Cancer-retina antigens as potential paraneoplastic antigens in melanoma-associated retinopathy

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Melanoma-associated retinopathy is a rare paraneoplastic neurological syndrome characterized by retinopathy in melanoma patients. The main photoreceptor proteins have been found to be expressed as cancer-retina antigens in melanoma. Here we present evidence that these can function as paraneoplastic antigens in melanoma-associated retinopathy. Sera and one tumor cell line of such patients were studied and ret-transgenic mice spontaneously developing melanoma were used as a murine model for melanoma-associated retinopathy. Splenocytes and sera were used for adoptive transfer from tumor-bearing or control mice to wild-type mice. Retinopathy was investigated in mice by funduscopy, electroretinography and eye histology. Expression of photoreceptor proteins and autoantibodies against arrestin and transducin were detected in melanoma-associated retinopathy patients. In tumor-bearing ret-transgenic mice, retinopathy was frequently (13/15) detected by electroretinography and eye histology. These pathological changes were manifested in degenerations of photoreceptors, bipolar cells and pigment epithelium as well as retinal detachment. Mostly these defects were combined. Cancer-retina antigens were expressed in tumors of these mice, and autoantibodies against arrestin were revealed in some of their sera. Adoptive transfer of splenocytes and sera from tumor-bearing into wild-type mice led to the induction of retinopathy in 4/16 animals. We suggest that melanoma-associated retinopathy can be mediated byhumoral and/or cellular immune responses against a number of cancer-retina antigens which may function as paraneoplastic antigens in melanoma-associated retinopathy.

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To date, many authors have described the presence of autoantibodies (AA) against various neuronal proteins, paraneoplastic antigens, in sera of patients with different kinds of malignant tumors located outside the nervous system (for review see Ref. 1 and 2). These AAs may cross-react with the corresponding paraneoplastic antigens or their epitopes, which are present in neurons, and thus initiate the development of a variety of neurological disorders and paraneoplastic syndromes, even though the primary tumor and its metastases have not invaded the nervous system. Melanoma-associated retinopathy (MAR) is a rare paraneoplastic syndrome in patients with metastatic melanoma characterized by visual disorders and the presence of serum AA against retinal proteins.8-14 MAR is closely related to cancer-associated retinopathy,15 which often occurs in lung cancer patients but differs in ophthalmological symptoms and probably in the antigens recognized, which are unknown today (for review see Ref. 16).

The ophthalmological components of both cancer-associated retinopathy and MAR are well understood. In case of MAR, the full field flash electroretinogram (ERG) shows a reduction of the b-wave, indicating a dysfunction of depolarizing bipolar cells. Reduced amplitudes of a-waves reflect degeneration in photoreceptors cells.5-11 Sera from cancer-associated retinopathy patients stain strongly photoreceptor cells but only weakly bipolar cells, while sera from MAR patients strongly react with bipolar cells.8-11 Suggesting that the MAR syndrome is induced by the degeneration of bipolar cells. Indeed, the histological examination of the retinas from MAR patients indicates the degeneration of second-order neurons—bipolar cells.12 This may explain why MAR patients often suffer from sudden visual loss and night blindness, whereas color vision is frequently normal.

However, sometimes the a-wave in the ERG of MAR patients has been reduced, and rare degenerative regions in neural retina and pigment epithelium, as well as retinal detachment, have been detected. Sera of MAR patients appear to show reactivity against photoreceptor cells.12 In addition, AA against two photoreceptor proteins, transducin (Tr)17 and rhodopsin (Rho),18 were found in the sera of patients with MAR. These data allow us to suggest that not only bipolar cells but also photoreceptor cells may be involved in the MAR syndrome (for review see Ref. 16). The paraneoplastic retina degeneration in cancer-associated retinopathy was shown to be linked to the apoptotic death of photoreceptors cells induced by AA directed against the paraneoplastic antigen recoverin (Rec).19,20 Another hypothesis attributes cancer-associated retinopathy to the presence of Rec-specific cytotoxic T cells.21-23 However, there is still a lack of comparable investigations in MAR.

