–specific antibody in the sera of cancer patients with ELISA methods. Surprisingly an anti-KM-HN-1 IgG was observed in 14 to 100% of all types of cancer patients tested, on the other hand this was not observed in healthy donors (Fig. 5; Table 2). Thereby quantification of anti-KM-HN-1 IgG in sera may provide us a new diagnostic method for various types of cancer. However, a statistically significant association between KM-HN-1 antibody status and clinical characteristics of patients (age, sex, stage) was not evidenced (data not shown).

In this study, we used synthetic KM-HN-1 peptides consisting of HLA-A24 binding motifs for the generation of CTLs from PBMCs, because the *HLA-A24* allele is the most frequent HLA class I allele in the Japanese population and is found in 60% of the Japanese (95% of these cases are genotypically *A*2402*), in 30% of Chinese, and in 20% of Caucasians (46). Of the 13 synthetic peptides used, three peptides (HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$) induced peptide-specific CTL lines. CTLs recognizing KM-HN-derived peptide were generated from all four SCCHN patients and two of four healthy donors. Although the KM-HN-1–specific CTL-precursor frequency of SCCHN patients seemed to be higher than that of healthy donors, peptide-specific CTLs were not detected by intracellular staining of IFN-γ with PBMCs that were stimulated with KM-HN-1 peptides *ex vivo*, in either cancer patients or healthy donors. Peptide-specific CTLs could be detected after two stimulations with antigenic peptide, but a statistically significant difference in frequency of IFN-γ producing CTLs (1–4% of $CD8^+$ T cell) between cancer patients and healthy donors was not observed (data not shown).

Further examination showed that CTL lines specific to only two (HN-$1_{196-204}$ and HN-$1_{499-508}$) of these peptides lysed *KM-HN-1* transfectant (HSC-4-HN-1), but all CTL lines reactive to peptide-pulsed C1R-A*2402 lysed HSC-4-HN-1 pretreated with IFN-γ (Fig. 6). Similar results are shown for CTL activities against cancer cell lines (Fig. 8). When cancer cell lines were treated with IFN-γ, the *KM-HN-1* mRNA level did not change (data not shown). In general, IFN-γ induces several changes in cancer cells, *e.g.*, up-regulation of MHC class I

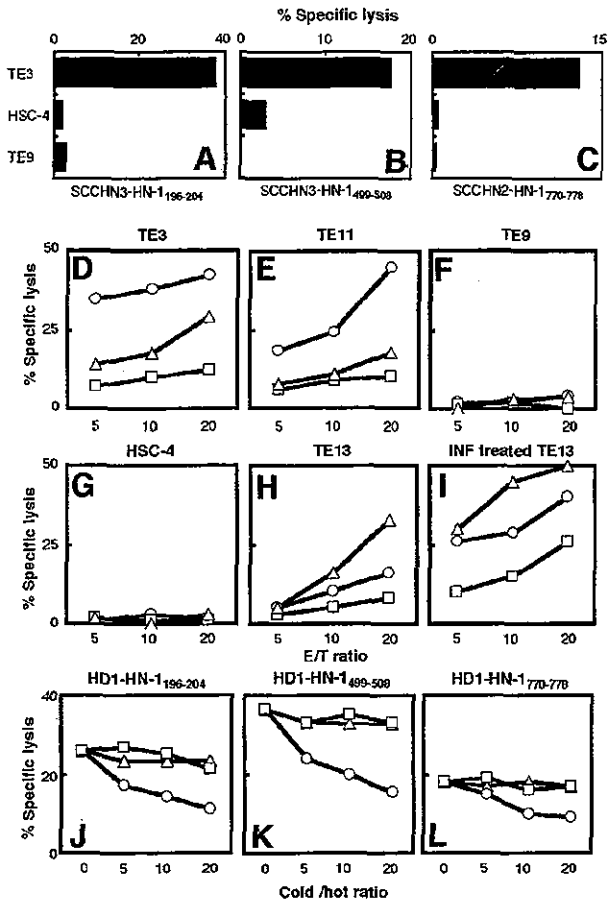

Fig. 8 ^{51}Cr release assay for cytotoxicity of CTL lines against cancer cell lines. *A–C*, cytotoxic activities of CTL lines generated from SCCHN patients against cancer cell line were detected by 4-hour ^{51}Cr release assay at effector-to-target ratio of 10:1. Peptides used for CTL generation were indicated in abscissa (*D–I*), HN-$1_{196-204}$ ([trio]), HN-$1_{499-508}$ (○), and HN-$1_{770-778}$ (□) peptide-specific CTL lines generated from healthy donor (*HD*) 1 were used as effector cells, respectively. Cytotoxic activities against TE3 (*D*) and TE11 (*E*) expressing both HLA-A24 and KM-HN-1, TE9 (KM-HN-1$^+$ and HLA-A24$^-$; *F*) and HSC-4 (KM-HN-1$^-$ and HLA-A24$^+$; *G*). Cytotoxic activities against TE13, expressing both HLA-A24 and KM-HN-1 (*H*) and TE13 pretreated with IFN-γ (*I*). *J–L*, cold target inhibition assay. ^{51}Cr-labeled PC-9 (*J* and *K*) and TE13 (*L*) cells pretreated with IFN-γ were mixed with various numbers of unlabeled C1R-A*2402 cells that had been unloaded (△) or loaded with the KM-HN-1 peptide indicated (○) or with irrelevant HIV peptide (□). The cytotoxicity of the ^{51}Cr-labeled target cells in the presence of unlabeled target cells was determined by 4-hour ^{51}Cr release assays at an effector-to-^{51}Cr-labeled-target-cell ratio of 10:1.

molecules and induction of expression of the immunoproteasome (47). Our results may be attributable to both of these mechanisms. First, after cancer cell lines used in this study were treated with IFN-γ, the expression of HLA-A24 molecules increased two (HSC-4-HN-1) and five (TE13) times (data not shown). Second, although HLA expression increased 2- and 5-fold, CTL lines specific to HN-$1_{770-778}$ showed an almost 10-fold increase of CTL activities when exposed to IFN-γ-treated target cells.

Identification of new CTL epitopes in different tumor antigens will allow for development of multiantigenic (epitope-based) tumor vaccines, which will probably be useful to circumvent tumor escape from immune systems by losing expression of antigen. In this study, we found a novel human cancer/testis antigen, KM-HN-1. *KM-HN-1* gene is expressed in many types of cancer, and a humoral immune response to KM-HN-1 protein is detected in many cancer patients. Furthermore, three peptides derived from KM-HN-1 could induce HLA-A24-restricted and tumor-reactive CTL lines. These results suggest that KM-HN-1 might be a good candidate for the development of a cancer vaccine applicable to various types of cancer patients.

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Degenerate recognition and response of human CD4+ Th cell clones: implications for basic and applied immunology

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Abstract

It was once considered that the T cell response is an all or nothing type event, but recent studies have clearly indicated that T cells show many different types of activation in recognition of altered ligands for T cell receptors (TCR). In this review, we summarize our recent findings on the response of human CD4+ helper T (Th) cell clones to altered peptide ligands (APL); peptides carrying single or multiple residue substitutions in antigenic peptides. The extensive analyses revealed that TCR-antagonism and partial agonism are frequently observed by the stimulation with APLs substituted at particular amino acid residues of antigenic peptides. We observed unique partially agonistic APLs inducing prolongation of T cell survival without cell proliferation. Superagonistic APLs stimulated enhanced proliferation and production of cytokines in Th cell clones reactive to tumor-associated antigens. The other APL induced enhanced production of interleukin-12 by antigen presenting cells and subsequent enhancement of IFN-γ production by T cells reactive to allergens. By utilizing an HLA-DR-restricted T cell epitope library generated by mutated invariant chain genes, it was revealed that human Th cell clones recognize a more diverse array of peptides with multiple and simultaneous amino acid substitutions in an antigenic peptide. APLs also induced altered intracellular signaling events including intracellular calcium increase and phosphorylation of signaling molecules. This information provides basic knowledge regarding the characteristics of antigen recognition by human Th cells and the subsequent activation, and a novel method for manipulation of human Th cell responses by APLs, as a possible candidate for antigen-specific immuno-potentiating or immunosuppressive therapy.

