Vaccination with recoverin, a cancer-associated retinopathy antigen, induces autoimmune retinal dysfunction and tumor cell regression in mice

Akiko Maeda1,2, Tadao Maeda1, Hiroshi Ohguro3, Krzysztof Palczewski1,4,5 and Noriyuki Sato2

1 Department of Ophthalmology, University of Washington, Seattle, USA
2 Department of Pathology, Sapporo Medical University, School of Medicine, Sapporo, Japan
3 Department of Ophthalmology, Hirosaki University, School of Medicine, Hirosaki, Japan
4 Department of Chemistry, University of Washington, Seattle, USA
5 Department of Pharmacology, University of Washington, Seattle, USA

Recoverin (Rec)-specific CTL present in peripheral blood recognize Rec-expressing tumor cells of patients with cancer-associated retinopathy (CAR), a paraneoplastic retinopathy syndrome. To evaluate the effects of Rec on retina and cancer cells, we generated an experimental mouse model, tested the induction of Rec-specific anti-tumor CTL, and analyzed retinal function using electroretinogram (ERG) in these animals. We observed a Rec-specific CTL response in BALB/c mice and significant growth inhibition of Rec-expressing syngeneic MethA fibrosarcoma cells in vivo. R64 (AYAQHVFRSF) peptide, derived from Rec that induces anti-tumor CTL in humans, produced anti-tumor effects in BALB/c mice. Furthermore, elevated anti-Rec antibodies correlated with decreased ERG amplitudes in Rec, Rec-expressing tumor and R64-treated mice. These data suggest that Rec contains amino acid sequences which cause retinal dysfunction, but they also induce anti-tumor CTL and tumor regression. These observations describe initial characterization of the CAR mouse model, a necessary step in developing new insight into immunological mechanisms of paraneoplastic syndromes and tumor immunity for potential immunotherapeutic approaches to cancer.

Key words: Cancer-associated retinopathy (CAR) / Recoverin / CTL / Paraneoplastic syndrome

1 Introduction

A variety of neurological disorders called paraneoplastic syndromes are known to be associated with malignant tumors [1–3]. The expression of a tumor antigen presumably triggers immunological responses, which in turn recognize the same antigen or shared epitope in the nervous system, resulting in neuronal cell damage. Cancer-associated retinopathy (CAR) is one of the paraneoplastic syndromes, and Rec-specific auto-Ab contributes to the pathogenesis of retinopathy [4, 5]. Rec is a photoreceptor and selected retinal bipolar-specific Ca2+-binding protein which is believed to play a regulatory role in phototransduction [6, 7]. Rec-specific CTL exist in the peripheral blood of CAR patients, and these CTL can recognize tumor expressing Rec [8]. Additionally, Rec induces uveoretinitis in LEW rats [9] and anti-Rec Ab injected into the eye cavity causes retinal cell death by apoptosis [10]. Therefore, Rec may contribute to the tumor cell destruction and the occurrence of CAR or uveoretinitis. If cancer rejection and retinal disease occur at the same time, this model may explain a molecular pathology of CAR. However, if only Rec-specific CTL can be elicited, Rec may serve as a valuable vaccine for cancer therapy against aberrantly Rec-expressing cancer cells [11], leading to tumor immunity and a favorable prognosis for cancer patients.

In this study, we investigated the anti-tumor and anti-retina effects of Rec in mice grafted with cancer cells. Experimental uveoretinitis (EAU)-sensitive (C57BL/6) and -resistant (BALB/c) mice were employed. Rec-specific CTL response was observed only in BALB/c mice. In Rec, Rec-expressing tumors, and R64 peptide-treated mice, anti-tumor activities were demonstrated. Furthermore, elevated anti-Rec Ab titers strongly correlated with retinal dysfunction. These data suggest that sensitivity against Rec is different between mice strains, and an anti-tumor epitope and anti-retina epitope exist in the Rec sequence. Studies on the CAR mouse model are

Abbreviations: CAR: Cancer-associated retinopathy EAU: Experimental autoimmune uveoretinitis ERG: Electroretinogram Rec: Recoverin

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important for the understanding of the molecular mechanisms of autoimmune retinal dysfunction and tumor immunity.

### 2 Results

Rec is a pathogen of both uveoretinitis and retinal degeneration [9, 10]. In a mouse model of uveoretinitis, inflammation occurs in B.10A and C57BL/6 strains which produce Th1 type cytokines, but not in BALB/c variety, which are considered to be strong Th2 responders against retinal antigens [12]. Rec-specific auto-Ab are suggested to contribute to the pathogenesis of retinopathy [4, 5]. Therefore, it appears that the genetic background of mice may influence their immunoreactivity and CTL induction.