We have recently shown that photoreceptor proteins could function as cancer-retina antigens (CRA) in melanoma.24-25 In addition, by screening retina and melanoma cDNA libraries with sera...
from melanoma patients with or without MAR syndrome, we could isolate 20 different antigens recognized by patients’ antibodies, including visual Rho and arrestin (Arr). In the present work, we address the question of whether CRA can function as paraneoplastic antigens in MAR. On the basis of their frequent expression, we focused on 6 CRA previously found to be expressed in melanoma tissues such as Rho, Tr, cGMP-phosphodiesterase 6 (PDE6), and cyclic GMP-phosphodiesterase 6 (PDE6), Rec, photoreceptor-specific guanylyl cyclase 1 (GC1), Arr. We report on AA in the sera of MAR patients, the frequent occurrence of retinal degeneration in melanoma-bearing, ret transgenic mice and the induction of retinal degeneration in normal mice by transferring splenocytes and sera from tumor-bearing, transgenic mice.

Material and methods

Patients

The tumor material used was surplus material after surgical removal of lymph node metastases. Tissue and serum specimens were investigated after informed consent of the patients and approval by the local ethical committee.

Patient MAR1 suffered from breast cancer at the age of 55 years, which was treated successfully by surgery and axillary lymphadenectomy. In 1994, 7 years later, a malignant melanoma (SSM-type, Breslow 1.0 mm, Clark level III; initial Stage IA, pT1a; S-100, HMB-45, and Melan A positive) was diagnosed on her back. Ophthalmological symptoms appeared since January 1999: acquired night blindness in the left eye, 1 month later also in the right eye. The conventional ERG from April 1999 demonstrated a diffuse loss of rod function in both eyes, while cone function was better preserved. The pattern ERG demonstrated a progressive loss of retinal function in the most central retina of both eyes. Palpable lymph nodes were found in the right axilla followed by radical lymphadenectomy, with 7 of 16 lymph nodes positive. In July 1999 subjective and psychophysical visual functions were unchanged, but a further deterioration of rod and cone responses was found by conventional ERG. After positive PET/CT findings (12/1999), lymphadenectomy of the right axilla showed malignant melanoma metastases in 4 of 7 lymph nodes (2/2000). Subjective or psychophysical visual functions remained unchanged. ERG 2001 unchanged. In 8/2001, numerous lymph node metastases were detected (under the kidney and in retroperitoneal, axillary, infracavicular and subportal lymph nodes). Patient received DTIC (decarbazine) chemotherapy in 2001/2002, but showed still progressing lymph node metastases. In 2002, distant metastases were detected and the patient died in 3/2002 with stage IV (T1aN3M1c).

Additional sera were obtained from previously described MAR cases.17,26 Patients MAR-2 and MAR-3 were male and suffered from night blindness, photopsias and problems with contrast sensitivity. Both patients had abnormal ERG and tritan defects on the FM 100-Hue test.27 Patient MAR-4 was a woman with bilateral night blindness and decreased visual acuity. Bilateral centrocecal scotomas with enlarged blind spots were noted.17

Cell culture

A melanoma cell line (MM990428) was established from a lymph node metastasis of patient MAR-1 as described earlier.27 Cells were cultivated in RPMI-1640 medium, supplemented with 10% fetal calf serum, l-glutamine (2 mM) and penicillin/streptomycin solution (5 U/mL) at 37°C and 5% CO2.

