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Head and neck cancer antigens recognized by the humoral immune system☆

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Abstract

Head and neck cancer in advanced stages are difficult to treat. Therefore, development of new treatment modalities and preventive measures are required. We now report the identification of human head and neck cancer antigens recognized by the humoral immune system. We used the serological analysis of recombinant cDNA expression libraries (SEREX) approach. cDNA libraries from cell lines of squamous cell carcinoma of head and neck (SCCHN) and a normal testicle tissue were screened using sera from six allogeneic SCCHN patients. Total 28 positive clones belonging to 19 different genes were identified, including 12 known genes and 7 unknown ones. Expression analysis on 13 normal tissues and 13 cancer tissues using reverse transcription-PCR (RT-PCR) revealed eight ubiquitously expressed genes, nine of which were expressed preferentially in cancer tissues and two cancer/testis antigens. These antigens we defined may be pertinent candidate antigens for future cancer-diagnosis and related immunotherapy.

Keywords: Serological analysis of recombinant cDNA expression libraries (SEREX); SCCHN; Cancer/testis antigen; Auto-antigen; Nuclear-antigen

Head and neck cancer has a worldwide incidence of ~400,000 new cases per year. Treatment is usually surgical, often in combination with chemotherapy or radiotherapy, however survival rate of patients with this cancer has not improved in over 30 years, remaining at 50–60% for a 5-year survival rate, despite advances in surgical procedures as well as various combinations of chemotherapeutic agents [1]. So novel and possibly more effective therapeutic approaches are necessary. Immunotherapy, one alternative mode of therapy, has been effective in a small number of patients with metastatic melanoma or renal cell carcinoma. Squamous cell carcinoma of head and neck (SCCHN) is generally infiltrated by T lymphocytes, and cytotoxic T cell lines with a specificity for autologous tumor were obtained from peripheral blood mononuclear cells (PBMC) and tumor infiltrated lymphocytes (TIL) of SCCHN patients [2]. Such being the case, immunotherapy might be an attractive candidate of alternative therapy for SCCHN patients. In comparison to what is known of other tumors, knowledge of tumor immunity directed against head and neck cancer is limited.

For tumor immunotherapy, it is important to identify new tumor-associated antigens. Sahin et al. [3] introduced a new approach for identification of these antigens, and this approach termed serological analysis of

☆ *Abbreviations:* SEREX, serological analysis of recombinant cDNA expression libraries; EST, expressed sequence tag; SCCHN, squamous cell carcinoma of head and neck; RT-PCR, reverse transcription-PCR; CTL, cytotoxic T lymphocytes; CT antigen, cancer/testis antigen; CK1, casein kinase 1; RPA2, replication protein A2; NUP107, nuclear pore complex protein 107; Nrf2, NF-E2 related factor 2; HIV-EP-1, human immunodeficiency virus type 1 enhancer-binding protein 1; UBE3A, ubiquitin-protein ligase E3; PBMC, peripheral blood mononuclear cells; TIL, tumor infiltrated lymphocytes.

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recombinant cDNA expression libraries (SEREX), makes use of diluted sera from cancer patients to detect prokaryotically expressed cDNA libraries prepared from tumor cells. Novel as well as previously defined tumor antigens have been identified using the SEREX method, including MAGE-1 and tyrosinase, both originally identified by expression cloning of epitopes recognized by cytotoxic T lymphocytes (CTL) [4,5].

Cancer germline genes, such as MAGE genes [4], are expressed only in spermatoids/spermatogones and in tumors. As the testis is an immunoprivileged site, these can be considered immunologically tumor specific. Because of their expression pattern, this group of genes has also been named cancer–testis antigens (CT antigens) and these are considered to be ideal targets for tumor-immunotherapy. NY-ESO-1, is one of the CT antigens, originally identified by SEREX in esophageal cancer patient and it is particularly immunogenic, eliciting both cellular and humoral immune responses in a high proportion of patients with advanced NY-ESO-1 expressing tumors [6]. Thus, SEREX can define immunogenic tumor antigens that elicit cellular as well as humoral immunity.

To date, there are two known methods of SEREX, one is using a tumor cDNA library and the other is using a normal testis cDNA library. CT antigens were found using each method [7,8]. In the current study, we applied SEREX analysis on SCCHN using a SCCHN library and a normal testis library.

Materials and methods

Patients and cell lines. Sera obtained from six SCCHN patients were used for SEREX analysis. Five SCCHN and two adenocarcinoma tissues from the head and neck region, two brain tumor tissues, two lung cancer tissues, one gastric cancer tissue, and one pancreatic cancer tissue were from cancer patients taken after a formal signed agreement. Five SCCHN cell lines: HO-1-u-1, SAS, HSC-3, HSC-4 (provided by Cell Resource Center for Bio medical Research Institute of Development, Aging and Cancer, Tohoku University), and Nakamura (provided by Dr. Yumoto, Department of Otolaryngology Head and Neck Surgery, Kumamoto University) were used to construct a cDNA library.

cDNA libraries. The method to construct SCCHN cDNA library has been described elsewhere [9]. In brief, poly(A) RNA was extracted from five SCCHN cell lines. A cDNA library of 1.3×10^6 primary clones was constructed in λ ZAP II phage vector. Normal testis cDNA library was purchased from CLONTECH.

Immunoscreening. The immunoscreening method used was a modification of our published methods [10]. In brief, to remove antibodies reactive with antigens related to the vector system, sera were absorbed by repeated passage through columns of Sepharose 4B coupled with lysates from *Escherichia coli* Y1090 and bacteriophage λ gt 11 (Bio Dynamics Laboratory). Final serum dilution (1:600–1:1200) was prepared in 1% bovine serum albumin/Tris-buffered saline (TBS). Recombinant phages at a concentration of 1×10^4/15cm plate were amplified for 4 h at 42 °C, covered with nitrocellulose filters, "Hybond"-c extra (Amersham, Buckinghamshire, England), pretreated with isopropyl β-D-thiogalactoside (Wako, Osaka, Japan), and incubated for an additional 3 h at 37 °C to transfer encoded proteins onto the filter membranes. Membranes were then blocked with 5% (w/v) nonfat dried milk/TBS for 15 h at 4 °C. Subsequently, membranes were incubated in diluted sera for 2 h at room temperature. After washing with TBS containing 0.05% Tween 20 (TBS-T), the membranes were incubated with horseradish peroxidase (HRP)-conjugated mouse anti-human IgG (Southern Biotechnology Associates, Inc, Birmingham, AL) for 1 h at room temperature. The membranes were washed in TBS-T and TBS and incubated with ECL RPN 2106 (Amersham) for 1 min, then exposed to autoradiographic film to detect antibody-reactive phage plaques. Positive clones were subcloned and retested for serum reactivity, as described above. A total of 5×10^6 recombinant clones were screened.

Sequence analysis of identified genes. Positive clones were subjected to in vivo excision of pBluescript phagemids, using the ExAssist helper phage/SOLR strain system (Stratagene) and of converting λ TriplEx2 phagemid clones to pTriplEx2 plasmid clone, using the BM25.8 strain system (CLONTECH). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, CA). cDNA inserts were sequenced using an ABI Prism (Perkin–Elmer, Norwork, CT) automated DNA sequencer. Sequence alignments were determined using BLAST, UniGene, and LocusLink on NCBI (http://www.ncbi.nlm.nih.gov/), SEREX database (http://www-ludwig.unil.ch/SEREX.html), Blast and Motif database on Genomenet (http://www.genome.ad.jp/), PROSITE database on ExPASy (http://www.expasy.ch/), PSORT website (http://psort.ims.u-tokyo.ac.jp/), and Proteome database (http://www.proteome.com/).

Reverse transcription-PCR (RT-PCR). Total RNA from 13 normal tissues (OriGene, Rockville, MD) and 13 cancer tissues was used (described above). Integrity of RNA was checked by electrophoresis in formalin/ MOPS gels. One microgram of each total RNA sample was subjected to cDNA synthesis, using random hexamer primer and Superscript reverse transcriptase (GIBCO/BRL, Rockville, MD). As a control for genomic DNA contamination, all cDNA synthesis reactions were set up in duplicate with additional samples lacking reverse transcriptase. Gene-specific PCR primers were designed to amplify fragments of 500–1500 bp and used in RT-PCR consisting of initial denaturation at 94 for 5 min, and 28–33 amplification cycles at an annealing temperature of 58–62 °C. KM-HN-1 PCR primer sequences were: sense 5′-CCATCCCAGATACATTCCGAGGAC-3′ and antisense 5′-GGTCGAG-GAAGGACAGTGT-GT-3′. KM-HN-2 PCR primer sequences were: sense 5′-CCATGTGCTAGAGACAGCCAAC AA-3′ and antisense 5′-ATCTTGCATGATCCAGGGAGGTTC-3′. KM-HN-3 PCR primer sequences were: sense 5′-ACCATCTGGCTC CTCATCGTCCTC-3′ and antisense 5′-CCCACCCCAATCATAGG CATAGTC-3′. KM-HN-4 PCR primer sequences were: sense 5′-CGG AGGAGGAACGAAAACGCAGAA-3′ and antisense 5′-TGCTTCC TTGAGAGATGGTCGTTG-3′. KM-HN-5 PCR primer sequences were: sense 5′-ACAGAGGAGCACCAAGGAGAAGAT-3′ and antisense 5′-TCTTCAGGGACTCGGAGGCTTTGC-3′. KM-HN-6 PCR primer sequences were: sense 5′-AAATCAAGAGCCCGAGCCCA GCAC-3′ and antisense 5′-TGAGGGCTGGCTGTTGGCTTTCT-3′. KM-HN-7 PCR primer sequences were: sense 5′-CTCCGCGCCCTG AACTCAATATGT-3′ and antisense 5′-CTTTGGCTTCTTGCTCTT GGAGTG-3′. KM-HN-8 PCR primer sequences were: sense 5′-GAG AGGAAGAAGAAGAGGCAGCAG-3′ and antisense 5′-ACCGCC CCTGGACTACTTATGGAG-3′. KM-HN-9 PCR primer sequences were: sense 5′-AGATGAGGGGAAGATGTCCGTGTC-3′ and antisense 5′-AAGCTCTGTCTGTGGCTCTTGTTC-3′. KM-HN-10 PCR primer sequences were: sense 5′-AGCCTGAGATCACAAAGCCAG TCA-3′ and antisense 5′-CTTCTGTTATACTGTGGGGCTCTG-3′. KM-HN-11 PCR primer sequences were: sense 5′-AGTCAGCGAC GGAAAGAGTATGAG-3′ and antisense 5′-GTGGAGAGGATGC TGCTGAAGGAA-3′. KM-HN-12 PCR primer sequences were: sense 5′-ATCCCACAAAAGATGGCGATGACG-3′ and antisense 5′-TGCTGTGCCGAGGTTTGTAAGAGG-3′. KM-HN-13 PCR primer sequences were: sense 5′-AGACGGGCATGGTGGTACACT

CCT-3′ and antisense 5′-AGGCAGTGTCCCAGGTAGTGTTGC-3′. KM-HN-14 PCR primer sequences were: sense 5′-GTGGCTTTA ATTTTCCCCTCTTGC-3′ and antisense 5′-GTAAAGGACGAGTT GTAAGCTTGG-3′. KM-HN-15 PCR primer sequences were: sense 5′-GTGAAGGGTGCCCCAGCAAATGGA-3′ and antisense 5′-CC TTCCCAACAGCCAGACCAATGA-3′. KM-HN-16 PCR primer sequences were: sense 5′-GGCAGGGATTGGGGAATTTTTCTG-3′ and antisense 5′-CTCAATGGAGCAGTTAGGGCAGAC-3′. KM-HN-17 PCR primer sequences were: sense 5′-TGTGGCCTTGGGC ACGAACTCAGT-3′ and antisense 5′-TGAAGTCGTCGGTGG GGTCGTTGT-3′. KM-HN-18 PCR primer sequences were: sense 5′-ACTTCTGTGTAGCACCTGGGTCAG-3′ and antisense 5′-CAT GGACGGAGGTTGAGATGAAGC-3′. KM-HN-19 PCR primer sequences were: sense 5′-CTGTGATCCTCATCCCTCCAAGAA-3′ and antisense 5′-CACAGACACATCATCAGGGCCTAA-3′. PCR products were visualized by ethidium bromide staining after separation over a 0.8% agarose gel. After normalization by β-actin mRNA as a control, the expression of mRNA in various cancer tissues and normal tissues was compared.