To determine if susceptibility to generate CTL is different between mouse strains, EAU-sensitive C57BL/6 mice and EAU-resistant BALB/c mice were tested. To avoid allogeneic responses, C57BL/6 and BALB/c mice were challenged with syngeneic cancer cells, EL4 and MethA, respectively. Bone marrow-derived dendritic cells were used for *in vivo* and *in vitro* stimulation. When BALB/c mice were challenged with MethA cells transfected with mouse Rec cDNA (MethA/Rec), a strong CTL activity was observed in the 51Cr-release assay (Fig. 1). However, when C57BL/6 mice were challenged with EL4 or Rec cDNA transfected EL4 (EL4/Rec) cells, cytotoxic activity was not detected. This indicates that Rec-specific CTL can be induced only in BALB/c mice using a combination of *in vivo* and *in vitro* stimulation.

Recently, anti-tumor epitopes were identified on Rec in CAR patients’ peripheral blood that correspond to R49.2 and R64 peptides [8]. Since these human anti-tumor CTL epitopes of Rec are restricted by HLA-A*2402, and because peptide-binding motifs of HLA-A*2402 and H-2Kd of BALB/c mice are similar, the immunogenicity of R49.2 and R64 peptides were examined in BALB/c mice. Cytotoxicity assays against MethA/Rec, MethA pulsed with R49.2 or R64 peptides, and MethA were examined. When R64 peptide was used for immunization, CTL that

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**Fig. 1.** Induction of the Rec-specific CTL in BALB/c and C57BL/6 mice. Lymphocytes from BALB/c mice were stimulated with irradiated syngeneic MethA fibrosarcoma cells or MethA/Rec as antigens, and cytotoxicity assays were carried out against MethA or MethA/Rec at different effector/target (E/T) ratios, as described in Sect. 4. C57BL/6 mice were immunized with irradiated syngeneic EL4 lymphoma cells or EL4/Rec. Bars represent mean ± SD.

**Fig. 2.** The CTL induction by peptides R49.2 and R64 corresponding to the Rec sequence in BALB/c mice. (A) Mice were immunized twice with 50 μg of R64 peptide, T cells isolated and stimulated with R64 peptide presented by syngeneic BALB/c bone marrow-derived dendritic cells. Cytotoxicity was tested against MethA, MethA/Rec, and MethA cells pulsed with R64 peptide at different effector/target (E/T) ratios. (B) Mice were immunized twice with 50 μg of R49.2, and T cells were isolated and stimulated with R64 peptide presented by syngeneic BALB/c bone marrow-derived dendritic cells. Cytotoxicity was tested against MethA, MethA/Rec, and MethA cells pulsed with R49.2 peptide at different effector/target (E/T) ratios.
reacted with MethA/Rec and R64 peptide pulsed MethA cells was observed (Fig. 2A). In a similar experiment, R49.2 peptide failed to generate R49.2 peptide-specific CTL (Fig. 2B). These results indicated that R64 is an immunogenic peptide in human and BALB/c mice.

To test whether endogenously expressing Rec induces CTL in vivo, MethA/Rec and Rec were challenged to BALB/c mice. Without extra vivo stimulation by bone marrow derived-dendritic cells, cytotoxic activity against target cells was not observed (data not shown). To boost response, anti-CTLA4 Ab, which facilitates the induction of the antigen-specific T cell response in vivo [13], was injected intraperitoneally. After three immunizations, both Rec and irradiated MethA/Rec challenges to BALB/c mice induced CTL response. Interestingly, these CTL recognized R64-pulsed MethA cells, but not R49.2-pulsed MethA cells (Fig. 3A and B). These data verified that R64 is Rec-specific CTL epitope in BALB/c mice.

Since CTL responses were observed in our in vivo stimulation, the influence of Rec immunization of tumor growth was examined in vivo. Rec, R49.2, R64, and irradiated MethA/Rec or MethA cells to BALB/c mice and tumor growth was measured. Anti-CTLA4 Ab was given intraperitoneally on days 0, 3 and 6 post-first antigen immunization. The subcutaneous growth of MethA/Rec cells was suppressed in Rec-, MethA/Rec- and R64-treated group and compared with R49.2- or PBS-treated or MethA transplanted groups (Fig. 4).