Keywords: HLA class II molecule; Human CD4+ Th-cells; Antigenic peptides; Altered peptide ligands; T cell recognition; TCR antagonism; Peptide partial agonism

1. Introduction

The human histocompatibility leukocyte antigen class-II (HLA-II) molecule has a peptide binding groove on top of the molecule and binds antigenic peptides processed by antigen presenting cell (APC) to present them to CD4+ helper T (Th) cells (Germain and Margulies, 1993). Three to five amino acid residues were separated by one to two intervening residue(s) and acted as anchor residue(s) for peptide binding to HLA-II molecules (Sette et al., 1993; Hammer et al., 1993; Matsushita et al., 1994). On the other hand, side chains of amino acid residues flanking anchor residues proved to be the main recognition sites by T cell receptors (TCR); this was clearly established in crystallographic analyses of the DR molecule bound by either self (Brown et al., 1993) or non-self peptides (Stern et al., 1994).

CD4+ Th cells usually recognize non-self peptides in the context of self HLA-DR molecules. Recognition and responses of T cells were once considered to be an on/off phenomenon, however recent findings obtained using altered peptide ligands (APLs) carrying single residue substitutions in antigenic peptides presented by one major histocompatibility complex (MHC) class II molecule or one specific peptide presented by different MHC class II molecules showing a limited polymorphism revealed that

Abbreviations: HLA-II, human histocompatibility class II; APC, antigen presenting cell; Th cell, helper T cell; TCR, T cell receptor; APL, altered peptide ligand; MHC, major histocompatibility complex; IFN-γ, gamma interferon; IL, interleukin; CLIP, class II-associated invariant chain peptide; ZAP-70, zeta-associated protein-70

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altered TCR ligands induce altered T cell responses in both mice and humans, including (1) T cell non-responsiveness, through TCR antagonism and (2) partial agonism inducing partial activation of T cells without cell proliferation (Sloan-Lancaster and Allen, 1996).

Previous analyses also revealed that the interactions of TCRs with MHC-wild-type peptides had stronger affinities and/or smaller off-rates than did those of TCRs with MHC-APL complexes (Lyons et al., 1996). These differences in characteristics of molecular interactions may induce insufficient engagements of TCR with MHC-APL complexes such that intracellular signals mediated by TCR through recognition of APLs are inadequate for full activation of T cells to induce cell proliferation. In some cases, inadequate signals induce unique altered T cell responses.

In this review, we will summarize our recent analyses on recognition by human $CD4^+$ Th cell clones of diverse peptides, and the heterogeneity of subsequent T cell responses and T cell activation signals induced, as summarized in Table 1.

2. Frequencies of agonistic and antagonistic single residue substituted APLs depend on position of substituted amino acid residues of the peptide

If there is a general rule for structures of APLs which stimulate or inhibit T cell responses to wild type antigenic peptides, it would be easier to generate peptides which augment or inhibit responses of human Th cells. We used a human Th1-cell like clone YN5-32 reactive to a streptococcal M12p54-68 peptide (^{54}NRDLEQAYNELSGEA^{68}) in the context of HLA-DR4 (DRB1*0406), and analyzed responses of YN5-32 to 156 independent APLs carrying single residue substitutions at residues 57 (P1)-65 (P9) of the peptide where P1 (position 1) means the putative most N-terminal DR anchor residue (Chen et al., 1996). As shown in Fig. 1, residues Leu-57 (P1), Ala-60 (P4) and Asn-62 (P6) were the most likely to be DR-anchor residues, and 30% (17/57) of APLs substituted at these residues exhibited full agonism to stimulate various magnitudes of proliferative responses in the T cell clone, whereas only 7.5% (3/40) of non-fully agonistic peptides exhibited TCR antagonism. On the other hand,

Table 1
Summary of our observations on responses of human $CD4^+$ Th-cell clones to APLs

Th-cell clone	Specificity	Observed immune responses to APLs	Reference
YN5-32	Streptococcal M12p54-68/DR4	TCR antagonism	
		Partial agonism; increases in cell size and expression levels of CD4, 11a, 28, 49d, 95 without anergy induction	Chen et al. (1996)
		Polymorphism at DRβ37 affected T cell recognition	Chen et al. (1997)
		Quantitative and qualitative alteration of intracellular calcium increase	Chen et al. (1998)
		Overexpression of partially agonistic TCR-ligand induced proliferation without phosphorylation of ZAP-70 and LAT	Irie et al. (2003)
SK2.11	AChRα p75-87/DQ6	TCR antagonism	Kanai et al. (1997).
BC20.7, BC33.5, BC42.1	BCGa p84-100/DR14	Partial agonism in recognition of artificial or natural self APLs; increased survival without antigenic stimuli or production of IL-4 and IFN-γ without cell proliferation	Matsushita et al. (1997)
C27	p21Ras p3-17/DR1	Superagonism; increased proliferation and production of IFN-γ and GM-CSF in recognition of cancer-associated mutated peptides and its APL	Yokomizo et al. (1997)
Y41.2	TEL/AML1 fusion peptide/DP17	Superagonism; increased proliferation and production of IFN-γ and GM-CSF in recodnition of APLs derived from leukemia-associated TEL/AML1 fusion peptide	Yun et al. (1999)
29.15.2	p21Ras p3-20/DR51	Superagonism; increased proliferation in recognition of APL identified by using a combinatorial peptide library and mass spectrometry	Tanaka et al. (1999)
ST1.9	*Cry j*1p335-346/DR52	Superagonism; increased production of IFN-γ	Ikagawa et al. (1996)
DT13.2	*Der f*1p18-31/DQ6	Superagonism; increased production of IFN-γ stimulated by increased production of IL-12 from antigen presenting cells	Matsuoka et al. (1996)
SA32.5, MK20.2	GAD65 p115-127/DR53	Generation of a multiple residue substituted epitope expression library by using CLIP-substituted invariant chain genes to identify agonistic APLs and mimicry microbial peptides	Uemura et al. (2003)

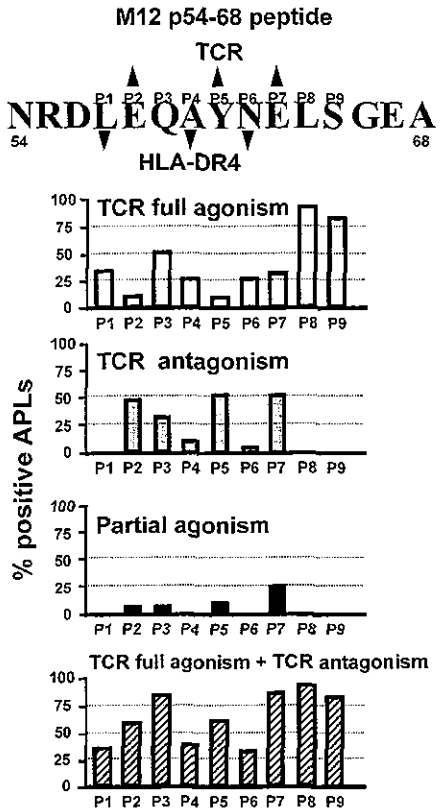

Fig. 1. Summary of responses of the human Th cell clone YN5-32 to 156 APLs carrying single residue substitutions in a streptococcal antigenic peptide M12p54-68. From P1 to P7 residues, residues were replaced with 19 other amino acids. The P8 and P9 residues were replaced with 10 and 11 other amino acids, respectively. Percentages of APLs exhibiting either full agonism (open bars), TCR antagonism (shedded bars) or partial agonism (closed bars) are indicated for each residue. APLs carrying substitutions at putative TCR contact residues, P2, P5 and P7, frequently exhibited TCR antagonism. Some of them, especially APLs substituted at P7, exhibited partial agonism. Because APLs with full agonism or TCR antagonism have to bind to MHC molecules, the frequencies of those peptides indicated by cross hatched bars represent the frequency of peptides with MHC-binding capacity.

residues Glu-58 (P2), Tyr-61 (P5) and Glu-63 (P7) were the most likely to be TCR-recognition sites and only 15.8% (9/57) of APLs stimulated proliferative responses in YN5-32 thereby indicating that substitutions at these residues frequently abrogate T cell recognition. Interestingly, as many as 60.4% (29/48) of non-fully agonistic APLs exhibited TCR antagonism to inhibit the proliferative response of YN5-32 to the wild-type peptide.

Eight (27.6%) of these antagonistic APLs carrying relatively conservative amino acid substitutions exhibited partial agonism to induce large increases in cell size and expression levels of CD4, CD11a (LFA-1α), CD28, CD49d (VLA-4α) and CD95 (Fas), on the T cell surface, as compared with responses to the wild-type peptide. This was the most prominent at Glu-63 (p7) where 5 of 10 antagonistic APLs exhibited partial agonism. These observations indicate that many APLs carrying substitutions at TCR recognition sites in the T cell epitope induce a partial agonism as well as TCR antagonism in YN5-32, as noted by other studies of mouse T cell clones. Differences, such as the absence of anergy induction or little increase in CD25 expression by partially agonistic APLs have been noted in human Th cells. The polymorphism (Ser-Tyr) at the DRβ^{37} residue induced conformational changes of peptides, which can be distinguished by YN5-32 TCR in some but not all peptides, providing further evidence for altered human T cell responses induced by minor modifications of TCR ligands (Chen et al., 1997).