Results

Screening

Approximately 5.0×10^6 recombinant clones of two cDNA libraries derived from SCCHN cell lines and normal testis tissue were screened by six individual sera from patients suffering from SCCHN to identify novel tumor-associated antigens. Twenty-eight positive clones representing 19 different ORFs or genes were detected and further analyzed for molecular distribution. Table 1 summarizes the characteristics of the 19 genes identified based on information derived from the NCBI BLAST, UniGene database, as well as the SEREX database of the Ludwig Institute for Cancer Research.

Functionally unknown genes

Seven out of 19 genes identified by serological screening were functionally unknown (Table 2). Analysis by PSORT predicted that four out of seven gene products localize in nucleus, two in cytoplasm and one in endoplasmic reticulum. KM-HN-1 represented a new gene, which was not found in any databases with the exception of short expressed sequence tag (EST) sequences. Analysis using a protein motif database (MOTIF) revealed that KM-HN-2 contains protein kinase ATP-binding region signature, serine/threonine kinase active site signature, leucine zipper pattern, microbodies C-terminal targeting signal, protein kinase domain, and kinase-associated domain 1; KM-HN-4 contains immunoglobulin domain and glutamic acid rich region; KM-HN-7 contains no motif; KM-HN-8 contains a bipartile nuclear localization signal, a lysine rich region, and a fork head domain; KM-HN-13 contains no motif; KM-HN-17 contains the somatomedin B domain, a Sushi domain, and the von Willebrand factor type D domain.

Already known genes

Twelve genes' products are already characterized (Table 3). KM-HN-3 is homologous to casein kinase 1

Table 1
Clones identified by SEREX and their homologous sequences

Clone	Homology to NCBI BLAST database	Homology to SEREX database	UniGene no.	Chromosome[a]	Library
KM-HN-1	No match	None	None	4	Testis
KM-HN-2	KIAA0175	None	Hs.184339	9p11.2	Testis
KM-HN-3	Casein kinase 1, gamma 1S	None	Hs.157777	15q22.1–q22.31	Testis
KM-HN-4	Homosapiens clone HH409 unknown mRNA	None	Hs.216381	1p31.1	Testis
KM-HN-5	MAGE4a	HOM-Ov1(No. 789)	Hs.37107	Xq28	Testis
KM-HN-6	Replication protein A2	None	Hs.79411	1p35.3	Testis
KM-HN-7	KIAA0729	None	Hs.180948	2	SCCHN
KM-HN-8	DKFZP564M182	NY-LU-5(No.83) and others	Hs.85963	16	SCCHN
KM-HN-9	NFR κB binding protein	90.1/T3(No. 386)	Hs.349688	11q24–25	SCCHN
KM-HN-10	Nuclear pore complex protein	None	Hs.236204	12q14.3	Testis
KM-HN-11	NF-E2-related factor 2	None	Hs.155396	2q31	SCCHN
KM-HN-12	Integrin α6	None	Hs.227730	2p14–q14.3	SCCHN
KM-HN-13	FLJ00016	None	Hs.57100	5q35	SCCHN
KM-HN-14	Ribosomal protein L35a	None	Hs.287361	3q29	Testis
KM-HN-15	Karyopherin (importin) β3	None	Hs.113503	13q32.1–32.3	Testis
KM-HN-16	Mitochondrial ribosomal protein S10	None	Hs.28444	6p21.1–12.1	Testis
KM-HN-17	BK65A6.2 Sushi domain (SCR repeat) containing	None	Hs.131819	22q11–12	SCCHN
KM-HN-18	Human immunodeficiency virus type 1 enhancer-binding protein 1	None	Hs.306	6p24–22.3	SCCHN
KM-HN-19	UBE3A	se-37-2(No.2028) and others	Hs.180686	15q11–13	SCCHN

[a] Chromosome location of SEREX-defined genes was determined using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and UniGene (http://www.ncbi.nlm.nih.gov/UniGene/).

Table 2
Predicted localization and motif search of products of genes of unknown function

Clone	Homology to NCBI BLAST database	Predicted localization[a]	Motif
KM-HN-1	No match	Nucleus (87%)	Leucine zipper
KM-HN-2	KIAA0175	Nucleus (56.5%)	Protein kinase ATP-binding region, serine/threoin kinase active site, leucine zipper
KM-HN-4	Homosapiens clone HH409 unknown mRNA	Nucleus (65.2%)	Immunoglobulin domain
KM-HN-7	KIAA0729	Cytoplasm (52.2%)	None
KM-HN-8	DKFZP564M182	Nucleus (82.6%)	Fork head domain
KM-HN-13	FLJ00016	Endoplasmic reticulum (44.4%)	None
KM-HN-17	BK65A6.2 Sushi domain (SCR repeat) containing	Cytoplasm (26.1%)	Somatomedin B domain, von Willebrand factor type D domain, Sushi domain (SCR repeat)

[a] Localization of these genes' products were predicted using PSORT web site (http://psort.ims.u-tokvo.ac.jp/).

(CK1) γ1S. CK1 is the most abundant serine/threonine protein kinase detected in eukaryotic cell extracts [11,12]. CK1 preferentially phosphorylates acidic proteins such as casein and phosvitin, using ATP. In vertebrates, seven isoforms (α, β, γ1, γ2, γ3, δ, and ε) have been isolated. CK1 γ1 has two known types of cDNA in humans [13]. One of them, named CK1 γ1S is expressed only in testis among normal tissues. The other, named CK1 γ1L, is expressed ubiquitously. KM-HN-5 is homologous to MAGE A4. MAGE A4 is a member of MAGE (Melanoma AntiGEn) gene family. These genes were found to encode tumor-specific antigenic peptides presented by HLA class I molecules to CTL [14]. KM-HN-6 is homologous to replication protein A (RPA) 2. RPA is a trimeric protein complex involved in many cellular processes, including DNA replication initiation and elongation, repair, and recombination. Human RPA is a heterotrimer comprised of Mr70000 (RPA1), Mr29000 (RPA2), and Mr14000 (RPA3) subunits. RPA2, which is a single-stranded DNA binding protein, is a phosphoprotein that becomes differentially phosphorylated throughout the cell cycle. Phosphorylation of RPA2 is first observed at the G1–S transition and is maintained through late mitosis [15].

KM-HN-9 is homologous to NFR κB. NFR κB protein binds preferentially to the related κB site of IL-2R α gene in T cells [16]. KM-HN-10 is homologous to Human Nup 107. Human Nup 107 is a human homolog of rat NUP 107. Rat NUP 107, one of the nuclear pore complex proteins, contains a leucine zipper motif in its C-terminal region. Nuclear pore complex functions in nucleo-cytoplasmic transport. It is a large modular structure composed of spokes, rings, a transporter, fibers, and a nuclear cage. Its mass has been estimated to be 1.25×10^8 Da, and it is composed of an estimated 100 or more distinct proteins [17]. KM-HN-11 is homologous to NF-E2 related factor 2 (Nrf2). Nrf2 was found to bind to the antioxidant response element with high

Table 3
Localization and function of products of known genes

Clone	Homology to NCBI BLAST database	Localization[a]	Function[a]
KM-HN-3	Casein kinase I γ1S	Unknown	Serine/threonin kinase
KM-HN-5	MAGE4a	Unknown	MAGE gene family
KM-HN-6	Replication protein A2	Nucleus	DNA replication, DNA repair, DNA recombination
KM-HN-9	NFR κB binding protein	Nucleus	Transcription factor, DNA binding protein, activator
KM-HN-10	NUP107	Nuclear membrane	Nuclear import/export protein
KM-HN-11	NF-E2-related factor 2	Nucleus	Transcription factor, DNA binding protein
KM-HN-12	Integrin α6	Plasma membrane, integral membrane	Signal transduction, cell adhesion
KM-HN-14	Ribosomal protein L35a	Cytoplasm, ribosome associated	RNA binding protein, ribosomal subunit
KM-HN-15	Karyopherin β3	Nucleus	Nuclear cytoplasmic transport, protein translocation
KM-HN-16	MRP S10	Mitochondria	Catalyze protein synthesis
KM-HN-18	HIV-EP1	Nucleus	Transcription factor, DNA binding protein
KM-HN-19	UBE3A	Cellular protein	Protein modification, protein degradation

[a] Using NCBI LocusLink database (http://www.ncbi.nlm.gov/LocusLink/).

affinity only as a heterodimer with a small Maf protein [18]. Nrf2 is essential for the transcriptional induction of phase II enzymes (such as GST and NAD(P)H) and the presence of a coordinate transcriptional regulatory mechanism for phase II enzyme genes [19]. KM-HN-12 is homologous to integrin alpha 6. Integrins are integral cell-surface proteins composed of an alpha chain and a beta chain. A given chain may combine with multiple partners resulting in different integrins. For example, integrin alpha 6 may combine with beta 4 in the integrin referred to as TSP180, or with beta 1 in the integrin VLA-6. Integrins are known to participate in cell adhesion as well as in cell-surface mediated signaling [20].

KM-HN-14 is homologous to ribosomal protein L35a. Ribosomes, the organelles that catalyze protein synthesis, consist of a small 40S and a large 60S subunit. Together these subunits are composed of four RNA species and approximately 80 structurally distinct proteins. Ribosomal protein L35a is a component of the 60S subunit. The protein belongs to the L35AE family of ribosomal proteins. The rat protein has been shown to bind to both initiator and elongator tRNAs, and thus, it is located at the P site, or P and A sites, of the ribosome [21]. KM-HN-15 is homologous to karyopherin beta 3. Karyopherin (importin) beta family plays a role in nuclear import of proteins. Karyopherin beta 3 is located mainly to the cytosol and the nucleus, particularly the nuclear rim. The rat protein is thought to be a nuclear transport factor that mediates the import of some ribosomal proteins into the nucleus [22]. KM-HN-16 is homologous to mitochondrial ribosomal protein S10. Mammalian mitochondrial ribosomal proteins are encoded by nuclear genes and catalyze protein synthesis within the mitochondrion. Mitochondrial ribosomal protein S10 is in a small 28S subunit of the ribosome, and the function of which is unknown [23]. KM-HN-18 is homologous to HIV-EP1. HIV-EP1 is a C2H2 type zinc finger protein which binds to the DNA κB site present in the long terminal repeat of HIV provirus to activate the HIV-1 gene expression. Inhibition of HIV-EP1 would lead to interference of the replication of HIV [24]. KM-HN-19 is homologous to UBE3A (also designated as E6-AP). UBE3A is a member of a family of putative ubiquitin-protein ligases and has a role in HPV-induced degradation of p53 [25].

RT-PCR analysis

The RNA expression patterns of the identified antigens were analyzed by RT-PCR analysis (Fig. 1 and Table 4). The RNA was derived from 13 cancer tissues and 13 normal tissues. Two genes (KM-HN-1 and -3) were expressed in cancer only, except for normal testis. The expression of three genes (KM-HN-2, -4, and -5) was highly restricted in several tissues among normal tissues. The expression of six genes (KM-HN-6, -7, -8, -9, -10, and -17) was observed in many normal tissues and the expression of eight genes (KM-HN-11, -12, -13, -14, -15, -16, -18, and -19) was observed in almost all the normal tissues investigated (Table 4). KM-HN-1 mRNA was detected in normal testis and 7/13 of cancer tissues. KM-HN-2 mRNA was expressed in normal testis, colon, and several cancer tissues, but expression in the normal colon was very low. KM-HN-3 mRNA was expressed only in testis among normal tissues and expressed in 4/13 cancer tissues (Fig. 1). The level of KM-HN-4 mRNA expression in the normal heart is higher than in the testis. The MAGE-A4 being homologous to KM-HN-5 was thought to be one of the CT antigens, but in our experiments KM-HN-5 mRNA was weakly expressed in normal brain and thyroid carcinoma, and expressed at a high level in normal testis, tongue cancer, and pancreatic adenocarcinoma. KM-HN-6 mRNA was expressed in lung, liver, and testis among normal tissues, and all of cancer tissues. KM-HN-7 mRNA was expressed in heart, lung, liver, and testis among normal tissues, and the expression level in cancer tissues was lower than in normal. KM-HN-8 mRNA was not expressed in SCCHN tissues. KM-HN-9 mRNA was expressed in brain, heart, lung, kidney, and testis among normal tissues and in the tongue submandibular gland, and pancreas among cancer tissues. KM-HN-10 mRNA was expressed in heart, lung, liver, kidney and testis among normal tissues and in tongue and pancreas among cancer tissues. KM-HN-11 mRNA was expressed in all tissues except normal brain. KM-HN-12 mRNA showed higher expression in cancer tissues than in normal tissues. KM-HN-13, -14, -15, and -16 mRNAs were ubiquitously expressed in both normal and cancer tissues. And KM-HN-17, -18, and -19 mRNAs were expressed ubiquitously in normal tissues, but they were not expressed in some cancer tissues.