Electroretinogram (ERG) recordings were used to determine whether immunization with Rec and its variants in the presence of anti-CTLA4 Ab also affects the retina. There were no significant changes in R49.2-treated mice at the intensity varying from 0.0002 to 0.02 cd·s/m² compared to PBS-treated mice. In contrast, b wave amplitudes of R64, irradiated MethA/Rec- and Rec-immunized mice as a function of light intensity were lower than those of control mice (Fig. 5). The strongest effect was observed for Rec-treated mice, suggesting that immunization with this antigen led to retinal dysfunction. This dysfunction correlated with serum titer of anti-Rec Ab in tested mice. The highest titer was observed for Rec-treated mice, followed by MethA/Rec-treated and R64-treated mice (Fig. 6A). Immunoblotting using recombinant Rec showed that these Ab from Rec-,
Fig. 5. Dark adapted ERG responses. The ERG responses were recorded at various intensities (0.0002–0.02 cd · s/m²) employing the UTAS E-3000 analyzer (LKC Technologies Inc., Gaithersburg, MD). (A) ERG responses at 0.0093 cd · s/m² for control mice (treated with PBS) and treated mice with MethA, MethA/Rec, R64-pulsed MethA, or R49.2 pulsed MethA cells are superimposed. (B) Intensity-responses curve of the amplitude of flash b wave from the dark adapted ERG. Five mice per each group were employed.

MethA/Rec- and R64-treated mice reacted with purified recombinant Rec (Fig. 6B). R49.2 peptide and PBS were not immunogenic (Fig. 6). No histological changes, such as invasion of immunocytes and retinal destruction related to uveoretinitis, were observed (data not shown).

3 Discussion

3.1 General remarks

Maddison et al. [14] reported that patients with Lambert-Eaton myasthenic syndrome have a more favorable clinical prognosis than cancer patients without neural disorder. In paraneoplastic cerebellar degeneration (PCD), anti-cdr2-, Yo- and Hu- specific CTL may also contribute to the pathogenesis of paraneoplastic syndromes [15–17]. In paraneoplastic syndromes of the eye, immunological mechanisms are also involved in a better clinical prognosis and therefore, CTL epitopes derived from Rec might be candidates for cancer immunotherapy [8, 18]. In this report, we investigated the antigen-specific immunoresponses that contribute to the retina dysfunction and cancer rejection in CAR paraneoplastic syndromes using the mouse experimental model.

3.2 Anti-tumor and retinal degeneration properties of Rec

This study showed that Rec has anti-tumor activities, but it causes retinal dysfunction in the mouse animal model of the disease. Epitopes of anti-tumor CTL encompass 49–58 and 64–73 regions of the Rec sequence in human, and the 64–73 region in BALB/c mice [8]. Additionally, since cancer cells produce many cytokines, the Th balance of cancer patients can easily transform to Th2 [19],...
Rec can also induce EAU, retinal degeneration like CAR, and anti-tumor CTL. EAU was observed only in Th1 background mice or LEW rats. The sequences corresponding to 65–79, 153–164 and 149–167 regions of Rec were identified as uveitisogenic epitopes in LEW rats [21, 22]. Adamus et al. [22] reported that CAR patients’ sera contain auto-Ab that react with a peptide that corresponds to the 61–82 sequence of human Rec. These data suggest that genetic sensitivity, distinct and overlapping epitopes of the Rec sequence, and immunological disruption in cancer patients can explain widely diverse immunological reactions.

R49.2 and R64 peptides are the immunogenic epitopes in human. However, R49.2 failed to induce Rec-specific CTL in BALB/c mice in this study. This observation could be explained by two Glu substitutions in the mouse sequence by Gln residues in the sequence of the human R49.2 peptide. The specificity and affinity of peptide binding to MHC class I molecules has been extensively examined. It was concluded that not only anchor motif, but also amino acid residues at the non-anchor position were revealed to be important for high affinity binding [23]. Preferable non-anchor sequences of R49.2 in mice might be different from that in humans. The similarity between peptide binding motifs of mouse H-2K\(^d\) and human HLA-A*2402 suggests common epitopes, such as epitopes corresponding to the R64 and HER2-derived peptides [24, 25]. However, the non-anchor motif influences affinity to MHC molecules between human and mice, and human epitopes may become non-immunogenic sequences in mice.