Mice

Mice (C57Bl/6 background) transgenic for the human ret gene under the control of metallothionein-1 promoter expressing ret in melanocytes28 were kindly provided by Dr. Nakashima (Japan). All mice were crossed and kept under specific pathogen-free conditions in the animal facility of German Cancer Research Center (Heidelberg). Experiments were performed in accordance with the institution’s guidelines. Spontaneous tumor development was assessed twice a week. After a short latency (20–90 days), around 30% of mice develop skin tumors on the face (nose, ears, eyes and neck), back or on the tail. Tumor-bearing mice developed metastases in lymph nodes and some distant organs like liver, lungs and brain. Histological analysis of primary tumors and metastases revealed the morphology of malignant melanoma. In addition, it was found that these tumors expressed melanoma-associated antigens tyrosinase, tyrosinase-related protein-1 and -2, as well as gp100 (Umsky, unpublished observations).

Transfer of splenocytes and serum

Single-cell suspensions from spleens of tumor-bearing ret transgenic (experimental group) or nontransgenic wild-type mice (control group) were prepared by mechanical dissociation. After the lysis of erythrocytes by a short NH4Cl hypotonic solution treatment, residual cells were washed twice, resuspended in PBS and used as a source of T- and B lymphocytes. In addition, serum samples were prepared as a source of immunoglobulins. Spleen cells (2 x 107 cells/200 µL PBS) and serum (100 µL) were transferred i.v. into wild-type mice. Three weeks later, mice were killed and eyes were isolated, and the retinal histology was examined.

Funduscopy

The posterior parts of both eyes were examined after pupil dilation with 1 drop of atropine (1%). The mouse is grasped firmly in one hand and clinically evaluated using a head-worn indirect ophthalmoscope (Sigma 150 K, Heine Optotechnik, Herrsching, Germany) in conjunction with a condensing lens (90D lens, Volk, Kronhausen, Unterhaching, Germany) mounted between the ophthalmoscope and the eye.

Electroretinography

Whole-field ERGs were recorded simultaneously from both eyes to examine the retinal function as described.29 In brief, mice were dark-adapted for at least 12 hr and anesthetized. After pupil dilation (1% atropine), individual mice were fixed on a sled, and gold wires (as active electrodes) were placed on the cornea. The ground electrode was a subcutaneous needle near the tail; a reference electrode was placed subcutaneously between the eyes. ERG was performed at 2 luminance levels, at 500 cd/m2 and 12,500 cd/m2. The mice were introduced into an ESPION ColorBurst Handheld Ganzfeld LED stimulator (Diagnosys LLC, Littleton, MA) on a rail to guide the sled (High-Throughput Mouse-ERG, STZ for Biomedical Optics and Function Testing, Tubingen, Germany). Responses were recorded with an ESPION Console (Diagnosys LLC) and stored. To determine the cutoff value for the ERG investigation,29 10 control mice were subjected to ERG by a luminance of 500 cd/m2 and 12,500 cd/m2. The cutoffs for the a-wave were set to –2.5 at 500 cd/m2 and –23.8 at 12,500 cd/m2. Transgenic mice were measured for 10 times at both illumination intensities. Mean values of the a-waves higher than cutoff were interpreted as a result of degenerated photoreceptor cells. The cutoffs for the b-wave were set to 109 (at 500 cd/m2) and 121 (at 12,500 cd/m2); lower mean values were interpreted as degeneration of bipolar cells.16

Histology of eyes

Eye balls were fixed for 24 hr in Davidson solution (mixture of 31.4% ethanol, 8.3% formaldehyde and 11.1% acetic acid in water), dehydrated and embedded in plastic medium (JB4-Plus; Polysciences, Inc., Eppelheim, Germany). Transverse 2-µm sections were cut with an ultramicrotome (Ultratom OMU3; Reichert, Walldorf, Germany) and stained with methylene blue and basic fuchsin. Sections were evaluated by light microscopy and images were taken. Absence of tumor cells in eyes was determined by 2 independent researchers.
Immunohistochemistry

Snap-frozen eye balls were embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands). Sections were first fixed in ice-cold absolute acetone and were then preabsorbed using the Avidin/Biotin Blocking solution (Vector Laboratories, Burlingame, CA) and subsequently incubated with 5% normal goat serum. The antibodies against Ret were applied at a concentration 2 μg/mL overnight. After incubation with a goat anti-rabbit IgG coupled to biotin for 1 hr and subsequent incubation with a streptavidin–biotin–phosphatase complex, specific staining was visualized by alkaline phosphatase substrate (all components from the Alkaline Phosphatase Rabbit IgG ABC Kit and AP substrate Kit; Vector Laboratories) as described by the manufacturer. Hemalun solution was used for counterstaining.