Based on this knowledge of Th cell responses to APLs, we identified many antagonistic APLs which can inhibit proliferation of Th-cell clones auto-reactive to the acetylcholine receptor α chain derived self peptide in the context of the disease-susceptible HLA-DQ6 molecule and established from a patient with infant-onset myasthenia gravis unique to Asian populations (Kanai et al., 1997).

3. Unique partially agonistic APLs inducing prolonged survival of Th cells in the absence of antigenic stimulus

By utilizing three other human Th cell clones with distinct TCR-Vβ recognizing the same non-self mycobacterial *Bacillus Calmette-Guérin* a (BCGa) peptide/HLA-DR14 complex, we found another type of unique partial agonism, as follows (Matsushita et al., 1997). Stimulation of T cells with a one-residue-substituted APL or a minimally homologous self-peptide fragment can prolong the in vitro survival of T cells in the absence of antigenic stimuli, in a clone specific-manner. This prolongation is associated with the up-regulation of Bcl-x_L, without proliferation and these peptide-clone combinations are capable of inducing lymphokine secretion. Thus, peptide partial agonism may play a role in the survival of not only thymocytes but also mature Th cells, in the absence of non-self peptide ligands.

4. Augmentation of T cell responses (superagonism) stimulated by APLs: implication to peptide-based cancer immuno-therapy

A T cell response to a tumor requires a tumor antigen processed into peptides which can be presented to $CD8^+$ cytotoxic T cells by MHC class I molecules, and to $CD4^+$ Th cells by MHC class II molecules. While cytotoxic T cells can kill tumor cells directly, some Th1 cells can mediate cytotoxicity to tumors, amplify responses of cytotoxic T cells, and activate APC, through secretion of lymphokines to augment anti-cancer immunity. We established a Th cell clone reactive to oncogenic and mutated p21 Ras proteins as well as mutated peptides, in an HLA-DR1-restricted manner. We provided evidence for augmentation of proliferation and production of gamma interferon (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-

CSF) by this T cell clone in recognition of APLs carrying a single residue substitution in the mutated P21 Ras peptide (Yokomizo et al., 1997).

We also identified superagonistic and single residue substituted APLs derived from leukemia-associated TEL/AML1 fusion peptide (IGRIA/ECILGMNPSR) (Yun et al., 1999). The APLs having Val or Leu substitutions at putative P8(Gly) or P9(Met) of the peptide respectively stimulated much stronger proliferation and production of Th1-type cytokines in a Th clone reactive to TEL/AML1 fusion peptide in the context of HLA-DP17. These superagonistic APLs can be given consideration for anti-leukemic immunotherapy.

To identify peptide superagonists in a systematic and sophisticated manner, we used a combinatorial peptide library and mass-spectrometry (Tanaka et al., 1999). The proliferative responses of a human $CD4^+$ T cell clone reactive to a self-K-Ras-derived peptide, Ras p3-20 (^{3}EYKLVVVGAGGVGKSALT^{20}), were tested using a set of X9 combinatorial peptide libraries containing the flanking residues (EYKLVXXXXXXXXXSALT, where X indicates random amino acids). Certain peptide libraries, such as EYKLVXXXXXXMXXSALT and EYKLVXXXXXXXHXSALT, stimulated a marked proliferation of T cells. However, no combinations of substitutions tested, such as EYKLVXXXXXXMHXSALT, exhibited additive effects. We subsequently synthesized peptides with degenerate sequences (a mixture of 480 species), where each position is composed of the wild-type residue or of amino acids that induced the proliferation of T cells, in positional scanning. Interestingly, one fraction of degenerate peptides, separated by reverse-phase HPLC, stimulated a much stronger proliferation than did the Ras p3-20; in addition, the retention time of this fraction was distinct from that of Ras p3-20. Mass spectrometry analysis of this fraction and flanking fractions identified five peptide species that exhibit strong signals in a manner that parallels the antigenic activity. Finally, 17 candidate peptide sequences were deduced from mass spectrometry and hydrophobicity scoring results, of which two peptides (EYKLVVVGAGGMLKSALT and EYKLVVVGAGGMIKSALT) did induce 52- and 61-fold stronger proliferation, respectively, compared with the Ras p3-20. These findings indicate that: (1) synthetic peptides that carry "the best" residue substitution at each position of combinatorial peptide libraries do not always exhibit superagonism, and (2) such a drawback can be overcome with the use of mass spectrometry. This approach provides new perspectives for accurate and efficient identification of peptide superagonists.

5. APL affects not only T cell responses but also APC responses to increase IL-12 production: Implication to peptide therapy inducing Th1-dominance

Human Th0 clone DT13.2 reactive to the group I allergen in *Dermatophagoides farinae* extracts (*Der f* I) p18-31 (^{18}RSLRTVTPIRMQGG^{31}) in the context of HLA-DQ6 (DQA1*0102/DQB1*0602) molecules was generated from a patient with bronchial asthma and DT13.2 produced both interleukin (IL)-4 and IFN-γ. Analysis of changes in DT13.2 responses to *Der f* I p18-31-derived APLs revealed that the substitution of ^{27}Arg to Lys resulted in a significant increase in IFN-γ production, with no remarkable changes either in proliferative response or in IL-4 production (Matsuoka et al., 1996). Interestingly, the selective enhancement of IFN-γ by the APL was accompanied by an increased production of IL-12 and this event was suppressed by an anti-IL-12 antibody down to the level of IFN-γ production induced by the wild-type peptide. The superagonistic APL derived from another Japanese Cedar pollen allergen (*Cry J*I) also augmented production of IFN-γ in a human Th0 clone reactive to *Cry J*I peptide/HLA-DR52 complex (Ikagawa et al., 1996).

Our observations suggest that the mode of interaction between TCR and MHC/peptide complex may determine the Th1-predisposing condition by controlling the IL-12 production by APC. Furthermore, this kind of Th1-response inducing APLs may provide peptide therapy for diseases caused by Th2 responses such as allergy.

6. Generation of a Th cell epitope expression library for extensive analysis of degeneracy in peptides recognized by human Th cell clones

Because we found that the systematic detection of cross-recognized epitopes considering the combinatorial effect of amino acids within the epitope is impossible in approaches using positional scanning synthetic combinatorial peptide libraries, we established an alternative method by utilizing molecular genetic approaches. A DNA-based randomized epitope library using class II-associated invariant chain peptide (CLIP)-substituted invariant chains was generated (Fujii et al., 1998; Fujii et al., 2001; Uemura et al., 2003). This approach, by which multiple residues of an antigenic peptide were simultaneously randomized, has the great advantage of producing several conformations of the peptide/HLA-II complexes, and increasing the possibility to identify degenerate sequences with agonistic properties. GAD65-autoreactive T cell clones restricted by disease-susceptible HLA-DR53 and established from patients with type I diabetes were utilized as models. Analysis of agonistic epitopes indicate that recognition by each TCR was significantly affected by combinations of amino acids in the antigenic peptide, although the degree of combinatorial effect differed between each TCR. Protein database searching based on the TCR recognition profile proved successful in identifying several microbial and self-protein-derived mimicry epitopes with limited sequence homology to the original GAD65 epitope. Some of the identified mimicry epitopes were actually produced from recombinant microbial proteins by APCs to stimulate T cell clones. Our data

demonstrate the importance of the combinatorial nature of amino acid residues of epitopes to investigate diversity of T cell recognition and molecular mimicry, and the Th cell epitope display library we established provides a useful tool for these objectives.

7. Altered intracellular signalings induced in a Th-cell clone by APLs

In mouse T cell clones, TCR antagonistic or partially agonistic APLs induce partial phosphorylation of CD3ζ chains leading to the absence of phosphorylation and activation of ZAP-70 (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). Studies of calcium signaling activity in mouse T cells stimulated with APLs indicated that the Ca^{2+} response induced by antagonistic APLs was smaller in amplitude and shorter in duration than that induced by fully agonistic ligands (Sloan-Lancaster et al., 1996; Wülfing et al., 1997).