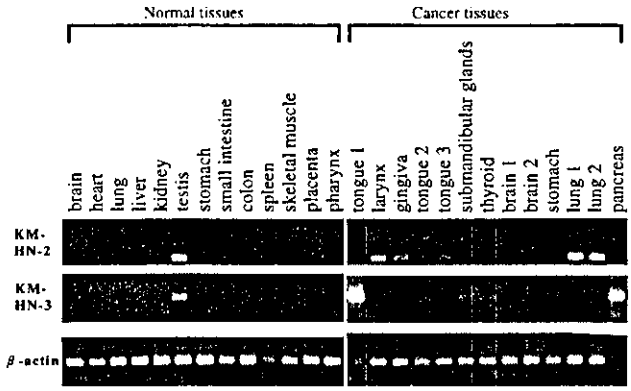

Fig. 1. RT-PCR analyses of KM-HN-2 and KM-HN-3 expression in various normal and cancer tissues. The same cDNA sample were tested for β-actin, as an internal control.

Table 4
Tissue specificity of mRNA expression investigated using RT-PCR of SEREX-defmed genes in various human tissues[a]

	Tissue	Clone no.[b]																		
		HN-1	HN-2	HN-3	HN-4	HN-5	HN-6	HN-7	HN-8	HN-9	HN-10	HN-11	HN-12	HN-13	HN-14	HN-15	HN-16	HN-17	HN-18	HN-19
Normal tissue	Brain	—	—	—	—	+	—	—	+	+	—	—	+	++	++	++	++	+	+	+
	Heart	—	—	—	++	—	—	++	+	+	+	+	++	+	++	+	++	+	+	+
	Lung	—	—	—	—	—	+	++	—	+	+	+	+	+	+	+	+	+++	+	+
	Lever	—	—	—	—	—	+	++	+	++	+	+	+	+	+	++	++	+	+	+
	Kidney	—	—	—	—	—	—	—	—	—	++	+	+	+	++	+	+	++	+	+
	Testis	+	++	+	+	++	++	++	+	+++	++	++	++	+	+	++	++	++	+	+
	Stomach	—	—	—					+			++								
	Small intestine	—	—	—					—			+								
	Colon	—	+	—					—			+								
	Spleen	—	—	—					+			+								
	Skeletal muscle	—	—	—					++			++								
	Placenta	—	—	—					+			+								
	Pharynx	—	—	—					+			+								
Cancer tissue	Tongue 1	+++	—	+++	—	++	++	—	—	+	+	+	+	+	+	+	+	—	+	—
	Tongue 2	+	—	—					—			++								
	Tongue 3	++	+	+					—			++								
	Larynx	—	++	—	—	—	+	+	—	—	—	++	++	+	+	+	+	—	+	+
	Gingiva	—	++	+					—			++								
	Submandibular gland	—	—	—	—	—	+	++	—	++	—	++	++	+	+	++	++	—	—	+
	Thyroid	+	—	—	++	+	++	+	—	—	—	++	++	+	+	++	++	—	—	+
	Brain 1	—	—	—					+			++								
	Brain 2	—	—	—					++			++								
	Stomach	+	—	—					++			++								
	Lung 1	—	+++	—					++			+++								
	Lung 2	+	+++	—					++			+++								
	Pancreas	++	—	++	—	++	++	+	—	+	+	+	+	+	+	+	+	+	++	+

[a] The level of expression was determined by the intensity of ethidium bromide stained RT-PCR products: +++, strong amplification; ++, moderate amplification; +, weak amplification; —, no amplification.

[b] The SEREX-defined genes are listed in Table 1 and "KM-" is deleted from gene designation.

Discussion

Few attempts have been made to induce CTL that recognize SCCHN [26–29]. Therefore, more tumor-associated antigens, expressed in SCCHN and induce specific CTL, need to be identified. We identified 19 SCCHN antigens recognized by IgG class antibodies. Two of these antigens (KM-HN-1 and 3) are expressed in various cancer tissues and normal testis, determined using RT-PCR analysis. So these are candidates of CT antigens recognized by CTL. Further analysis is required to determine if other antigens detected in this study are useful for a serological diagnosis or humoral immunotherapy or not.

In this study, four of seven functionally unknown genes' (KM-HN-1, -2, -4, and -8) products were predicted to locate in the nucleus, using the PSORT web site, and six of 12 known genes' (KM-HN-6, -9, -10, -11, -15, and -18) products were reported to locate in the nucleus (KM-HN-15, -16, -17, -18, -22, and -24). One of the reasons why we identified many nuclear-antigens in this study might be that an excess amount of nuclear proteins that released from apoptotic or necrotic tumor cells provoke humoral immunity.

As for the several known genes identified in the current study, other investigators have reported an elevated level of expression in cancer tissues. MAGE A4 being homologous to KM-HN-5 is one of CT antigens recognized by CTL. It has been reported that MAGE A4 gene is expressed in carcinomas of the oesophagus, head and neck, lung and bladder, in metastatic melanomas, and in the Reed–Sternberg cells of Hodgkin's disease [14]. In this study, we observed expression of this gene in tongue, thyroid, and pancreatic cancers. MAGE-A4 is currently used in clinical trials of therapeutic vaccination against cancer in metastatic melanoma patients. Integrin alpha 6, being homologous to KM-HN-12, beta 4 was identified initially as an antigen associated with carcinoma progression. Van Waes et al. reported that expression of this integrin is associated with the progression of SCCHN [30]. The expression of KM-HN-12 was almost ubiquitous, but the expression was increased in cancer tissues in our study. The expression of ribosomal gene L7a and L35a being homologous to KM-HN-14 is increased in the high grade gliomas. These findings support the recent hypothesis that components of the translational machinery mediate critical alterations in cell growth, proliferation, and in tumor formation. Tumor-specific overexpression of distinct subsets of more than 80 ribosomal proteins necessary for efficient translation has also been noted in colon, liver, breast, and prostate tumors. Ribosomal proteins also possess extraribosomal functions unrelated to protein synthesis [31].

UBE3A being homologous to KM-HN-19 was originally identified in a study examining the association of the human papilloma virus E6 oncoproteins with the tumor suppressor p53. This antigen was also identified by another several SEREX approaches suggesting strong immunogenicity. Furthermore a SEREX study on cutaneous T cell lymphoma revealed that anti-UBE3A antibodies were detected only in cutaneous T cell lymphomas patients' sera, but not in healthy donors [32].

Taken together, the antigens we identified in this study may be of further interest for use as tumor makers for diagnosis, prognostic purpose or for monitoring. Further investigations are awaited to add the new tumor antigens, especially KM-HN-1 and -3, into the growing list of potential targets for immunotherapy.

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Potentiation of Tumor Eradication by Adoptive Immunotherapy with T-cell Receptor Gene-Transduced T-Helper Type 1 Cells

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ABSTRACT

Adoptive immunotherapy using antigen-specific T-helper type 1 (Th1) cells has been considered as a potential strategy for tumor immunotherapy. However, its application to tumor immunotherapy has been hampered by difficulties in expanding tumor-specific Th1 cells from tumor-bearing hosts. Here, we have developed an efficient protocol for preparing mouse antigen-specific Th1 cells from nonspecifically activated Th cells after retroviral transfer of T-cell receptor (TCR)-α and TCR-β genes. We demonstrate that Th1 cells transduced with the TCR-α and -β genes from the I-A^d-restricted ovalbumin (OVA)$_{323-339}$-specific T-cell clone DO11.10 produce IFN-γ but not interleukin-4 in response to stimulation with OVA$_{323-339}$ peptides or A20 B lymphoma (A20-OVA) cells expressing OVA as a model tumor antigen. TCR-transduced Th1 cells also exhibited cytotoxicity against tumor cells in an antigen-specific manner. Moreover, adoptive transfer of TCR-transduced Th1 cells, but not mock-transduced Th1 cells, exhibited potent antitumor activity *in vivo* and, when combined with cyclophosphamide treatment, completely eradicated established tumor masses. Thus, TCR-transduced Th1 cells are a promising alternative for the development of effective adoptive immunotherapies.

INTRODUCTION

T-helper type 1 (Th1)-dominant immunity, which is regulated by interleukin (IL)-12 and IFN-γ, plays a crucial role for the eradication of tumors *in vivo* (1, 2). However, the production of Th1 cytokines such as IL-2 and IFN-γ is markedly suppressed in the majority of tumor-bearing hosts (3, 4). Such defects in Th1-mediated immunity in tumor-bearing hosts have made it difficult to induce tumor-specific CTLs that promote tumor rejection (5). Therefore, it is critically important to develop methods that promote Th1-dominant immunity at the local tumor site.

In a previous study (6), we demonstrated that adoptively transferred Th1 cells exhibit strong antitumor activity *in vivo* and can eradicate an established tumor mass. Moreover, in contrast to Th2 cells, Th1-cell therapy is beneficial for inducing immunological memory in tumor-specific CTLs (6). These findings indicate that Th1-cell therapy is an effective strategy to introduce local T-cell help that overcomes strong immunosuppression in tumor-bearing hosts. However, the application of Th1 cells to adoptive tumor immunotherapy has been hampered by difficulties in inducing sufficient numbers of tumor antigen-specific Th1 cells.

Recently, it has become possible to introduce T-cell receptor (TCR)-α and -β genes into T cells by retroviral transduction (7–10). We used this technology to introduce TCR transgenes specific for the model tumor antigen ovalbumin (OVA) into anti-CD3-activated Th1-polarized CD4$^+$ T cells. We show that TCR-transduced Th1 cells produce IFN-γ but not IL-4 in response to OVA$_{323-339}$ peptides. Moreover, we show that adoptively transferred TCR-transduced Th1 cells, when combined with cyclophosphamide (CY) treatment, can completely eradicate A20-OVA tumor masses. These findings indicate that TCR transduction of nonspecific Th1 cells is an effective strategy for introducing local T-cell help in the tumor-bearing host.

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Charles River Japan (Yokohama, Japan). OVA$_{323-339}$-specific I-A^d-restricted TCR-transgenic mice (DO11.10) maintained on the BALB/c background were kindly donated by Dr. K. M. Murphy (Washington University School of Medicine, St. Louis, MO; Ref. 6). All of the mice were female and were used at 5–6 weeks of age.

Cytokines, mAbs, and Antigen. IL-12 was kindly donated by Genetics Institute (Cambridge, MA). IL-2 was supplied by Takuko Sawada (Shionogi Pharmaceutical Institute Co. Ltd., Osaka, Japan). Anti-IL-4 monoclonal antibody (mAb; 11B11) was purchased from American Type Culture Collection (Manassas, VA). PE-anti-CD4 mAb, FITC-anti-CD45RB mAb, FITC-anti-CD8 mAb, purified anti-CD3 mAb, PerCP-anti-CD3 mAb, FITC-anti-IFN-γ mAb, and anti-IFN-γ mAb (R4–6A2) were purchased from PharMingen (San Diego, CA). KJ1–26 mAb was kindly donated by Dr. K. M. Murphy (Washington University School of Medicine, St. Louis, MO). Recombinant IFN-γ was purchased from Pepro Tech EC Ltd. (London, England). OVA$_{323-339}$ peptide was kindly supplied by Dr. H. Tashiro (Fujiya Co. Ltd., Hadano, Japan).