Why Rec-specific CTL was obtained only from BALB/c mice is still unclear. To induce antigen-specific CTL, the interaction between an antigen, MHC molecule and T cell receptor is essential. Since the MHC class I molecules present 8–13 mer peptides, Rec must be proteolized into pieces. In this study, because Rec-specific CTL responses could be observed in Rec-challenged BALB/c mice, immunized Rec was successfully digested, processed, presented, and recognized in vivo. The CTL epitopes of Rec have been previously examined only in HLA-A*2402 positive humans, but the existence of additional epitopes for other HLA types have not been reported. Theoretically, there are some 8–10 mer peptides derived in the Rec sequence that seem to have high affinity to H-2K\(^d\) of C57BL/6 mice [26, 27], but they failed to induce CTL responses. This H-2K\(^d\) epitope may not be present within Rec, but the balance between Th1 and Th2 might be a critical means for CTL induction, similar to the EAU model induced by retinal antigens [12].

### 3.3 Immunogenicity of Rec and tumor cell-expressing Rec

In this study, CTL activity decreased, as expected, with dilution but it was weaker than in similar studies to other antigens. However, this low titer of CTL activity might be rescued by stimulation with dendritic cells in vivo. Because Rec-specific CTL activity was also generally low in human, immunogenicity of Rec could be lower than immunoreactivity of other cancer antigens such as cancer testis antigens [8]. Lower CTL activities were observed also in other tumor-neural antigens [15–17].

CTL4A is a suppressive signal of T cell activation, and Leach et al. suggested that in mice the anti-CTLA4 Ab treatment induces rejection of tumor cells in vivo [13]. The mechanism of inhibition of T cell activation is a result of enhanced signals through B7/CD28 pathways. Therefore, blocking of CTL4A activity might be important in observing tumor regression in vivo as previously proposed [28]. Our study showed that tumor-expressing Rec was sufficient to induce immunoresponse, although a CTL4A modulation was required. Interestingly, Rec immunization could induce anti-tumor activity in vivo. This reaction also required an anti-CTLA4 signal, but the protein injection was also effective in BALB/c mice. Since MethA/Rec injections could also cause CTL activity in the mouse system, processed Rec in tumor cells and Rec proteolized in immune system could provide CTL epitopes in vivo. Although immunization with the whole protein to elicit cellular immunity is rare and sometimes difficult, MUC1 protein successfully primed CTL in several mice strains, and Ab production in MUC1 transgenic mice and in humans were reported [29, 30]. Additionally, because whole Rec immunization can induce EAU and one of the epitopes of EAU and the R64 CTL epitope overlap, it is highly likely that Rec generates R64 epitope in vivo.

In CD4\(^+\) T cell mediated disease models such as EAU or experimental autoimmune encephalomyelitis (EAE), CTL4A-Ig is therapeutically effective [31, 32]. These results indicate that blocking CTL4A signaling leads to activation of T cell response. In our study, anti-CTLA4 Ab was used to boost T cells’ responses; conversely, the activated immune system might accelerate retinal dysfunction. However, in histological examination, no obvious signs of uveoretinitis and retinal degeneration were detected. This silent immunoreaction to retina is similar to rejection of allogeneic retinal tissue implanted in the subretinal space. At the early stage of CAR, no retinal degeneration is observed, and only ERG abnormality is recorded [33]. These phenomena might result from the existence of immune privilege in the subretinal space [34].
Immunization with R64 induces a strong anti-tumor response and a Rec-specific antibody titer. However, the retinal function proven by ERG is not significantly reduced compared to PBS-treated animals and better preserved than in those animals immunized with the whole protein or Rec-expressing tumor cells. R64 offers a possibility for a cancer vaccine that is not or only minimally destructive to the retina.

3.4 Concluding remarks

In summary, we evaluated effects of Rec, an antigen in CAR, on the retina and cancer cells. Rec-specific CTL response was observed in BALB/c mice, resulting in significant growth inhibition of Rec-expressing syngeneic MethA fibrosarcoma cells in vivo. R64 (AYAQHVFRSF) peptide, derived from the Rec sequence that induces anti-tumor CTL in human, produced anti-tumor effects in BALB/c mice. Although clinical treatment of uveoretinitis and retinal degeneration need to be developed, our observation implicates a possibility that epitopes from paraneoplastic syndrome antigens can be used for cancer therapy by employing effective in vitro activation, such as an adoptive transfer, without elevation of antibody titer that may affect the retina.

4 Materials and methods

4.1 Mice

All experiments with mice employed procedures approved by the Sapporo Medical University or the University of Washington Animal Care Committee and conformed to recommendations by the American Veterinary Medical Association Panel on Euthanasia. Female BALB/c (H-2Kd) and C57BL/6 (H2-Kb) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and only 6–12-week-old animals were used.