To determine if APLs affect intracellular activation signals in human Th cells, we investigated changes in intracellular calcium concentrations ($[Ca^{2+}]_i$) in the Th cell clone YN5-32 stimulated with either fully agonistic peptide M12p54-68 or partially agonistic APL E63V (standing for APL having Val-substitution at amino acid residue 63 Glu), or simply antagonistic APL E58M as described in the Section 1 (Chen et al., 1998). Both E63V and E58M stimulated a Ca^{2+} response in ~40% of the T cells, whereas M12p54-68 did so in ~70% of T cells. The most predominant pattern of a Ca^{2+} increase induced by M12p54-68 was a small sinusoidal peak followed by a sustained high response. The most frequent pattern of calcium response induced by E63V was a continuous high response without a preceding sinusoidal peak, whereas that induced by E58M was large with frequent oscillations. Furthermore, our results suggest that the Ca^{2+} response induced by the fully agonistic peptide depends on activation of the genistein-sensitive signaling pathway, including protein tyrosine kinases, whereas the Ca^{2+} response to a simple antagonistic APL completely depends on activation of the GF109203X-sensitive signaling pathway, including protein kinase Cs and extracellular Ca^{2+}. These differences in the $[Ca^{2+}]_i$ response in recognition of different APLs may parallel the unique T cell activation patterns induced by APLs in human T cells.

We then asked whether forced overexpression of partially agonistic TCR-ligands on APCs provides high-avidity TCR-ligands to stimulate T cell proliferation, we generated L cell transfectants expressing various numbers of HLA-DR4 covalently linked with APLs derived from M12p54-68 peptide and observed responses of the cognate T cell clone YN5-32. Some overexpressed HLA-DR4/partially agonistic APL complexes induced T-cell proliferation in a density-dependent manner, however tyrosine-phosphorylation of ZAP-70 and linker for activated T cells (LAT) and kinase activity of ZAP-70 were not detectable (Irie et al., 2003). Our data suggest the presence of an unique signaling pathway coupling TCR-ligation with T cell proliferation in a ZAP-70 less dependent manner, and this activation pathway is observed when TCRs are engaged with relatively low affinity TCR ligands expressed in high density on the surface of APC. This suggests that T cell activation signals are not uniform and they can be alternatively activated depending on binding characteristics between TCRs and their ligands.

8. Conclusions

In conclusion, we observed various kinds of responses to APLs in human Th cell clones, as summarized in Table 1, and the implications of our findings are as follows. (1) It is so far difficult to predict degeneracy of Th cell recognition in a given TCR by analyzing the past literature, and our Th cell epitope expression library using CLIP-substituted invariant chain genes will provide a breakthrough in this field. (2) Our findings may support the following ideas, (1) maintenance of Th cell survival (memory ?) by self APLs in the absence of stimuli with non-self peptides, (2) triggering of autoreactive Th cells by non-self agonistic APLs (molecular mimicry), and (3) a possible application of APLs to augmentation of desirable anti-microbial or anti-tumor immunity, or to inhibition of pathological immune responses such as allergy and autoimmunity. Our analyses of human Th cell responses to APLs have provided pertinent information on the basic immunology of human Th cell biology and also on the strategy for new methods for manipulation of antigen-specific responses of human Th cells.

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Detection of the novel autoantibody (anti-UACA antibody) in patients with Graves' disease

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Abstract

Uveal autoantigen with coiled coil domains and ankyrin repeats (UACA) is an autoantigen in patients with panuveitis such as Vogt–Koyanagi–Harada disease. The prevalence of IgG anti-UACA antibodies in patients with uveitis is significantly higher than healthy controls, suggesting its potential role as an autoantigen. Originally, UACA was cloned from dog thyroid tissue following TSH stimulation. So, we presumed UACA could be a novel autoantigen in autoimmune thyroid diseases. We measured serum anti-UACA antibody titer using ELISA in patients with autoimmune thyroid diseases (Graves' disease, Hashimoto's thyroiditis, subacute thyroiditis, and silent thyroiditis). The prevalence of anti-UACA antibodies in Graves' disease group was significantly higher than that in healthy group (15% vs. 0%). Moreover, the prevalence of anti-UACA antibodies in Graves' ophthalmopathy was significantly higher than that in Graves' patients without ophthalmopathy (29% vs. 11%). Especially, 75% of severe ocular myopathy cases showed high UACA titer. Immunohistochemical analysis revealed that UACA protein is expressed in eye muscles as well as human thyroid follicular cells. Taken together, UACA is a novel candidate for eye muscle autoantigens in thyroid-associated ophthalmopathy.

Keywords: Uveal autoantigen with coiled coil domains and ankyrin repeats; Graves' disease; Graves' ophthalmopathy; Autoantigen; Ocular myopathy; FRTL5; Vogt–Koyanagi–Harada disease; Thyroid–eye shared autoantigen

Uveal autoantigen with coiled coil domains and ankyrin repeats (UACA) is an autoantigen associated with panuveitis. Anti-UACA antibody appears in patients sera of Vogt–Koyanagi–Harada disease (VKH), sarcoidosis, and Behçet disease with uveitis. Although UACA is expressed in various tissues such as skeletal muscle and melanocyte, the appearance of anti-UACA antibody seems to reflect the autoimmune reaction against uveal melanocyte [1]. Interestingly, UACA was originally

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identified from dog thyroid as one of TSH regulated genes with unknown function [2]. The dual expression of UACA in thyroid and skeletal muscle led us to the idea that UACA could be an autoantigen associated with Graves' disease, since patients with Graves' disease frequently suffer from ophthalmopathy with ocular myopathy [3–15].

Thyroid-associated ophthalmopathy (TAO) is considered to be an autoimmune disorder of eye muscle and surrounding orbital connective tissue and fat, and the current dogma tells that TAO is induced by autoimmune reaction against thyroid and orbital tissue shared antigens [3–21]. One such candidate is TSH receptor, which is expressed in the orbital preadipocyte and fibroblast [8–11]. Several eye muscle and thyroid shared antigens also have clinical relevance in TAO; flavoprotein [16,17], 1D [18], and G2s protein [22–24]. The primary reaction in ocular tissue is thought to be T-cell-mediated autoimmunity against TSH receptor (TSHR) expressed in ocular fibroblasts. The appearance of antibodies against Fp, G2s, and 1D seems to be the secondary event in TAO process, reflecting the release of sequestered cytoskeletal proteins from damaged eye muscles [23,24]. These eye muscle proteins are expressed in eye muscles as well as skeletal muscles. Since UACA is expressed in skeletal muscle as well as thyroid tissue, we presumed that the appearance of anti-UACA autoantibody could be linked to the autoimmune response associated with Graves' ophthalmopathy.

In this study, we measured serum UACA antibody titer in patients with autoimmune thyroid diseases; healthy controls, Graves' disease, Hashimoto's disease, silent thyroiditis, and subacute thyroiditis. The mean value of UACA antibody titer in the Graves' disease group was significantly higher than healthy controls, but other group was not. Moreover, high UACA titer was observed in Graves' ophthalmopathy patients with severe ocular myopathy.

This is the first report describing the presence of anti-UACA autoantibodies in patients with Graves' disease. Especially, high UACA titer appears to be associated with eye muscle damage of Graves' ophthalmopathy.

Materials and methods

Study patients. We studied 159 Graves' disease, 26 Hashimoto's thyroiditis, 20 silent thyroiditis, 11 subacute thyroiditis, and 43 controls. We explained the purpose of this study to all subjects and obtained their informed consent. Graves' disease patients consisted of untreated 122 females and 37 males. Diagnosis of Graves' disease was confirmed by elevated free T_3 level (13.61 ± 6.67 ng/dl), undetectable TSH level, and positive TSH binding inhibitory immunoglobulin and/or thyroid stimulating antibody. They had hyperthyroidism symptoms such as palpitation and body weight loss. Silent thyroiditis group consisted of 18 females and 2 males with a mean age of 38 year. Diagnosis of silent thyroiditis was confirmed by elevation of free T_3 levels (7.51 ± 2.39 ng/dl), suppressed ^{123}I uptake, and elevated thyroglobulin level. Subacute thyroiditis patients had neck pain and tenderness. They had elevated free T_3 level (8.15 ± 5.48 ng/dl), CRP and ESR level, and suppressed TSH level. Hashimoto's thyroiditis group had elevated TSH levels (69.1 ± 38.2 μU/ml) and positive thyroid TGAb (antithyroglobulin antibody). Forty three normal individuals of similar age and gender were used as controls.