Generation of OVA-Specific Th1 Cells from DO11.10 Mice. CD4$^+$ CD45RB$^+$ naive T cells were isolated from nylon-passed spleen cells from DO11.10 TCR-transgenic mice using FACSVantage (Becton Dickinson, San Jose, CA) as reported previously (6). Purified CD4$^+$ CD45RB$^+$ cells were stimulated with 10 μg/ml OVA$_{323-339}$ peptide in the presence of mitomycin C-treated BALB/c spleen cells, 20 units/ml IL-12, 1 ng/ml IFN-γ, 50 μg/ml anti-IL-4 mAb, and 20 units/ml IL-2 for Th1 development. At 48 h, cells were restimulated with OVA$_{323-339}$ under the same conditions and were used at 9–12 days of culture.

Construction of Retrovirus Vectors. Complementary DNAs encoding MHC class II-restricted OVA-specific TCR-α and -β genes were amplified from cDNA of CD4$^+$ T cells of DO11.10 mice by reverse transcription-PCR as described previously (7). TCR-α and -β cDNAs were inserted into *Eco*RI and *Not*I sites within the multiple cloning site of the pMX vector, and were designated as pMX-DOTAE and pMX-DOTBE, respectively (7).

Preparation of Retroviruses and Infection of Nonspecific Th1 Cells. Recombinant retroviruses were produced as described previously (7). Briefly, pMX-DOTAE or pMX-DOTBE vector, which were kindly donated by Dr. T. Kitamura (Institute of Medical Science, University of Tokyo, Tokyo, Japan), were transfected into PLAT-E by using FuGENE 6 transfection reagent (Roche, Indianapolis, IN). Twenty-four h later, supernatants were replaced with fresh medium. After incubation for an additional 24 h, supernatants containing the retroviruses were harvested and stored at −80°C until use. Nonspecific Th1 cells were induced from isolated CD4$^+$CD45RB$^+$ naive T cells of BALB/c mice by stimulation with 2 μg/ml anti-CD3 mAb in the presence of mitomycin C-treated BALB/c spleen cells in Th1 cytokine conditions, as described above. Twenty-four h after stimulation, these CD4$^+$ T cells were coinfected by retroviruses carrying pMX-DOTAE and pMX-DOTBE in 12-well plates coated with retronectin and anti-CD3 mAb. This

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infection procedure was carried out three times at 8-h intervals. At 4 days, the expression of DO11.10 TCR was examined by FITC-conjugated KJ1-26 mAb, which is a clonotypic mAb to DO11.10 TCR. These KJ1-26$^+$ cells were isolated with MACS beads (Miltenyi Biotec Inc., Auburn, CA) at over 98% purity and were expanded under Th1 conditions until day 6. More than 90% of Th cells, expanded in this Th1 condition, showed IFN-γ-producing ability but no IL-4-producing ability. As a control, polyclonally activated Th1 cells were transduced with pMX-internal ribosomal entry site (IRES) green fluorescent protein (GFP) vector and used as Mock-GFP gene-transduced Th1 cells.

Flow Cytometry. The phenotypic characterization of Th1 cells was carried out using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and Cell Quest software. Fluorescence data were collected with logarithmic amplification. For each sample, data from 10,000 volume-gated viable cells were collected. Mean fluorescence intensity was calculated using the Cell Quest program.

Intracellular Cytokine Expression. For the detection of cytoplasmic cytokine expression, cells stimulated with immobilized anti-CD3 mAb for 6 h in the presence of brefeldin A were first stained with FITC-conjugated anti-KJ1-26 mAb and PerCP-anti-CD4 mAb, fixed with 4% paraformaldehyde, treated with permeabilizing solution [50 mM NaCl, 5 mM EDTA, 0.02% NaN_3, and 0.5% Triton X-100 (pH 7.5)], and these cells were then stained with PE-conjugated anti-IL-4 mAb or anti-IFN-γ mAb for 45 min on ice. The percentage of cells expressing cytoplasmic IL-4 or IFN-γ was determined by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA).

Cytokine Levels. The levels of IFN-γ or IL-4 in culture supernatants were measured by OptEIA mouse IFN-γ and OptEIA mouse IL-4 (PharMingen), respectively.

Cytotoxicity Assay. The cytotoxicity mediated by Th1 cells was measured by 4-h ^{51}Cr-release assays as described previously (11). Tumor-specific cytotoxicity was determined using A20-OVA (H-2^d) as target cells. As a control, parental A20 cells (H-2^d) were used. The percentage of cytotoxicity was calculated as described previously (11).

Tumor Cell Therapy Using Th1 Cells Combined with CY. A20-OVA cells (2×10^6) were inoculated intradermally into BALB/c mice. When the tumor mass became palpable (6–8 mm), the tumor-bearing mice were treated with none, CY, CY + TCR-transduced Th1 cells, CY + mock-transduced Th1 cells, TCR-transduced Th1 cells, or mock-transduced Th1 cells. Th1 cells (5×10^6 cells/mouse) were i.v. transferred into tumor-bearing mice 1 day after i.p. injection of CY (80 mg/kg). The antitumor activity mediated by the transferred cells was determined by measuring tumor size in perpendicular diameters. Tumor volume was calculated by the following formula: tumor volume = 0.4 × length (mm) × [width (mm)]^2 (12). Tumor-bearing mice that survived for more than 60 days after therapy were considered completely cured. The mean of five mice per group is indicated in graphs.

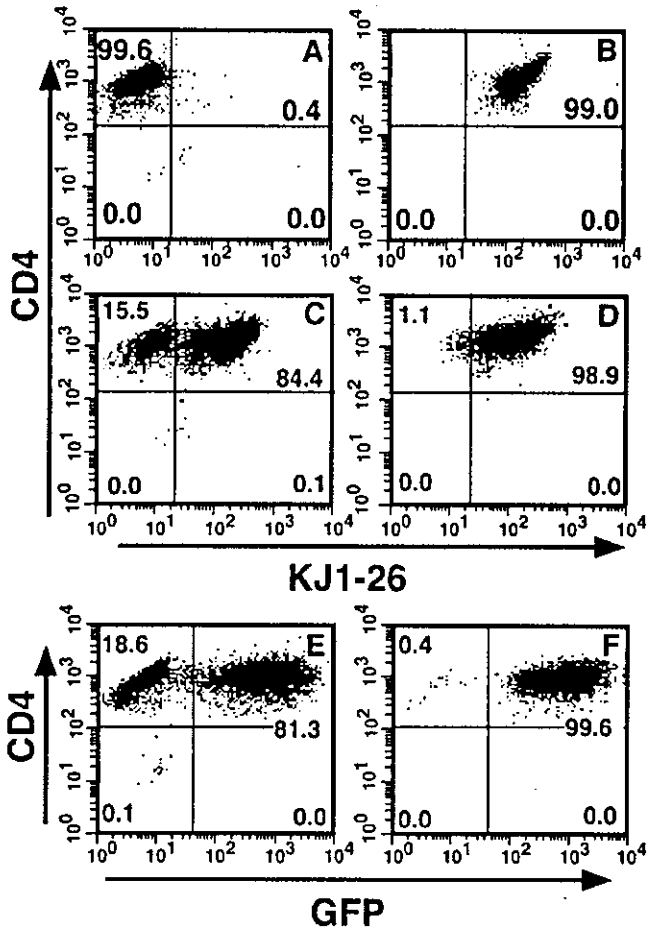

Fig. 1. Retroviral transfer of DO11.10 T-cell receptor (TCR)-α and -β chain genes into anti-CD3 monoclonal antibody (mAb)-activated nonspecific T-cell helper (Th) cells. Naïve Th cells prepared from BALB/c mice were activated with anti-CD3 mAb for 24 h, and these cells were then retrovirally transduced with DO11.10 TCR genes (*C, D*) or with a mock gene containing green fluorescent protein (*GFP; E, F*), and expanded under Th type 1 (Th1)-inducing conditions, as described in "Materials and Methods." Four days after initiation of the culture, expression of the DO11.10 TCR recognized by KJ1-26 clonotypic mAb (*C*) or of the mock gene labeled with GFP (*E*) was determined by flow cytometry. Then, gene-transduced KJ1-26$^+$ Th (*D*) or GFP$^+$ Th cells (*F*) were isolated by MACS. As negative and positive controls for DO11.10 TCR expression, anti-CD3 mAb-activated Th cells derived from wild-type BALB/c mice (*A*), and Th1 cells derived from DO11.10 TCR transgenic mice (*B*) were used, respectively. Similar data were obtained in three independent experiments.

RESULTS AND DISCUSSION

We have previously developed an adoptive tumor immunotherapy protocol, using Th1-polarized TCR transgenic T cells specific for the model tumor antigen ovalbumin (OVA) presented by I-A^d. To evaluate the utility of retroviral gene transduction as a tool for generating tumor antigen-specific Th1 cells, we introduced the TCR-α and -β chain genes from the DO11.10 T-cell clone into polyclonally activated T cells by retroviral vectors. Specifically, we isolated CD4$^+$CD45RB$^+$ naive CD4$^+$ T cells from wild-type BALB/c mouse spleen cells and activated these cells with anti-CD3 mAb under Th1-inducing conditions (IL-12 + IFN-γ + anti-IL-4 mAb) in the presence of mitomycin C-treated spleen cells as antigen presenting cells, which is essential for the cross-linking of soluble anti-CD3 mAbs. After 24 h of culture, the cells were retrovirally transduced with DO11.10 TCR-α and -β genes or a mock-GFP gene (three successive gene transductons 8 h apart). TCR- or mock-transduced Th cells were cultured for another 3 days in Th1-inducing conditions. Then, the expression of DO11.10 TCR on expanded cells was examined by flow cytometry. TCR expression was evaluated with the clonotypic KJ1-26 mAb, which stains most CD4$^+$ T cells in DO11.10 transgenic mice (Fig. 1*B*), but very few T cells from normal mice (Fig. 1*A*). The majority (84%) of anti-CD3-activated CD4$^+$ T cells successfully expressed DO11.10 TCR detected by KJ1-26 mAb (Fig. 1*C*) or a mock gene detected by GFP expression (Fig. 1*E*) after retroviral transduction.

To investigate the function of DO11.10 TCR-transduced Th cells, KJ1-26-expressing cells were enriched by MACS beads (Fig. 1*D*). As a control, GFP-expressing Th cells were isolated by fluorescence-activated cell sorting (Fig. 1*F*). As shown in Fig. 1, *D* and *F*, we could prepare retrovirally modified Th cells expressing DO11.10 TCR and mock-GFP Th cells that were >98% pure. We examined the cytokine producing ability of DO11.10 TCR gene-transduced Th cells by intracellular staining after stimulation with the cognate I-A^d-binding $OVA_{323-339}$ peptide. As shown in Fig. 2, *B* and *F*, DO11.10 TCR-transduced Th cells detected by KJ1-26 mAb produced IFN-γ but not IL-4 in response to OVA-peptide. In sharp contrast, mock-GFP-transduced Th1 cells produced neither IFN-γ nor IL-4 in response to stimulation with $OVA_{323-339}$ (Fig. 2, *D* and *H*). We further demonstrated that DO11.10 TCR-transduced Th1 cells exhibit high levels of