4.2 Cell lines and antigens

MethA, a fibrosarcoma cell line derived from BALB/c mice, and EL4, a lymphoma cell line from C57BL/6, were obtained from American Type Culture Collection. MethA/Rec and EL4/Rec cells were obtained by transfection of MethA and EL4 cells, respectively, with mouse Rec cDNA inserted into pIREShygro (CLONTECH, Palo Alto, CA), employing electroporation with a Gene Pulser (Bio-Rad, München, Germany) [35]. Rec cDNA was obtained by PCR from the mouse retina cDNA library. Mouse Rec protein was expressed in E. coli using the pET21a bacterial expression vector (Novagen, Darmstadt, Germany). Recombinant Rec was purified using the Phenyl-Sepharose column chromatography [36] (Amersham, Piscataway, NJ). R49.2 (QFQSIYAKFF) and R64 (AYAQHVFRSF) peptides that correspond to human Rec 49–58 and 64–73, respectively, were synthesized using a solid-phase peptide synthesizer PSSM-8 (Shimadze Co, Kyoto, Japan). The peptides were purified using C18 reverse-phase high-performance liquid chromatography (HPLC) (Millipore/Waters, Milford, MA).

4.3 Induction of CTL

CTL induction in vitro was performed according to a previously published procedure, with some modifications [37]. In brief, 50 μg peptides, or 5×10⁶ irradiated cancer cells in emulsion with CFA containing Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) (1:1, v/v), were injected subcutaneously three times on day 0, 7 and 14. On day 21, spleen cells were collected and cultured in vitro in 24-well plates by weekly stimulation with syngeneic BALB/c bone marrow-derived dendritic cells as antigen presenting cells, and 50 μg of the Rec peptide or irradiated (200 Gy) tumor cells (2×10⁹/well), as a source of cognate antigen. Bone marrow-derived dendritic cells were generated as described [38]. In brief, the bone marrow was flushed from femurs and tibias and then cultured overnight in RPMI1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol to eliminate adherent macrophages. Next, the cells were cultured with recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF) (1000 U/ml) (Genzyme, Cambridge, MA) and recombinant murine IL-4 (Genzyme) for 7 days. Nonadherent and loosely adherent cells were used as bone marrow-derived dendritic cells.

4.4 Tumor growth and anti-CTLA4 treatment

Fourteen days post-immunization, mice were injected subcutaneously with 5×10⁶ MethA/Rec cells in PBS. Three-dimensional tumor growth was measured every 3 days using a caliper. Tumor volume was calculated as described [39]. Anti-CTLA4 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was given intraperitoneally on day 0, 3 and 6 post-first immunization, at the concentration of 100 μg/mouse.

4.5 Killing assay

The target cells were labeled with 100 μCi sodium ⁵¹chromate (⁵¹Cr) for 2 h at 37°C, harvested, washed, and re-suspended. Peptide-pulsed targets cells were prepared by incubating the target cells at 5×10⁶ cells/ml with 50 μg/ml of the Rec peptide for 18 h at 37°C and then labeling them with ⁵¹Cr. The effector cells were placed in each well of the V-bottom microtiter plates. The labeled target cells were then added to the wells at a concentration of 5×10⁶ cells/well and a total volume of 0.2 ml. After 4-h incubation, the release of
4.6 Electroretinogram (ERG)

Mice were dark adapted overnight before the experiments and anesthetized by intraperitoneal injection with 15 μl/g body weight of 6 mg/ml of ketamine and 0.44 mg/ml of xylazine diluted in PBS. The pupils were dilated with 1% tropicamide. A contact lens electrode was placed on the eye with a drop of methylcellulose, and a ground electrode placed in the ear. ERGs were recorded and analyzed with the UTAS E-3000 analyzer (LKC Technologies Inc., Gaithersburg, MD). The mice were placed in a Ganzfeld chamber and ERG recordings were obtained from one eye.

4.7 Serum titer of anti-Rec Ab

Serum titer of anti-Rec Ab was analyzed by immunoblot and ELISA [40]. Briefly for ELISA, 30 μg of recombinant mouse Rec were adsorbed on the wall surface of a 96-multwell plate. Serum samples from mice were diluted 400 times with PBS and added to each well. After 1-h incubation, unbound serum was washed away and horseradish peroxidase-conjugated anti-mouse IgG was added. The excess of conjugated IgG was washed away, and the substrate solution was added to each well and the absorbance at 405 nm was measured in each well employing MPR-A4i (TOSOH, Tokyo, Japan).

4.8 Histological assessment

Eyes were enucleated and fixed for 1 h in 4% phosphate-buffered glutaraldehyde and transferred into 10% phosphate-buffered formaldehyde until processed. Fixed tissues were stained with hematoxylin-eosin and analyzed, as reported [41].

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References


MHC class I molecules.