Graves' group included 31 patients with ophthalmopathy (24 females and 8 males, 21–58 years old), 128 patients without ophthalmopathy. The eye changes were classified according to an activity index (AI, 0–7) proposed by a committee of the International Thyroid Associations. Patients with ophthalmopathy were defined as >AI, and patients without ophthalmopathy were defined as A0. Ophthalmologic examination, including measurement of eye muscle function and performance of orbital MRI, was carried out on patients with ophthalmopathy. The congestive changes were defined as >AI, with or without eye muscle involvement. Ocular myopathy was defined as: diplopia and reduced eye movement associated with marked increase of eye muscle volume on orbital MRI. Congestive ophthalmopathy was defined as: nil or minimal eye muscle enlargement with, usually, a fibrotic appearance, as described by Ossoinig [25], features of periorbital inflammation (e.g., chemosis, lid swelling, and conjunctival injection), and no diplopia or reduced eye movements.

Human subjects. Human thyroid tissues were obtained by the University of Tottori committee for the protection of human subjects and in accordance with the Declaration of Helsinki. Thyroid tissue sample was obtained at surgery from a Graves' disease patient. Normal thyroid tissue was obtained at autopsy from a patient without thyroid disease. Human eye muscle tissue with Graves' ophthalmopathy was obtained at surgery from a Graves' disease patient (kindly provided by Dr. Yoichi Inoue, Olympia Eye Hospital).

Preparation of glutathione-S transferase fusion protein. A 783-bp DNA fragment digested from *Homo sapiens* cDNA clone IMAGE 608930 (Embank Accession No. AA197064) corresponding to nucleotide position 3462–4245 of UACA cDNA was inserted into pGEX4T-2 vector to produce glutathione-*S* transferase (GST) UACA fusion protein. This UACA fragment covers C-terminal 261 amino acids (18.0%) of whole UACA consisting of 1449 amino acids. Plasmids with this construct were transformed in *Escherichia coli* and incubated in 500 ml Luria broth medium for 8 h at 37 °C with shaking. Then, IPTG was added at a final concentration of 0.1 mM and the preparation was incubated for 16 h at 25 °C with shaking. This suspension was centrifuged and the pellet was suspended in 20 ml lysis buffer [50 mM Tris–HCl (pH 7.5), 25% sucrose]. Then, we added 100 μl of 10% Nonidet P-40, 1 M $MgCl_2$ on ice. The lysate was sonicated, centrifuged, and then the supernatant was incubated with 2 ml of slurry of glutathione–Sepharose 4B for 2 h at 4 °C. This suspension was centrifuged and the pellet was washed in WE buffer [20 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, and 1 mM DTT] 10 times. The fusion protein was eluted with G buffer [5 mM GSH, 50 mM Tris–HCl (pH 9.6)] and eluted protein concentration was estimated by Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

Enzyme-linked immunosorbent assay. Detection and titration of antibody to a fragment of UACA were examined using indirect enzyme-linked immunosorbent assay (ELISA). GST–UACA fusion protein and GST protein were prepared and used as antigens. Microtiter plates (96-well) (NUNC, Denmark) were coated with GST–UACA fusion protein in PBS (pH 7.4) for 15 h at 4 °C. GST protein was simultaneously coated in different wells as control. The plates were then washed with 5% skim milk/PBS for 2 h at room temperature. The plates were washed with PBS-T and incubated for 15 h at 4 °C with serum samples diluted at 1:50 with 1% skim milk/PBS. The plates were washed in PBS-T, and 100 μl of HRP-conjugated mouse anti-human IgG diluted at 1:2000 with 1% skim milk/PBS was added to each well followed by incubation at room temperature for 2 h. The plates were washed with PBS-T, and 100 μl solution of *o*-phenylenediamine (Sigma Fast; Sigma Chemical, St. Louis, MO) was added to each well. After

30 min, the reaction was stopped by adding 50 μl of 3 M H_2SO_4, and OD 490 nm was determined using a Model 550 microplate reader (Bio-Rad, Hercules, CA). The specific corrected OD value of an individual sample was calculated by subtracting the OD value of GST protein coated well from that of GST–UACA fusion protein.

Cell culture. FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD; ATCC No. CRL 8305) were a fresh subclone (F1) that had all properties previously detailed. All cells were grown in 6H medium consisting of Coon's modified F12 (Sigma Chemical, St. Louis, MO) supplemented with 5% calf serum, 1 mM non-essential amino acids (Gibco, Grand Island, NY), and a mixture of six hormones:bovine TSH (1×10^{-10} M), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). Fresh medium was replaced every 2 or 3 days, and cells were passaged every 7–10 days. In different experiments, cells were maintained in 5H medium without TSH and then exposed to TSH for appropriate time period (0, 3, 6, 12, and 24 h). In dose course analysis, FRTL5 cells were incubated with various concentrations (0, 10^{-3}, 10^{-2}, 10^{-1}, and 1 mU/ml) of TSH for 24 h.

The following human thyroid cancer cell lines were obtained from Dr S. Kosugi (Department of Laboratory Medicine and Clinical Genetics Unit, Kyoto University School of Medicine); NPA [26] and FRO [27] thyroid cancer cell lines were grown in RPMI medium 1640 (31800-022, Gibco-BRL, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. FRO cells, derived from a poorly differentiated follicular thyroid carcinoma, were characterized by the presence of wild-type p53 alleles for exons 5–8 [24]. 8505C [28] and HTC [29] thyroid cancer cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (12800-017, Gibco-BRL, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. Culture medium was changed every 2 days and cells were passaged every 5–6 days.

Western blot analysis. Cells were lysed on ice in 0.6 ml lysate mix containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride in PBS. For immunoblotting, 10 μg of each sample was electrically transferred to Immunobilon PVDF (polyvinylidene difluoride) Transfer Membranes (Millipore, Bedford, MA). Membranes were incubated in blocking buffer; Tris-buffered saline (TBS; Tris-HCl 10 mM, pH 8.0, and NaCl 150 mM) containing 0.05% [vol/vol] Tween 20 and 5% [wt/vol] non-fat dried milk for overnight. Membranes were then incubated in blocking buffer with rabbit polyclonal anti-UACA antibody (kindly provided by Dr. K. Yamada) (1:500 dilution) or goat polyclonal anti-actin antibody (sc-1616, Santa Cruz Biotechnology, USA) for 45 min, and then washed twice with TBS containing 0.05% Tween 20. Membranes were incubated in blocking buffer with horseradish peroxidase-conjugated anti-rabbit IgG antibody for UACA or horseradish peroxidase-conjugated anti-goat IgG antibody (Amersham, UK) for actin, respectively, washed three times with TBS with 0.05% Tween 20, and then detected with enhanced chemiluminescence reagents (Amersham, UK).

Reverse transcription-PCR. Poly(A)$^+$ RNA were purified from 10 μg of each total RNA and subjected to cDNA synthesis, using random primers and Superscript reverse transcriptase. Gene-specific PCR primers were designed to amplify fragments of 505 bp and used in the reverse transcription-PCR (RT-PCR) (94 °C 30 s, 56 °C 30 s, and 74 °C 4 min, 30 cycles). Forward and reverse primer sequences for PCR amplification of UACA were 5′-GAGAAAAGAAGTTGGAATCATAA-3′ and 5′-TTGTGTAGGTGAGTTGGGAAAG-3′, respectively.

Immunohistochemical evaluation of UACA expression. UACA expression was analyzed by immunocytochemical staining of Graves' thyroid tissues, eye muscle tissue obtained from a patient with Graves' ophthalmopathy. We immunostained the ocular tissue including extraocular muscles. Paraffin-embedded tissue section, 4-μm thick, was deparaffinized in xylene, rehydrated through a graded alcohol series to deionized water. The endogenous peroxidase activity was blocked with H_2O_2. The tissue section was incubated with rabbit polyclonal anti-UACA antibody (1:10) for 12 h at 4 °C, then washed and incubated with biotinylated horse anti-rabbit IgG (1:3000) for 30 min at room temperature. The sections were immersed in a solution with the avidin–biotin complex (Vector Laboratories, USA) for 30 min, developed with diaminobenzidine, and counterstained with eosin. The sections were scanned at magnification (200×, 400×) using light microscopy. Normal thyroid sample was obtained at autopsy from a patient without thyroid disease.

Immunofluorescent staining and microscopy. FRTL5 cells were plated on coverslips and cultured in Coon's modification HamF 12 with 5% fetal calf serum, then washed twice with PBS, and fixed with 2% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100, incubated with rabbit polyclonal anti-UACA antibody (1:100), and then visualized using FITC-conjugated anti-rabbit IgG antibody. In order to observe the fine localization of UACA protein within cells, we used confocal microscopy system (FLUOVIEW-OLYMPUS).

Statistical analysis. We used the χ^2 test (with Yeasts' correction for small numbers) and Fisher's exact test for categorical comparisons of the data. Differences in the means of continuous measurements were statistically analyzed using ANOVA. P value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed on a personal computer with the statistical package StatView 5.0 for Macintosh (SAS Institute, Cary, NC).