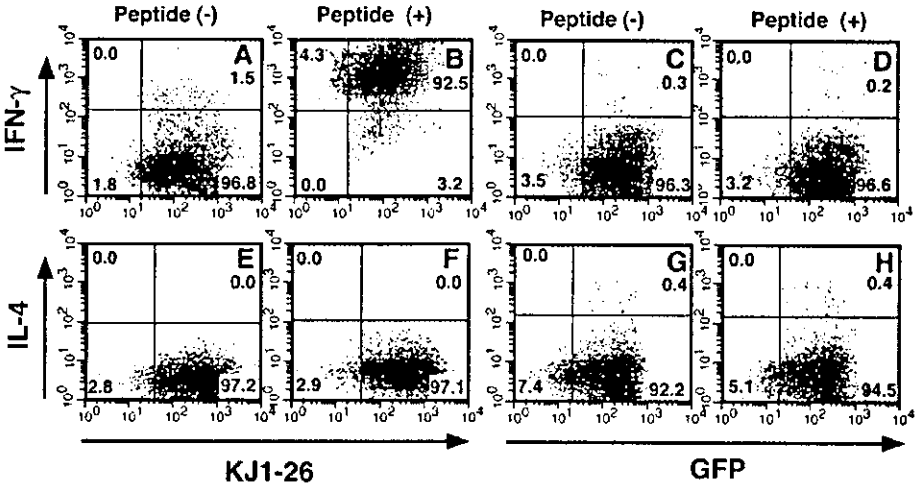

Fig. 2. DO11.10 T-cell receptor (TCR)-transduced T-helper type 1 (Th1) cells produce IFN-γ but not interleukin (IL)-4 in response to stimulation with the I-A^d-restricted ovalbumin $(OVA)_{323-339}$ peptide. Th cells were transduced with DO11.10 TCR genes or with a mock green fluorescent protein (GFP) gene and induced into Th1 cells, as described in "Materials and Methods." After culture for 7 days, KJ1-26$^+$ TCR-transduced Th1 cells (*A, B, E,* and *F*) and GFP$^+$ mock gene-transduced Th1 cells (*C, D, G,* and *F*) were stimulated with (*B, D, F,* and *H*) or without (*A, C, E,* and *G*) $OVA_{323-339}$ peptide. After 24 h of culture, cytokine-producing ability of cells was determined by intracellular staining: IFN-γ producing ability of TCR-transduced Th1 cells (*A, B*) or mock gene-transduced Th1 cells (*C, D*); IL-4 producing ability of TCR-transduced Th1 cells (*E, F*) or mock gene-transduced Th1 cells (*G, H*). The figures in data represent the percentage of cytokine-producing cells. Similar results were obtained in three separate experiments.

IFN-γ but not IL-4 production in response to stimulation with OVA-peptide-pulsed A20 tumor cells (Fig. 3, *A* and *B*). In addition to cytokine production, TCR-transduced Th1 cells, but not mock-transduced Th1 cells, exhibited strong cytotoxicity against antigenic peptide-pulsed A20 cells (Fig. 3*C*). When compared with Th1-polarized DO11.10 cells (DO11.10-Th1) from transgenic animals, the response of TCR-transduced Th1 cells to the cognate OVA peptide was lower than DO11.10-Th1 cells, although they exhibited a very similar response at a higher dose (2 μg/ml) of peptide (Fig. 3*D*). Such different responsiveness was also demonstrated when we used A20-OVA tumor stimulator, which exhibited lower stimulation activity compared with peptide-pulsed A20 tumor cells. As shown in Fig. 3, *E* and *F*, TCR-transduced Th1 cells showed lower IFN-γ production and cytotoxicity in response to A20-OVA tumor compared with DO11.10-Th1 cells. As shown in Fig. 1*D*, the expression intensity of genetically modified TCR is always lower than that of physiologically expressing DO11.10 TCR (Fig. 1*B*). Therefore, the lower responsiveness of TCR-transduced Th1 cells may be derived from lower expression intensity or affinity of genetically modified TCR compared with physiologically expressing TCR. In contrast to TCR-transduced Th1 cells, mock-transduced Th1 cells exhibited no significant IFN-γ production and cytotoxicity by stimulation with I-A^d-binding OVA-peptide (Fig. 3, *A–C*).

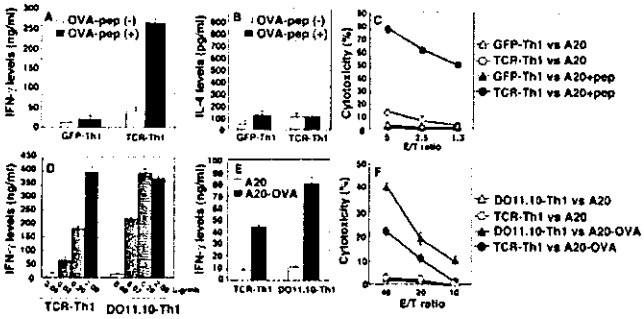

Fig. 3. T-cell receptor (TCR)-transduced T-helper type 1 (Th1) cells demonstrate both IFN-γ production and cytotoxicity in response to ovalbumin $(OVA)_{323-339}$ peptide-pulsed A20 tumor cells and A20-OVA tumor cells. Th1 cells transduced with DO11.10 TCR genes were induced as described in "Materials and Methods." After culture for 7 days, KJ1-26$^+$ TCR-transduced Th1 cells (*TCR-Th1*) were harvested and cultured with (■) or without (□) $OVA_{323-339}$ peptide [with, *OVA-pep (+)*; without, *OVA-pep (−)*] in the presence of mitomycin C-treated spleen cells to determine their ability to produce IFN-γ (*A*) or interleukin (IL)-4 (*B*). As negative control cells, Th1 cells transduced with a mock green fluorescent protein (GFP) gene (*A, B*; *GFP-Th1*) were used. *C*, cytotoxic activity of TCR-Th1 (○, ●) and GFP-Th1 cells (△, ▲) against unpulsed A20 tumor cells (○, △) or $OVA_{323-339}$ peptide-pulsed A20 tumor cells (●, ▲) was measured by 4-h ^{51}Cr-release assay. *D*, TCR-Th1 or Th1 cells induced from DO11.10 transgenic mice (DO11.10-Th1) were stimulated with three different doses of peptide (0, □; 0.02,[gbox]; 0.2,[rhbox]; 2 μg/ml, ■). After 24 h, IFN-γ levels of culture supernatants were measured by ELISA. *E*, IFN-γ-producing ability of TCR-Th1 or DO11.10-Th1 by stimulation with A20-OVA (■) or A20 (□) tumor cells. *F*, cytotoxicity of TCR-Th1 (○, ●) or DO11.10-Th1 (△, ▲) against A20-OVA (●, ▲) or A20 (○, △) tumor cells was measured by 4-h ^{51}Cr-release assay. The *bars*, mean ± SE of triplicate samples. Similar results were obtained in three separate experiments.

Finally, we investigated the therapeutic activity of TCR-transduced Th1 cells against tumor-bearing mice. BALB/c mice were inoculated with A20-OVA tumor cells and, when the tumor mass became palpable (6–8 mm), TCR-transduced Th1, mock-transduced Th1 cells, or DO11.10-derived Th1 cells were i.v. transferred. Consistent with previous results (6), transfer of 2×10^7 DO11.10-derived Th1 cells induced complete cure of all tumor-bearing mice. However, neither TCR-transduced Th1 cells nor mock-transduced Th1 cells exhibited significant antitumor activity *in vivo*, even when 2×10^7 cells were transferred (Fig. 4*A*). This lower antitumor activity of TCR-transduced Th1 cells might be due to lower intensity or affinity of genetically modified TCR as described above. Alternatively, down-modulation of retrovirally transduced TCR expression by transcriptional silencing mechanisms (13) might influence the antitumor activity of TCR-transduced Th1 cells *in vivo*. As shown in Fig. 4*B*, we found that the therapeutic efficacy of Th1 cells was augmented by combination therapy with CY. Namely, the transfer of 5×10^6 DO11.10-Th1 cells combined with CY pretreatment caused complete rejection of tumor, although Th1 cells alone exhibited no significant antitumor activity. Such potentiation by CY treatment was observed even when tumor-bearing mice were transferred with 2×10^6 Th1 cells.[4] Therefore, we examined whether therapeutic efficacy of TCR-transduced Th1 cells could be potentiated by combination with CY treatment. Neither TCR-transduced Th1 cells (5×10^6) nor mock-transduced Th1 cells showed significant antitumor activity *in vivo*. However, when tumor-bearing mice were pretreated with CY (80 mg/kg) 1 day before the transfer of TCR-transduced Th1 cells (5×10^6), tumor growth was strongly inhibited (Fig. 4*C*). All of the mice treated with CY plus TCR-transduced Th1 cells were completely cured from tumors (Fig. 4*D*). Such potentiation by CY was not observed when 2×10^6 TCR-transduced Th1 cells were transferred into tumor-bearing mice. Therefore, physiological DO11.10 TCR-expressing Th1 cells appeared to exhibit >2-fold stronger antitumor activity *in vivo* compared with TCR-transduced Th1 cells. Thus, combined therapy with

[4] Unpublished data.

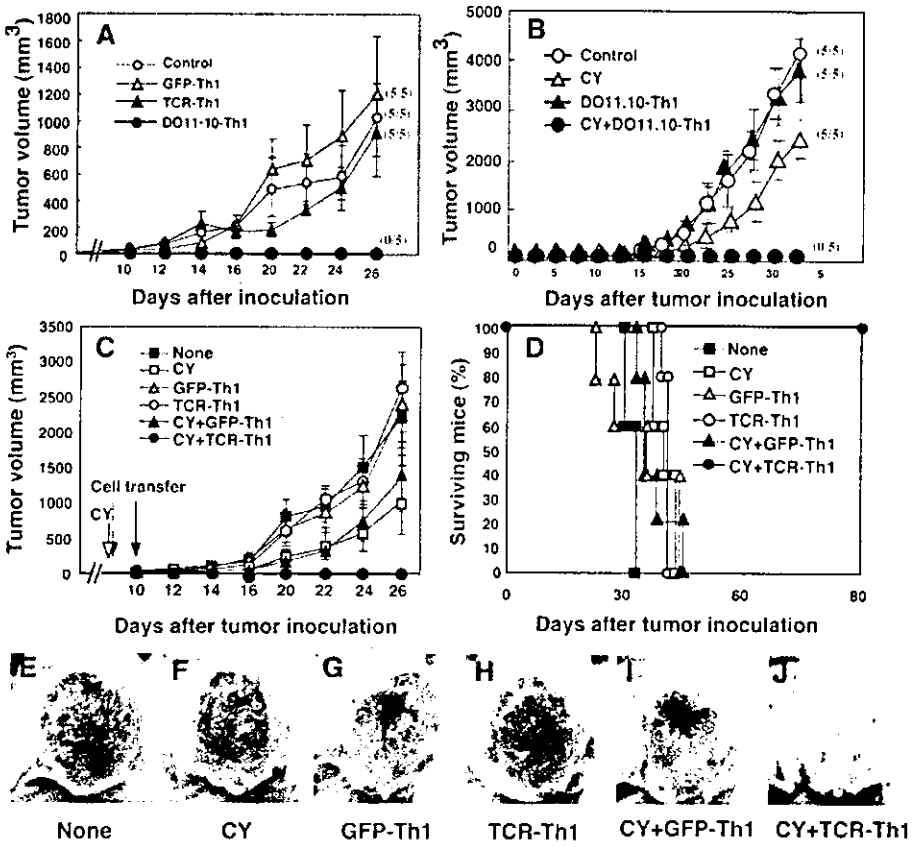

Fig. 4. T-cell receptor (*TCR*)-transduced artificial T-helper type 1 (*Th1*) cells, in combination with cyclophosphamide (*CY*) treatment, eradicate established tumors. A20-OVA cells (2×10^6) were intradermally inoculated into BALB/c mice. When the tumor mass became palpable (6–8 mm), the tumor-bearing mice were treated with various protocols. *A*, tumor-bearing mice were treated with none (○) or cell transfer (2×10^7/mouse) of DO11.10-Th1 (●), TCR-Th1 (▲) or green fluorescent protein (*GFP*)-Th1 cells (△). *B*, tumor-bearing mice were transferred with none (○), CY (△), DO11.10-Th1 (▲) or CY plus DO11.10-Th1 (●). Th1 cells (5×10^6) were transferred into tumor-bearing mice 24 h after CY (80 mg/kg) treatment. *C–J*, tumor-bearing mice were treated with none (■ in *C* and *D*; *photo E*), CY (□ in *C* and *D*; *photo F*), mock GFP-transduced Th1 cells (△ in C and D; photo G), TCR-transduced Th1 cells (○ in *C* and *D*; *photo H*), CY + mock GFP-transduced Th1 cells (▲ in *C* and *D*; *photo I*) or CY + TCR-Th1 cells (● in *C* and *D*; *photo J*). In experiments shown in *C–D*, Th1 cells (5×10^6 cells/mouse) were i.v. transferred into tumor-bearing mice 1 day after i.p. injection of CY (80 mg/kg). The antitumor activity mediated by the transferred cells was determined by measuring tumor size in perpendicular diameters. Tumor volume was calculated as described in "Materials and Methods." The *fractional numbers in parentheses* in *A* and *B*, dead mice/total mice within 60 days after tumor inoculation. The *bars*, mean ± SE of five mice in each experimental group. Similar results were obtained in three separate experiments.

CY might be better to induce the efficient therapeutic effect of genetically modified Th1 cells, although the transfer of large numbers of TCR-transduced Th1 cells might be effective. CY treatment alone or combined therapy with mock-transduced Th1 cells and CY failed to cure tumor-bearing mice, although these treatments showed a slight inhibition of tumor growth *in vivo*. Such antitumor activity by combination therapy with TCR-transduced Th1 cells and CY was not observed in A20 tumor-bearing mice (data not shown). Thus, these results clearly demonstrate that TCR-transduced Th1 cells induced from nonspecific Th1 cells exhibit specific antitumor activity *in vivo*. That combination therapy with TCR-transduced Th1 and CY can induce immunological memory *in vivo* was indicated by the fact that (*a*) all cured mice by treatment with TCR-transduced Th1 cells and CY could reject rechallenged A20-OVA tumor cells and (*b*) $CD8^+$ and $CD4^+$ CTLs were demonstrated in cured mice.[4]

Recently, $CD4^+$ T cells were demonstrated to play a crucial role not only in the induction of $CD8^+$ CTLs via dendritic cell activation (14–16) but also for maintaining the number and function of CTLs (14–18). Coadministration of $CD4^+$ T cells with $CD8^+$ T cells resulted in increased CTL activity and enhanced infiltration of these cells into tumors; both facilitate CTL-mediated tumor eradication *in vivo* (19). The rationale for introducing $CD4^+$ T-cell help for promoting antitumor immunity is supported by the fact that $CD4^+$ T cells augment the therapeutic effect of tumor vaccines that are based on MHC class I-binding peptides or dendritic cells (19). An important role for $CD4^+$ T-cell help has also been reported in tumor chemotherapy using CY (20). CY, in addition to its direct antitumoricidal activities, appears to act as an immunomodulator that facilitates the generation of antitumor effector T cells combined with $CD4^+$ T-cell help.

In a prior report (6), we demonstrated that adoptive cell transfer of a large number ($>2 \times 10^7$ cells) of Th1 cells is beneficial for inducing tumor-specific CTLs *in vivo* and for curing mice of established tumors. However, adoptive transfer of $<10^7$ Th1 cells was insufficient to cure tumor-bearing mice. This Th1-dependent antitumor immunity is completely dependent on the generation of host-derived tumor-specific CTL, because Th1 cell-therapy was unable to eradicate tumors in RAG-$2^{-/-}$ mice unless $CD8^+$ T cells were cotransferred. Therefore, when adoptive immunotherapy using Th1 cells was combined with dendritic cell-based tumor vaccine therapy that promotes CTL generation, the number of required Th1 cells decreased to 5×10^6 cells per mouse (19). Here, we further demonstrate that the therapeutic effect of CY is greatly augmented by combination therapy with a small number (5×10^6) of TCR-transduced Th1 cells as well as DO11.10-derived Th1 cells (Fig. 4). The immunopotentiating mechanisms of CY remains unclear, but CY treatment appeared to enhance the migration of Th1 cells into tumor local site including draining lymph node.[4] Then, the transferred Th1 cells exhibited proliferation and cytokine production by interacting with APC, which processed antigen of tumor cells destroyed by CY and/or Th1 cells. Thus, transferred Th1 cells introduce tumor local help that is essential for generating tumor-specific CTLs to eradicate established tumor.

The strategy used here for promoting tumor antigen-specific Th1 responses holds significant promise for tumor immunotherapy in human patients. A number of human tumor-antigen-specific T cells have been established. For example, several Th clones reactive against the class II-binding tumor rejection antigen peptide have been established (21–24). We have already cloned the TCR genes from a tumor-specific Th cell clone and introduced the TCR gene into polyclonally activated human T cells. These transduced T cells were able to produce cytokines in response to the relevant antigen.[4] These findings illustrate the feasibility of applying antigen-specific Th1 cells

to adoptive tumor immunotherapy, either by inducing tumor-specific Th1 cells from patient samples or by TCR transduction, as described here.

Th1-dominant immunity plays a critical role in the induction of antitumor immunity at the local tumor site via multiple helper functions such as their capacity to enhance CTL priming of APC and to promote migration, proliferation, and cytotoxicity of tumor-specific CTLs (14–19, 25–27). Therefore, adoptive immunotherapy using tumor-specific Th1 cells combined with other therapeutic modalities such as (*a*) dendritic cell-based tumor vaccine therapy; (*b*) $CD8^+$ T-cell therapy; (*c*) chemotherapy; or (*d*) radiation therapy (28, 29), may provide a more efficient strategy for tumor immunotherapy. Our protocol for inducing TCR-transduced tumor-specific Th1 cells should enable us to prepare tumor-specific Th1 cells even when tumor-specific Th1 cells from the patient cannot be obtained. This strategy should prove valuable for developing effective tumor immunotherapies. For application to clinical trial, the use of TCR-transduced Th1 cells comodified with thymidine kinase (TK) gene might be better strategy to prevent unwanted immune disorders induced by long-lived transferred Th1 cells (30).

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Aire downregulates multiple molecules that have contradicting immune-enhancing and immune-suppressive functions

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Abstract

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a systemic disease with autoimmune characteristics caused by mutations in a single gene called *AIRE*. Although a defect in negative selection has been emphasized for the pathogenesis of the autoimmune symptoms on the basis of studies of *Aire*-targeted mice, the function of the gene in the peripheral immune system and the cause of immunodeficiency noted in the disease have not been clarified yet. In this study, we demonstrated using murine *Aire* transfectants that Aire downregulates IL-1 receptor antagonist (IL-1Ra), which is important for immune suppression, and major histocompatibility complex (MHC) class II molecules, which are critical for acquired immunity. It was surprising to learn that Aire, which has been supposed to positively regulate transcription, downregulates multiple molecules. This downregulation of IL-1Ra and MHC class II molecules seems to be caused by the competition for transcriptional coactivator, CREB-binding protein (CBP), and may explain part of the contradictory (i.e., both autoimmune and immunodeficient) nature of APECED.

Keywords: AIRE; APECED; IL-1 receptor antagonist; MHC class II molecule; CREB-binding protein

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED; OMIM 240300) or autoimmune polyglandular syndrome type I (APS I) is an autosomal recessive disease characterized by multiple organ damage probably caused by autoimmune mechanisms [1]. The damage mainly affects endocrine organs and results in pathological conditions, such as hypoparathyroidism, adrenocortical failure, diabetes mellitus, gonadal failure, pernicious anemia, hypothyroidism, and hepatitis. Concurrently, this disease has an immunodeficiency aspect, chronic mucocutaneous candidiasis.

This disease is caused by mutations of a single gene, called *AIRE* (autoimmune regulator), on chromosome 21 [2–4]. Finns, Sardinians, and Iranian Jews were reported to have a high prevalence of the disease [1,5,6]. The protein coded by this gene has a nuclear localization signal (NLS), an HSR dimerization domain, a SAND domain, four LXXLL motifs, two PHD-type zinc-finger motifs, and a proline-rich region, suggesting that the protein can function as a transcription factor [7,8]. Indeed, AIRE exhibits a transcriptional activity [9,10].

The expression of AIRE is restricted to certain limited cells, such as thymic medullary epithelial cells, peripheral macrophages, and dendritic cells [11,12]. Recently, the failure to delete some organ-specific T cells in the thymus (negative selection) has been claimed to be

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the main cause of the disease ([13,14], see Discussion). However, the target genes of AIRE in the peripheral immune system and the mechanism(s) of immunodeficiency are almost unknown. In this study, we analyzed the target genes of murine Aire using stable transfectants and investigated the mechanisms underlying the disease.

Materials and methods

Cells and reagents. Raw 264.7 cells were kindly provided by Dr. Hiroshi Takayanagi. Lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 was purchased from Sigma. Recombinant mouse interferon-γ (IFN-γ) was purchased from Genzyme/Techne. FITC-labeled mAb against MHC class II molecule (AMS-32.1) was purchased from BD PharMingen. Polyclonal Ab against murine CBP (A-22) was purchased from Santa Cruz Biotechnology. CBP-expressing vector was kindly provided by Dr. Akiyoshi Fukamizu [15].

Production of stable transfectants. Mouse Aire cDNA was obtained by RT-PCR using total RNA from the mouse spleen. The primers used were as follows.

Sense: 5′-TCTAGACAGCCCCTGTGAGGAAGATGGCAGGT GGGG-3′

Antisense: 5′-TCTAGAGTCATCAGGAAGAGAAGGGTGGT GTCTCGG-3′ (mouse *Aire* gene, Accession No. AJ243821)

The subcloned genes were sequenced and one clone with no mutation was selected. It was excised from its host plasmid by *Xba*I restriction and was religated into the *Xba*I site of the pBK-CMV phagemid vector (Stratagene). A mutant Aire-expressing vector (Aire(1–293)) was constructed from this vector by PCR [16] using the following primers.

Sense: 5′-TGATGACTCTAGAGCGGCCG-3′

Antisense: 5′-AACCTGGGGCTCACTGGGGA-3′

Raw 264.7 cells were transfected with the Aire-expressing vector and control empty vector using FuGENE 6 (Roche) according to the manufacturer's instructions. Stable transfectants were selected using G418 (500 μg/ml, Invitrogen) for 2 weeks until individual colonies were formed on the plate. They were used either as clones or in bulk.

Ribonuclease protection assay. RiboQuant RNase Protection Assay Systems (BD Biosciences) were used according to the manufacturer's instructions. The mouse cytokine/chemokine sets, mCK-2b and mCK-5, were used.

Luciferase assay. Genomic DNA corresponding to the 5′ upstream region of the mouse *IL-1Ra* gene (Accession Nos. L32838 and AL732528) was cloned by RT-PCR, using the primers listed below.

Sense (1): 5′-GAGCTCCACTTTTCCATGATTTGGG-3′

Sense (2): 5′-GAGCTCTAGGGCAGAGGTCAGCA-3′

Sense (3): 5′-GAGCTCGGGACAGGGGGATCAGCA-3′

Sense (4): 5′-GAGCTCTCTGACAGTGGAACGGAATG-3′

Antisense: 5′-AAGCTTCCGAGGCCACAGCAAGGCAA-3′

The four sequences were excised from its host plasmid by *Hin*dIII and *Sac*I restriction sites and were religated into the gap between the *Hin*dIII and *Sac*I sites of the Picagene luciferase reporter plasmid (Wako Pure Chemical). A mutant reporter construct was generated in the same way as the mutant Aire(1–293) vector. The used primers were as follows.

Sense: 5′-ATAGGCTCTTGTCTGCAAACATAACAGAGTATC ATTAATGTTTTCAGGGA-3′

Antisense: 5′-TGTGGTCCTCTGTGAAGTTGGAGTCCTTTAG AGGTGTTTTCATTCCC-3′

Transient transfection and luciferase assays were performed basically as described previously [17]. Briefly, cells were seeded into a 48-well plate (2×10^5 cells/well) and transfected with the reporter plasmid at 0.5 μg/well. After 36 h, 100 ng/ml LPS was added to the medium and 12 h later, the cells were harvested and luciferase activity was measured. An empty pBK-CMV vector was used to adjust the transfected DNA quantity. An empty luciferase reporter plasmid was used as control to calculate relative luciferase unit.

Chromatin immunoprecipitation. The Aire transfectants or control cells (2×10^6/well) were cultured with or without 100 ng/ml LPS for 6 h. After that, cells were harvested and processed using a Chromatin Immunoprecipitation Assay kit (Upstate Biotechnology) according to the manufacturer's instructions. The primers used for PCR were sense (1) described above and the antisense primer 5′-TGCTGACCTCT GCCCTAGAG-3′ (complementary sequence of sense (2)).