Results

ELISA

We measured serum UACA antibody titer in patients with autoimmune thyroid diseases in ELISA, using recombinant C-terminal 18% fragment of human UACA protein. We measured titer of healthy controls (43 cases), Graves' disease (159 cases), Hashimoto's disease (26 cases), silent thyroiditis (20 cases), and subacute thyroiditis (11 cases). To exclude the effect of reactivity against GST protein, we used GST–UACA fusion protein and GST protein for ELISA, simultaneously. Evaluation of IgG anti-UACA autoantibodies was determined by subtracting the reactivities against GST from those against GST–UACA. The mean OD value of anti-UACA autoantibodies in Graves' patients was significantly higher than that in healthy controls (ANOVA; $P < 0.01$) (Fig. 1A). This group patient did not accompany VKH disease or other uveitis. In contrast, Hashimoto's thyroiditis, silent thyroiditis, and subacute thyroiditis group did not show any statistical significance compared with control. The cutoff OD value for positivity of anti-UACA IgG antibodies was defined as the mean value +3 SD of healthy controls (0.53). We found anti-UACA IgG antibodies in 15% (24/159) of Graves' patients and 0% (0/43) of healthy control (Table 1). The prevalence of IgG anti-UACA antibodies in Graves' patients was significantly higher than that in healthy control (Fisher's exact test; $P < 0.05$). Anti-UACA antibodies were found in 4% (1/26) of Hashimoto's thyroiditis, 5% (1/20) of silent thyroiditis, and 8% (1/11) of subacute thyroiditis group. The differences in prevalence of anti-UACA antibodies

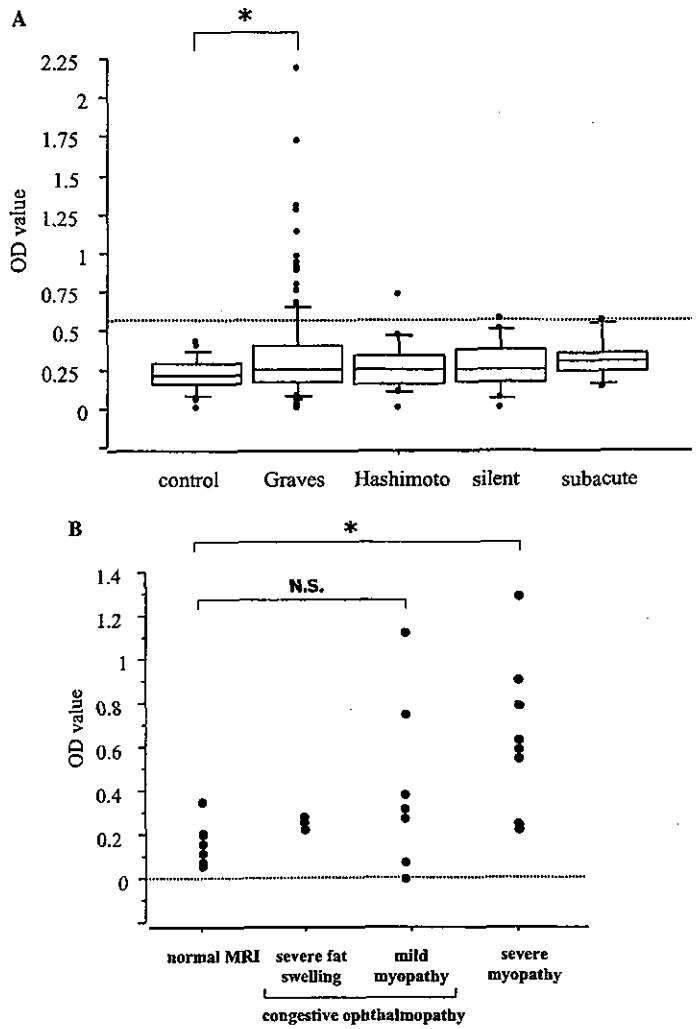

Fig. 1. (A) Distribution of anti-UACA IgG autoantibodies titer in healthy controls (control), in patients with Graves' disease (Graves), Hashimoto's disease (Hashimoto), silent thyroiditis (silent), and subacute thyroiditis (subacute). The titers of autoantibodies are expressed in the OD units. Broken line indicates a cutoff level for the positivity of autoantibody. The OD value subtracted GST protein from GST–UACA fusion protein in 159 Graves' disease samples was 0.339 ± 0.305 (mean ± SE), in 26 Hashimoto's thyroiditis samples was 0.269 ± 0.151 (mean ± SE), in 20 silent thyroiditis samples was 0.269 ± 0.156 (mean ± SE), in 11 subacute thyroiditis samples was 0.315 ± 0.130 (mean ± SE), and in 43 healthy control samples was 0.218 ± 0.103 (mean ± SE). The OD value for GST–UACA fusion protein to GST protein in 27 positive samples was 0.865 ± 0.384 (mean ± SE), and in negative samples was 0.240 ± 0.125 (mean ± SE). *Significant difference ($P < 0.01$) compared with control (ANOVA). (B) Distribution of anti-UACA IgG autoantibodies titer in patients with Graves' ophthalmopathy. The clinical manifestations of Graves' ophthalmopathy are classified into the following four groups; normal MRI, congestive ophthalmopathy with severe fat swelling, congestive ophthalmopathy with mild myopathy, and severe myopathy. The OD value subtracted GST protein from GST–UACA fusion protein in seven normal MRI samples was 0.167 ± 0.098 (mean ± SE), in three congestive ophthalmopathy with severe fat swelling samples was 0.255 ± 0.029 (mean ± SE), in seven congestive ophthalmopathy with mild myopathy samples was 0.418 ± 0.395 (mean ± SE), and in eight severe myopathy samples was 0.656 ± 0.349 (mean ± SE). *Significant difference ($P < 0.005$) compared with normal MRI (ANOVA). NS: not significant.

were not statistically significant between Hashimoto's thyroiditis and healthy control, silent thyroiditis and healthy control, and subacute thyroiditis and healthy control. More than half of Graves' patients with positive titer showed higher titer than patients with VKH disease. Positive patient sera with VKH disease showed about 0.5 OD value in the same ELISA.

Clinical manifestation

We investigated clinical manifestation of Graves' patients with high anti-UACA titer in detail. We found 37.5% (9/24) cases had ophthalmopathy. Especially, patient samples with severe eye muscle inflammation showed high anti-UACA titer. Nine cases with Graves'

Table 1
Prevalence of IgG anti-UACA autoantibodies evaluated by ELISA in sera from patients with thyroid diseases and healthy controls

Disease	Anti-human UACA IgG positive donors		P value
Graves' disease	24/159	15%	$P < 0.01$
Hashimoto's disease	1/26	4%	
Silent thyroiditis	1/20	5%	
Subacute thyroiditis	1/11	9%	
Healthy controls	0/43	0%	

Differences in prevalence of IgG anti-UACA autoantibodies are statistically significant between patients with Graves' disease and healthy controls using Fisher's exact test (2 × 2 table).

ophthalmopathy showed high titer (OD value >0.53) within 31 Graves' ophthalmopathy cases. The prevalence of anti-UACA antibodies was 29% in Graves' ophthalmopathy cases. But, the prevalence of anti-UACA antibodies in patients without ophthalmopathy was 11% (15/128). The prevalence of Graves' ophthalmopathy cases was significantly higher than that of Graves' patients without ophthalmopathy (Fisher's exact test; $P < 0.05$) (Table 2). Within nine cases of high titer, six cases showed severe eye muscle inflammation in MRI study. Graves' ophthalmopathy is classified into the following four groups; severe ocular myopathy, congestive ophthalmopathy with mild myopathy, congestive ophthalmopathy with severe orbital fat swelling and without myopathy, and normal MRI. The prevalence of anti-UACA antibodies was 75% (6/8) in severe ocular myopathy, which had severe eye muscle enlargement and high intensity signal within eye muscle in T2WI MRI study. In contrast, the prevalence of anti-UACA antibodies was 28% (2/7) in congestive ophthalmopathy cases with mild myopathy, who had mild eye muscle enlargement and high intensity signal in T2WI MRI study. The prevalence of anti-UACA antibodies was 0% (0/3) in congestive ophthalmopathy case, who had severe orbital fat swelling, no eye muscle enlargement, and no high intensity in T2WI MRI study. UACA titer of seven patients with normal MRI study was all normal.