Flow cytometric analysis. Stable transfectants and control cells were stimulated with 100 U/ml IFN-γ for 24 h. After that, the cells were harvested and nonspecific staining was blocked with anti-CD16/CD32 mAb (Fc block, 2.4G2). The cells were then incubated with Class II Ab for 20 min, after which they were washed twice with PBS containing 0.5% bovine serum albumin. The cells were analyzed on a FACSCalibur using CellQuest software (Beckton–Dickinson). Viable cells were gated on the basis of forward and side scatters and by propidium iodide staining.

Results

First, we established two lines of the murine Aire transfectant from macrophage-lineage Raw 264.7 cells. Two control lines were also established into which an empty vector was introduced. Aire mRNA expression was confirmed by Northern blotting (Fig. 1). We obtained total RNA from these cells with or without lipopolysaccharide (LPS) stimulation and analyzed the mRNA expression levels of cytokines and chemokines by a ribonuclease protection assay (Fig. 2A). Neither cytokine nor chemokine was induced more in the Aire-expressing cells than in the control cells. This was surprising because Aire has been considered to be a positive transcription factor [9,10]. On the contrary, IL-1 receptor antagonist (IL-1Ra) mRNA level was significantly decreased in the Aire-expressing cells compared with the control cells after LPS stimulation. The expression levels of other cytokines, such as macrophage migration inhibitory factor (MIF), and chemokines we investigated exhibited little difference between the Aire-expressing cells and the control cells (Fig. 2B).

Next, we sought in silico the 5′ flanking region of the murine *IL-1Ra* gene that was most affected by Aire expression (Fig. 3A) and noted a NF-κB-binding sequence

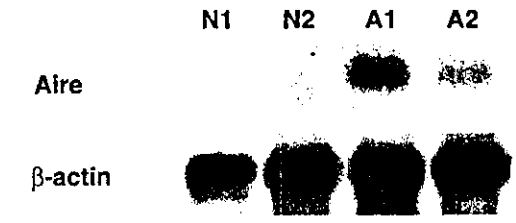

Fig. 1. Expression levels of Aire and β-actin mRNA. Five micrograms each of total RNA obtained from two Aire transfectants (clones A1 and A2) and two control cells (clones N1 and N2) was analyzed by Northern blotting. No intrinsic Aire mRNA was detected in the control cells.

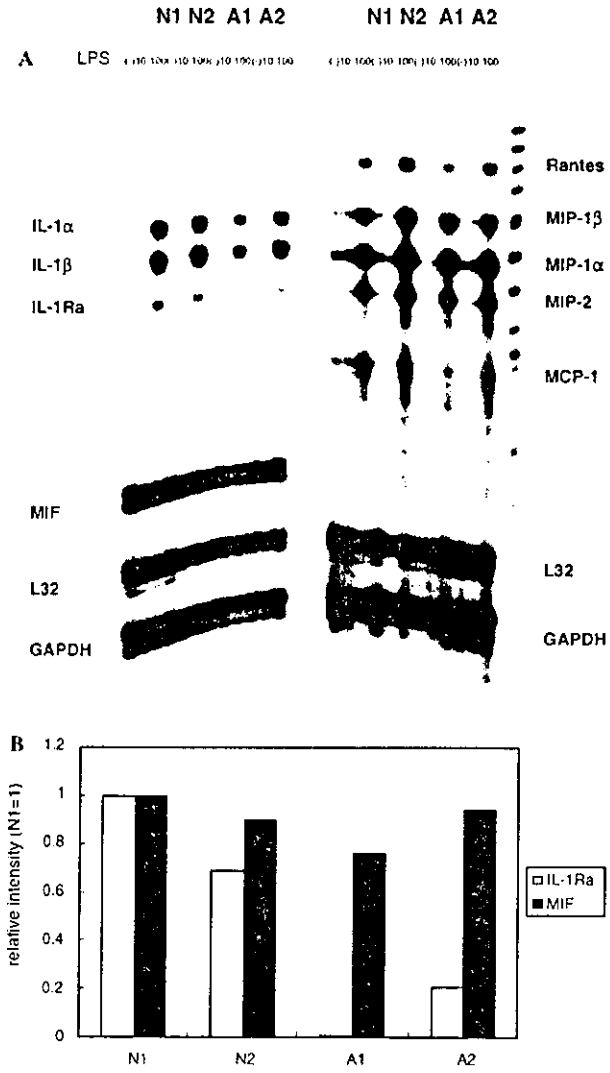

Fig. 2. Ribonuclease protection assay of cytokine and chemokine mRNA expressed by Aire transfectants and control cells. (A) Three micrograms each of total RNA obtained from these cells with or without 8-h LPS stimulation (10 or 100 ng/ml) was analyzed. The analyzed cytokines and chemokines were: IL-12p35, IL-10, IL-1α, IL-1β, IL-1Ra, IL-18, IL-6, IFN-γ, and MIF (left side) and Ltn, RANTES, Eotaxin, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, and TCA-3 (right side). L32 and GAPDH were used as controls. The lanes on the extreme left and right represent probe-only (i.e., without RNA) control lanes. (B) Quantification of expression levels of IL-1Ra and MIF. The intensity of the blots of the above data was quantified and standardized using clone N1 as 1. Data shown are representative of three independent experiments. Hereafter, clones A1 and N1 were used as the Aire-expressing cells and control cells, respectively, if not mentioned otherwise.

and an interferon-stimulated response element (ISRE), to which interferon regulatory factor (IRF) family transcriptional factors bind [18], near the translation initiation site. This region probably has a capacity to respond to inflammatory stimulation. At about 1000 bp upstream of this region, two cyclic AMP response elements (CREs) appeared in tandem, which are supposed to be bound by CRE-binding proteins (CREBs). Considering the observation that AIRE can bind to a CREB-binding protein (CBP) [10], which can be recruited to CRE via CREB, Aire overexpression may affect this region and cause IL-1Ra downregulation.

Therefore, we generated a luciferase reporter construct that contained the region including two CREs, ISRE, and NF-κB-binding sequence (construct A) and also three shorter constructs (B–D). The shortest one (D) contained no CREs, ISRE nor NF-κB-binding sequence (Fig. 3B). We transiently transfected these vectors into the Aire-expressing cells and control cells, then stimulated them with LPS and analyzed luciferase activity. As expected, construct D did not respond to LPS stimulation irrespective of Aire expression. On the other hand, the longest construct (A) exhibited less luciferase activity induction after LPS stimulation in the Aire-expressing cells than in the control cells. The other two constructs exhibited similar response to LPS stimulation in both cells (Fig. 3C). The fact that construct A exhibited less luciferase activity than construct B in Aire-expressing cells irrespective of LPS stimulation indicates that construct A contains negative element(s), which becomes apparent in the presence of Aire. These results also suggest that Aire expression reduces IL-1Ra promoter responsiveness to LPS stimulation through a CRE-related mechanism. To confirm this, we constructed a mutant-Aire expression vector (Aire(1–293)), which lacks a proline-rich region and both of the PHD-type zinc finger domains and has no transcriptional transactivation property [10], and a mutant luciferase reporter gene which has mutations in both of the CREs and did similar experiment, this time not using clones but using transfectants in bulk. LPS stimulation did not exhibit an Aire-mediated repression of luciferase activity when Raw cells were transfected with Aire(1–293) (Fig. 4A). Moreover, when the mutant luciferase reporter construct was used, very little luciferase activity was detected either with wild type Aire or mutant Aire. These results strongly favor the hypothesis of CRE-dependent luciferase downregulation by Aire. Then what is the underlying mechanism of the downregulation? CBP competition may be one possibility. That is, the binding of Aire to CBP leads to the lack of this transcriptional coactivator binding to the IL-1Ra promoter, thus causing a decrease in the extent of IL-1Ra induction.

To assess whether the lack of CBP causes this reporter's unresponsiveness to LPS, we attempted to rescue the luciferase induction using a CBP expression vector. As expected, CBP overexpression rescued the responsiveness to LPS in a dose-dependent manner (Fig. 4B). On the other hand, when mutant Aire was introduced into the cells, the responsiveness to LPS was not affected by the overexpression of CBP, further indicating that the lack of CBP is indeed the cause of the

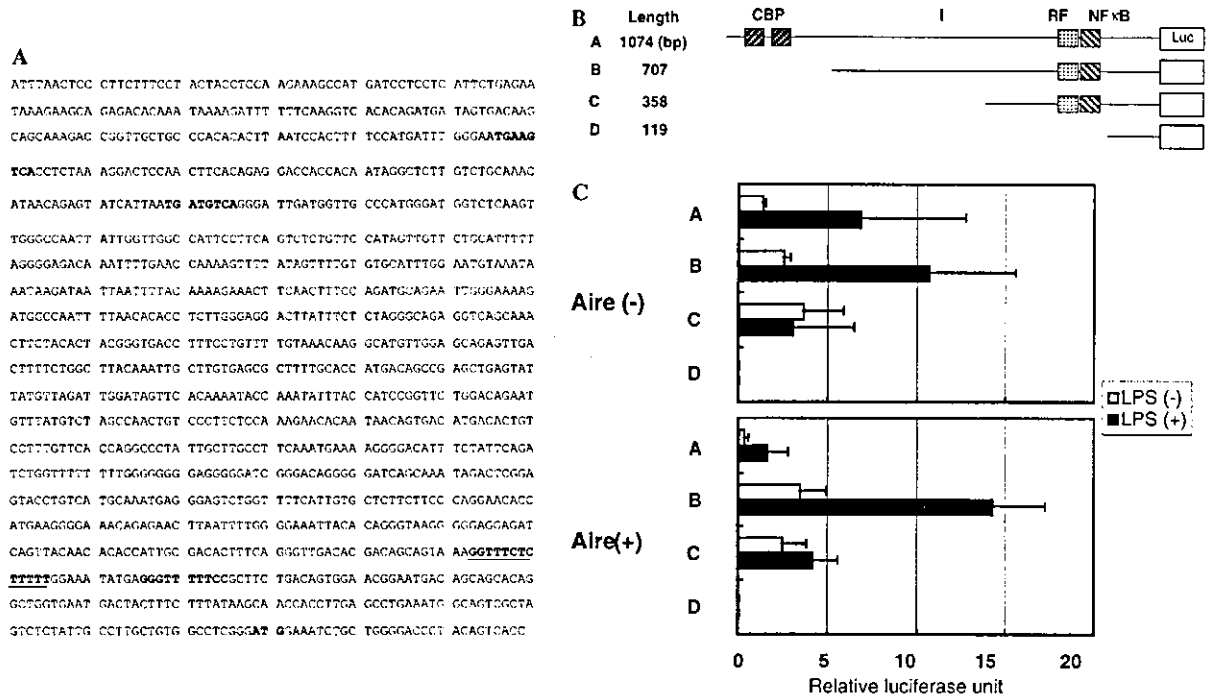

Fig. 3. Promoter analysis of IL-1Ra. (A) 5′-flanking region of the murine IL-1Ra gene (Accession Nos. L32838 and AL732528). Double underlines indicate CRE, to which the CRE-binding protein binds. A single underline indicates ISRE, to which IRF family transcription factors bind and the dotted underline indicates the site to which NF-κB binds. The bold ATG indicates the translation initiation site. (B) Four constructions of IL-1Ra-Luc reporter. (C) IL-1Ra-Luc reporter assay in Aire transfectants and control cells. These cells were transfected with each of the reporters with or without 12-h LPS stimulation (100 ng/ml) prior to the estimation of luciferase activity.

reporter construct's unresponsiveness to LPS. To clarify whether CBP is actually recruited to the IL-1Ra promoter, we performed chromatin immunoprecipitation (ChIP) assay using an anti-CBP antibody. As shown in Fig. 4C, the IL-1Ra-specific sequence was amplified only in the immunoprecipitated sample from LPS-stimulated control cells. This indicates that after LPS stimulation, the transcriptional complex containing CBP is recruited to the IL-1Ra promoter. On the other hand, no band was detected from the Aire-expressing cells irrespective of LPS stimulation, indicating that Aire expression inhibits the recruitment of CBP to this promoter. A similar mechanism has been proposed in the induction of MHC class II expression [19]. Two factors, CIITA and CBP, are necessary for the induction of this molecule [20]. It is postulated that glucocorticoid treatment leads to a reduction in class II molecule expression level due to the squelching of CBP by the glucocorticoid receptor. Thus, it is expected that the class II molecule expression is also downregulated by Aire in the same manner as in the presence of glucocorticoid. This was indeed the case. Raw 264.7 cells express class II molecules after IFN-γ treatment [21]. Although both Aire-expressing cells and control cells showed a low class II molecule expression level on the cell surface without IFN-γ stimulation, the former cells showed a much lower expression level of class II molecules than the latter cells after IFN-γ stimulation (Fig. 5).