The mean UACA titer of severe ocular myopathy cases was significantly higher than that of seven normal

Table 2
Prevalence of IgG anti-UACA autoantibodies evaluated by ELISA in sera from patients with Graves' ophthalmopathy and without Graves' ophthalmopathy

Group	Anti-human UACA IgG positive donors		P value
Graves' ophthalmopathy (+)	9/31	29%	$P < 0.05$
Graves' ophthalmopathy (−)	15/128	11%	

Statistical analyses refer to differences between patients with Graves' ophthalmopathy and without Graves' ophthalmopathy determined using χ^2 test (2 × 2 table, Yeasts' correction for small numbers).

Table 3
Prevalence of IgG anti-UACA autoantibodies evaluated by ELISA in sera from patients with Graves' ophthalmopathy and MRI study

Group	Anti-human UACA IgG positive donors		P value
Severe ocular myopathy	6/8	75%	$P < 0.01$
Congestive ophthalmopathy (with mild myopathy)	2/7	28%	
Congestive ophthalmopathy (with severe orbital fat swelling)	0/3	0%	
Normal MRI	0/7	0%	

Statistical analyses refer to differences between patients with severe ocular myopathy and Normal MRI group determined using Fisher's exact test (2 × 2 table).

cases in MRI study (ANOVA; $P < 0.005$) (Fig. 1B). The prevalence of anti-UACA antibodies was 75% (6/8) in patients with severe ocular myopathy. The prevalence was significantly higher than that of normal MRI group (Fisher's exact test; $P < 0.01$) (Table 3). But, any other group did not show the significant difference compared with normal MRI group.

Expression of UACA in thyroid

To examine mRNA expression of UACA in thyroid, we performed RT-PCR analysis (Fig. 2C). Gene-specific PCR primers were designed to amplify 505 bp fragments of C-terminal portion of human UACA cDNA. The expression of UACA mRNA was observed in all human thyroid cancer cell lines (HTC, 8505C, FRO, and NPA), as well as human thyroid tissue of Graves' disease, Hashimoto's thyroiditis, and normal control. This result indicates UACA mRNA is expressed in human thyroid follicular cells.

To examine the expression profile of UACA protein in FRTL5 cell, we performed Western blot analysis. UACA encoded 160kDa protein (Figs. 2A and B). The amount of UACA protein was augmented in a time (0, 3, 6, 12, and 24h) (Fig. 2A) and dose-dependent manner following TSH stimulation (0, 10^{-3}, 10^{-2}, 10^{-1}, and 1mU/ml) (Fig. 2B). In a time course, UACA protein increased after 3h following TSH stimulation. The strongest signal was observed after 6 or 12h following TSH stimulation, and the signal decreased after 24h following TSH stimulation. In a dose course of TSH, the minimum concentration of TSH to increase UACA protein was 10^{-3}mU/ml.

In order to study the cytochemical localization of UACA, we estimated the expression of UACA in FRTL5 cells. The UACA protein was weakly expressed both in nucleus and cytoplasm of cells in the absence of TSH (Fig. 3A). Interestingly, TSH stimulation recruited UACA into nucleus (Fig. 3A; 24h). In order to observe the fine localization of UACA within TSH-stimulated

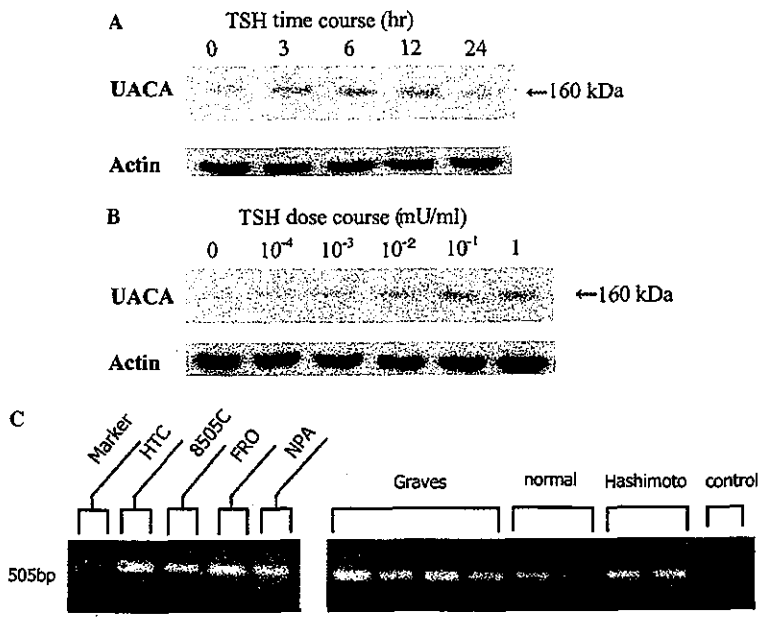

Fig. 2. TSH potentiates UACA protein expression in FRTL5 thyroid cells: (A) time sequence, (B) dose dependency. FRTL5 cells were preincubated in 5H medium with 5% CS for 5–6 days and then incubated with appropriate concentrations of TSH. To ensure the total amount of protein in each lane was identical, membranes were simultaneously incubated with anti-actin antibody (1:250 dilution). (C) RT-PCR analysis using human UACA-specific primers revealed the expression of UACA mRNA in human thyroid cancer cell lines (HTC, 8505C, FRO, and NPA), and the thyroid tissue of Grave's disease, Hashimoto's disease, and normal subject. Control lane indicates PCR product when any template was not included in PCR.

FRTL5, we used a differential interference contrast image. Most of the UACA fluorescence was localized within nucleus, whereas less was localized in cytoplasm (Fig. 3B). To examine UACA expression in human thyroid tissue, we then carried out an immunohistochemical analysis using rabbit polyclonal anti-UACA antibody. In thyroid tissue of Graves' disease, UACA appeared to be expressed in the nucleus of thyroid follicular cells (Fig. 3C).

Expression of UACA protein in eye muscle

In order to investigate the association of Graves' ophthalmopathy and anti-UACA antibody production, we examined UACA expression in human eye muscle derived from a patient with Graves' ophthalmopathy. In human eye muscle tissue with Graves' ophthalmopathy, UACA protein was exclusively expressed in eye muscle fiber (arrow), but UACA expression was relatively weak in surrounding orbital connective tissue and fat (Fig. 3D). The eye muscle sample was derived from a patient with Graves' ophthalmopathy, who was already treated by methimazole and corticosteroid. Since this pretreatment may modify the UACA expression in eye muscle, we simultaneously examined UACA expression in normal rat eye muscle. UACA protein was expressed in normal rat eye muscle fiber as observed in the human sample (data not shown). This result indicates UACA is expressed in eye muscle fiber as well as thyroid cells, which are the autoimmune target tissues in Graves' disease.

Discussion

UACA is a protein cloned by serological analysis of recombinant cDNA expression libraries (SEREX) method with serum samples obtained from patients with VKH disease, to identify the target autoantigens in VKH disease [1]. VKH disease is recognized as an autoimmune systemic disorder. In VKH, inflammatory disorders in multiple organs include melanocytes, uvea (resulting in acute bilateral panuveitis), skin (vitiligo and alopecia), central nervous system (meningitis), and inner ears (hearing loss and tinnitus). These inflammatory aspects are attributed to the immunological destruction of melanocytes. The prevalence of IgG anti-UACA autoantibodies is 19.6% in patients with VKH, and 0% in the healthy controls, 28.1% in patients with Behçet disease, and 21.1% in patients with sarcoidosis, so anti-UACA autoantibodies are considered as one of the autoantibodies in these panuveitis diseases. Originally, UACA was cloned from dog thyroid tissue following TSH stimulation, so we presumed UACA could be a novel candidate of autoantigen in autoimmune thyroid diseases [2]. Then, we analyzed the