Discussion

Aire has been supposed to be a transcription factor [7–10]. Recently, the gene chip analysis of Aire-knock-out mice has revealed that the transcription of peripheral organ-specific genes is reduced in *Aire*-deficient thymic medullary epithelial cells [13]. The authors of the above paper and others claimed that the failure to delete some organ-specific T cells in the thymus (failure of negative selection) is the main cause of the inflammation of multiple organs in the Aire-knock-out mice (and probably in APECED patients) [13,14]. However, this theory does not explain all aspects of APECED. For example, it does not explain the function of Aire expressed in the periphery, such as in macrophages and dendritic cells. Immunodeficiency observed in APECED patients cannot be explained by this mechanism, either. The fact that both APECED patients and Aire-knock-out mice show significant phenotypic variability indicates that external factors, such as infection, may play important roles in the pathogenesis of the disease [22].

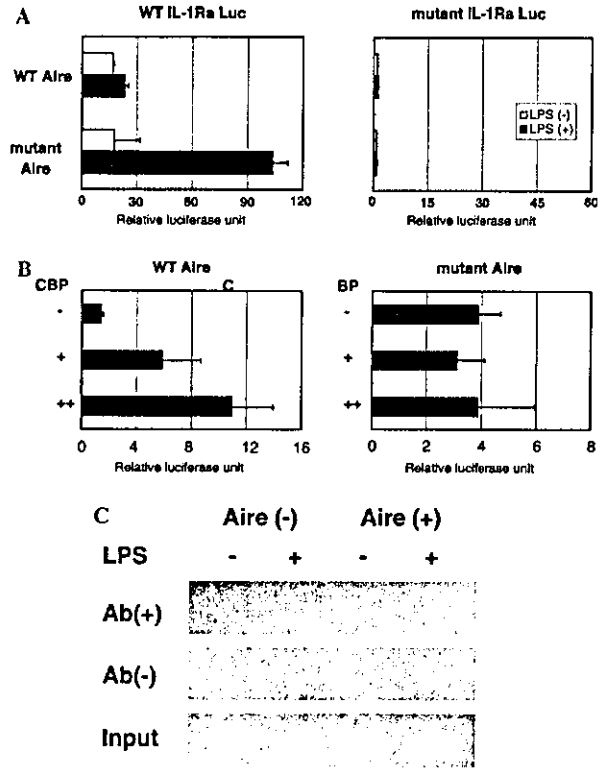

Fig. 4. Aire-mediated dampened response of the IL-1Ra promoter to LPS was dependent on CREs and rescued by CBP. (A) Mutant Aire showed little effect of dampening the IL-1Ra promoter responsiveness to LPS stimulation. The mutant IL-1Ra promoter that lacked two CREs showed little promoter activity in response to LPS irrespective of the cotransfection of wild-type or mutant Aire. (B) The cotransfection of CBP rescued the responsiveness of the IL-1Ra promoter to LPS in a dose-dependent manner (CBP+: 0.625 μg expression vector/well, ++: 1.25 μg expression vector/well). In the case of mutant Aire, CBP cotransfection did not affect the responsiveness of the IL-1Ra promoter. (C) The anti-CBP antibody could immunoprecipitate the IL-1Ra promoter region of the control cells after LPS stimulation. No band for the region was detected by PCR in the absence of the antibody. In the case of the Aire-expressing cells, the region was not immunoprecipitated even after LPS stimulation.

In this report, we have observed that Aire can negatively regulate multiple genes, contrary to previous reports arguing that Aire is a positive transcription factor [9,10]. Considering that IL-1Ra is supposed to prevent immunological responses by competitively inhibiting the biological effects of IL-1 [23,24] and that MHC class II molecules are critical for adaptive immune responses, the inhibition of the induction of both IL-1Ra and MHC class II molecules by Aire may explain the dual aspects of APECED, i.e., autoimmunity and immunodeficiency. This finding is even more interesting because IL-1 has been considered to be one of the important cytokines in the defense against *Candida albicans* infection [25], and candidiasis is among many traits of APECED [1]. Therefore, it would be of interest to

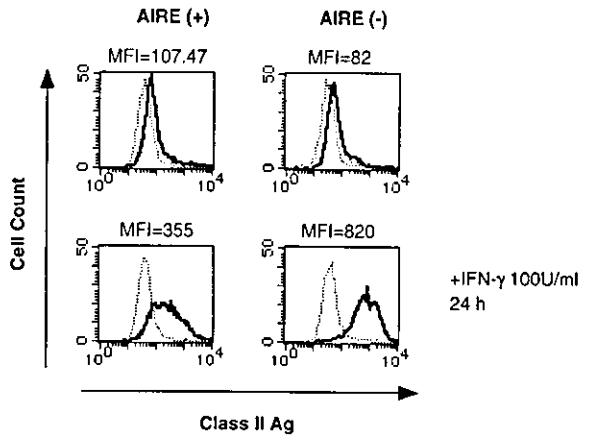

Fig. 5. Induction of class II molecule expression on Aire transfectant and control cells by IFN-γ. These cells were cultured with or without 24-h IFN-γ stimulation (100 U/ml) prior to flow cytometric analysis. Dotted lines represent background staining with control rat IgG. MFI, mean fluorescence intensity.

investigate the expression level of these genes in APECED patients and two Aire-targeted mice lines [13,22].

As for the mechanisms underlying Aire-mediated downregulation of the above-mentioned molecules, the promoter assay showed that dampened response to LPS by Aire is dependent on the two CREs in the IL-1Ra promoter (Fig. 4A) and that CBP overexpression rescues the responsiveness (Fig. 4B). Moreover, the ChIP assay showed that Aire expression inhibits the recruitment of CBP to the IL-1Ra promoter containing the two CREs (Fig. 4C). This suggests that the squelching of the transcriptional coactivator by Aire may explain the downregulation of IL-1Ra. MHC class II molecules are downregulated by the squelching of CBP [19] and, as expected, they are also downregulated in the Aire-expressing cells (Fig. 5). We are now screening the genes that are differentially expressed depending on the existence of Aire, using the exhaustive cloning method of suppression subtractive hybridization (SSH). It seems that more genes are positively regulated than negatively regulated by Aire (data not shown). We are now identifying the genes to investigate whether some of the differentially regulated genes further explain the mechanisms underlying the downregulation of the gene expression described in this paper and the pathogenesis of APECED.

Acknowledgment

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Transendothelial Migration of Human Basophils[1]

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During allergic reactions, basophils migrate from the blood compartment to inflammatory sites, where they act as effector cells in concert with eosinophils. Because transendothelial migration (TEM) represents an essential step for extravasation of cells, for the first time we have studied basophil TEM using HUVEC. Treatment of HUVEC with IL-1β significantly enhanced basophil TEM, which was further potentiated by the presence of a CCR3-specific ligand, eotaxin/CCL11. In addition to CCR3 ligands, MCP-1/CCL2 was also active on basophil TEM. Although stromal cell-derived factor-1/CXCL12, a CXCR4 ligand, failed to induce TEM in freshly isolated basophils, it caused strong TEM in 24-h cultured cells. IL-3 enhanced basophil TEM by increasing the chemokinetic response. Spontaneous TEM across activated HUVEC was inhibited by treatment of cells with anti-CD18 mAb, but not with anti-CD29 mAb, and also by treatment of HUVEC with anti-ICAM-1 mAb. Anti-VCAM-1 mAb alone failed to inhibit TEM, but showed an additive inhibitory effect in combination with anti-ICAM-1 mAb. In contrast, eotaxin- and IL-3-mediated TEM was significantly inhibited by anti-CD29 mAb as well as anti-CD18 mAb. These results indicate that β_2 integrins play the primary role in basophil TEM, but β_1 integrins are also involved, especially in TEM of cytokine/chemokine-stimulated basophils. In conclusion, the regulatory profile of basophil TEM is very similar to that reported for eosinophils. Our results thus support the previous argument for a close relationship between basophils and eosinophils and suggest that the in vivo kinetics of these two cell types are similar. *The Journal of Immunology*, **2004, 173: 5189–5195.**

Although basophils are the least abundant circulating leukocytes, an increasing body of evidence has demonstrated that these cells play an active pathogenic role in allergic inflammation by releasing diverse proinflammatory mediators, including vasoactive amines, cysteinyl leukotrienes, and cytokines (1). The biological significance of basophils in allergic disorders has become more apparent with the recognition and understanding of allergic dual-phase reactions; analyses of chemical mediators have revealed increases in basophil-derived mediators at the sites of late-phase reactions (2–4).

The results of experimental allergen challenge in various organs have revealed the influx of basophils to inflammatory sites several hours after Ag exposure (5–9), indicating the existence of a mechanism for recruitment of basophils from the blood compartment to inflamed tissue sites during allergic reactions. Like other types of leukocytes, the entire process of basophil influx to inflamed tissue sites comprises three essential sequential steps: adhesion to the vascular endothelium, transendothelial migration (TEM),[3] and locomotion toward inflammatory sites in extravascular tissues. To date, a considerable number of studies have outlined the mechanisms of both the adhesion and locomotion processes in basophils (10–14), demonstrating that adhesion molecules and/or several cytokines and chemokines are critically involved in these processes. In contrast to neutrophils, basophils express $\alpha_4\beta_1$ integrins in addition to β_2 integrins. Basophil-activating cytokines such as IL-3 are capable of up-regulating the expression of β_2 integrins (CD11b and CD18), thereby potentiating the β_2 integrin-mediated adherence of basophils to the endothelium (14). These basophil-activating cytokines also enhance basophil locomotive responses by inducing random movement (chemokinesis) (11). Regarding the basophil migration process, however, basophil-directed chemokines such as eotaxin play more critical roles by virtue of inducing strong directional movement (chemotaxis) (15, 16).

The molecular aspects of both the adhesion and migration processes of basophils have thus become increasingly clear, but those of basophil TEM, another essential process that links the adhesion process and the locomotion process, have not been studied to date. Given the importance of the TEM process for the recruitment of basophils to allergic inflammatory sites, for the first time we have studied basophil TEM using vascular endothelial cell monolayers.

Materials and Methods

Reagents and mAbs

Murine mAbs with blocking activity against adhesion molecules were used. Anti-CD49d mAb (IgG1, clone HP2/1) and anti-CD29 mAb (IgG1, clone 4B4) were purchased from Coulter Immunotech (Marseille, France). Anti-CD11a mAb (IgG1, clone TS1/22) was obtained from Endogen (Woburn, MA). Anti-CD49e mAb (IgG1, clone IIA1), anti-CD11b mAb (IgG1, clone 44), and anti-CD18 mAb (IgG1, clone L130) were purchased from BD Pharmingen (San Diego, CA). The $F(ab')_2$ of anti-CD54 mAb (IgG2a, clone MEM-111) and anti-CD106 mAb (IgG1, clone 1G11B1) were purchased from Caltag Laboratories (Burlingame, CA). The $F(ab')_2$ of mouse IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse IgG1 (MOPC-21) with irrelevant specificity was purchased from Sigma-Aldrich (St. Louis, MO). Murine anti-CCR1 mAb (IgG1, clone 141) and anti-CCR3 mAb (IgG1, clone 444) were donated by Dr. H. Kawasaki (Institute of Medical Science, University of Tokyo, Tokyo, Japan) (17). Other reagents used in the experiments were previously described (13).

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[3] Abbreviations used in this paper: TEM, transendothelial migration; MCP, monocyte chemoattractant protein; SDF, stromal cell-derived factor.

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