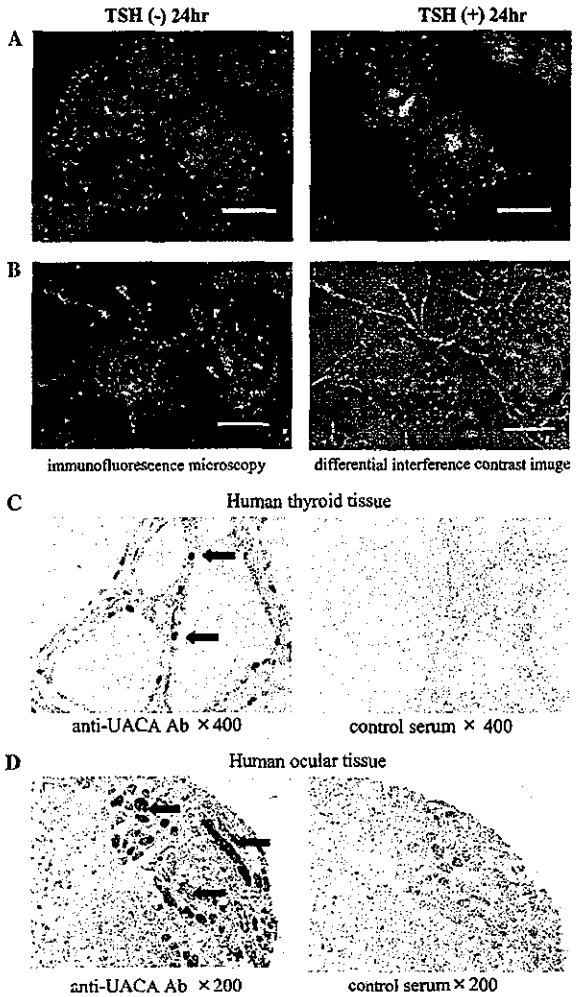

Fig. 3. (A) The cytochemical localization of UACA protein in FRTL5 cells in the absence or presence of TSH (1 mU/ml, 24 h). Inserted bar indicates 10 μm. (B) The fine cytochemical localization of UACA protein in FRTL5 cells followed by TSH stimulation (TSH 1 mU/ml, 24 h). Differential interference contrast image (right panel), and immunofluorescence microscopy image of FRTL5 cells (left panel). Inserted bar indicates10 μm. (C) The expression of UACA protein in human thyroid tissue of Graves' disease. Immunohistochemical analysis was done using rabbit anti-UACA antibodies. The sections were scanned at magnification (400×) using light microscopy. The arrows indicate nucleus of thyroid follicular cell expressing UACA protein. (D) The expression of UACA protein in human eye muscle tissue with Graves' ophthalmopathy. The sections were scanned at magnification (200×) using light microscopy. The arrows indicate eye muscle fibers expressing UACA proteins.

presence of anti-UACA antibodies in autoimmune thyroid diseases.

In ELISA study, the prevalence of anti-UACA antibodies in Graves' disease was significantly higher than that in healthy controls (15% vs. 0%). Moreover, the prevalence of anti-UACA antibodies in Graves' ophthalmopathy was significantly higher than that in Graves' disease without ophthalmopathy (29% vs. 11%). We then investigated clinical manifestation of these patients with high UACA titer in detail. The prevalence of anti-UACA antibodies was 29% (9/31) in Graves' ophthalmopathy cases. These results clearly suggest that the appearance of anti-UACA antibody is strongly associated with eye muscle inflammation in patients with Graves' ophthalmopathy.

Thyroid-associated ophthalmopathy is considered to be an autoimmune disorder of eye muscle and surrounding orbital connective tissue and fat [3–7]. The eye symptoms associated with TAO can be classified into two subtypes, congestive ophthalmopathy (CO), in which inflammatory changes in periorbital tissues predominate, and ocular myopathy (OM), in which eye muscle is mainly damaged [30]. The current dogma tells that TAO is best explained by reactivity against thyroid and orbital tissue shared autoantigens [6,7]. One of such shared antigens is TSH receptor (TSHR), which is expressed in orbital preadipocytes. TSHR is mainly associated with the development of Graves' ophthalmopathy [8–11]. Several shared eye muscle and thyroid autoantigens have been investigated in eye muscle component in TAO, such as 63–67 kDa eye muscle membrane antigens and 55 kDa protein [12–24]. The flavoprotein (Fp) subunit of the mitochondrial enzyme succinate dehydrogenase is the so-called 64-kDa protein. Antibodies against Fp seem to be the best clinical marker of ophthalmopathy in patients with Graves' hyperthyroidism, and they are sensitive predictors for the development of eye muscle dysfunction in ophthalmopathy patients treated by antithyroid drugs [16,17]. The "55-kDa protein" was identified as G2s protein, eye muscle shared autoantigen with unknown function [22–24]. The primary reaction in eye muscle may be T-cell-mediated autoimmunity against TSHR of fibroblasts. The antibodies against Fp and G2s are produced secondary during the ophthalmopathy process, reflecting the release of sequestered cytoskeletal proteins from damaged eye muscles. Our observation indicates that UACA could be a novel candidate for thyroid and orbital shared autoantigen such as Fp in Graves' ophthalmopathy.

We also showed that UACA is expressed in eye muscle of patients with TAO as well as thyroid follicular cells in Graves' disease by immunohistochemical analysis. UACA was highly expressed in human eye muscle fibers of Graves' disease (Fig. 3D). This result indicates that UACA is simultaneously expressed in orbital eye muscle as well as thyroid follicular cells. High prevalence of anti-UACA antibodies is observed in patients with Graves' ophthalmopathy (Fig. 1). In particular, patients with severe ocular myopathy showed high UACA titer. Taken together, we presume that the appearance of anti-UACA antibodies could be a clinical marker for severe ocular myopathy, especially when its titer is high.

Wall and co-workers [16,17] suggested that anti-flavoprotein antibodies are produced by secondary immunoregulatory event resulting from eye muscle necrosis. They also showed that the prevalence of anti-G2s antibodies was 50% in Graves' ophthalmopathy, and anti-G2s antibodies appear in early phase of TAO [22–24]. In our study, the prevalence of anti-UACA antibodies in Graves' ophthalmopathy is lower (29%) compared with anti-G2s antibodies. If UACA is the primary autoantigen in Graves' ophthalmopathy, the prevalence should be higher. It is more likely that anti-UACA Ab is produced by secondary immunoregulatory process resulting from eye muscle necrosis like anti-flavoprotein antibodies. The low prevalence of anti-UACA Ab may raise the possibility that UACA is not relevant for the development of TAO. But, the prevalence of anti-UACA antibodies is only 20% in VKH disease, the original disease with anti-UACA antibodies. Because of the difficulty to produce recombinant UACA protein as a whole molecule (160 kDa), we used the C-terminal 18.0% portion of UACA to detect anti-UACA antibodies in patients' sera. A relatively lower prevalence of anti-UACA antibodies in Graves' patients may be due to the limited usage of C-terminal fragment of UACA protein for ELISA. To evaluate the presence of autoantibody against the whole UACA molecule, it is necessary to analyze patients' sera using other N-terminal fragments of UACA protein.

At present, the recognition of TSHR on the retro-ocular preadipocytes by TSHR autoantibodies and TSHR-specific T cells could be the initial event that drives the "homing" of the lymphocytes to the retro-orbital tissue. Then, the eye muscle inflammation is activated, resulting in the appearance of eye muscle autoantibodies including G2s, Fp, and UACA. Consequently, our observation about anti-UACA Ab is not contrary to this theory explaining the development of TAO. Since UACA is expressed in eye muscle, the appearance of anti-UACA antibodies may reflect immunological damage of eye muscle fiber, as observed in flavoprotein. We presume that not thyroid destruction but eye muscle destruction is directly associated with the production of anti-UACA antibody. If we examine more TAO cases with anti-UACA antibodies, we can identify the clinical relevance of anti-UACA antibodies for the development of TAO.

Although the physiological function of UACA protein is still unclear, UACA contains six ankyrin repeats and coiled coil domains, including a motif of leucine zipper pattern. Ankyrin repeat is 31–33 amino acid motif present in a number of proteins and contributing to protein–protein interactions [31]. In FRTL5 thyroid cells, the amount of UACA protein increased in a time- and dose-dependent manner following TSH stimulation. In the absence of TSH, UACA protein was diffusely distributed both in nucleus and cytoplasm of FRTL5 cells. Following TSH stimulation, UACA protein was exclusively recruited into nucleus of FRTL5 cell (Fig. 3A). Consequently, TSH augments UACA expression and simultaneously converts the localization of UACA within FRTL5 thyroid cells. Interestingly, UACA protein was highly expressed in nucleus of thyroid follicular cell in human thyroid tissue of Graves' disease. These results suggest that UACA protein may play a potential role for thyroid cell proliferation, since TSH drives the growth of thyroid follicular cells. Further study is necessary to reveal the physiological relevance of UACA in thyroid cell proliferation.

In summary, we demonstrate the high prevalence of anti-UACA autoantibodies in patients with Graves' disease. We confirmed that patients with Graves' ophthalmopathy (especially, with severe ocular myopathy) showed high UACA titer. UACA protein is expressed in autoimmune target tissues of Graves' disease, such as thyroid follicular cells and ocular eye muscles, indicating UACA is a novel thyroid–eye shared autoantigen. Although the sequence of autoantibodies production such as anti-G2s, anti-Fp or anti-UACA remains unknown, anti-UACA antibodies could be a clinical marker of ocular myopathy in patients with Graves' ophthalmopathy.

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