Melanoma tissue specimen were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Sections were dewaxed, rehydrated and preabsorbed using the Avidin/Biotin Blocking solution (Vector Laboratories) and subsequently incubated with 5% normal goat serum. The application of the antibodies against Rho (0.3 μg/mL), Tr (0.3 μg/mL), Rec (4.5 μg/mL) and Arr (0.3 μg/mL) and further procedures were done as described for the cryo-sections mentioned earlier.

RNA and proteins

RNA and proteins were extracted with TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) for RNA and lysate buffer (25 mM Tris-HCl, pH 7.5 with 0.05% Triton X-100) for protein, as described by the manufacturers. Normal human retina RNA was obtained from a commercial source (BD Biosciences Clontech, USA). Bovine photoreceptor proteins were obtained as described.24 Polyclonal, monospecific rabbit antibodies against Rho, Tr, Rec and Arr were obtained as described.24 Polyclonal, monospecific rabbit antibodies against PDE6 and anti-human c-Ret were purchased from Affinity BioReagents (Golden, CO) and IBL (Gunma, Japan), respectively. Polyclonal, monospecific rabbit antibodies against GC1 were kindly provided by Prof. K.-W. Koch (Oldenburg, Germany).

RT-PCR analysis

One microgram of the total RNA from cell line MM990428 or from murine tumor tissues was reverse-transcribed by using the 1st strand cDNA synthesis kit (Roche Diagnostics) at 42°C for 50 min as described by the manufacturer. PCR-amplification was performed using 1 μL from the RT-reaction mixture in 25 μL of the PCR-mixture containing 50 pmol of sense and antisense primers. After the initial incubation at 94°C for 90 s, 33 cycles of amplification were carried out for genes of interest, and 25 cycles for GAPDH. Primer sequences for visual genes and GAPDH, as described.24 Primer sequences for visual genes and GAPDH are listed in Supporting Table S1. A sample was judged as being positive, if a clear band was visible in an ethidium bromide stained gel in at least 2/3 of independent PCR experiments. The amplified PCR products were electrophoresed on 1.5-3% agarose gels and subsequently stained with ethidium bromide.

Western blot analysis

After SDS-PAGE in a 12.5% polyacrylamide gel, separated proteins were electrotransferred to nitrocellulose membranes (Hybond-C: Amersham Bioscience, Buckinghamshire, UK) in Tris-glycine methanol buffer (pH 8.3). After blocking with 10% (w/v) delipidated dry milk in PBS containing TWEEN-20, the membranes were incubated with antibodies against photoreceptor proteins. Membranes were then incubated with an anti-rabbit peroxidase conjugated secondary antibody (Santa Cruz Biotechnol-ogy, Santa Cruz, CA). Immunoreactive bands were visualized by an enhanced chemiluminescence system (Amersham Bioscience) according to the method described by the manufacturer.

Results

CRA are expressed in melanoma cells of a MAR patient and AA against CRA are detected in sera of MAR patients

Since MAR is extremely rare, we only had access to sera of 4 patients with clinical symptoms of MAR. From one of these patients (MAR1), a stable tumor cell line (MM990428) could be established, and paraffin-embedded tumor tissue was available.

The cell line MM990428 was tested positive for Tr, Rec and Arr at mRNA and protein levels, while PDE6 and GC1 genes were found to be transcribed, but no protein could be detected by Western blotting. Rho, CGC (cGMP-dependent channels) and rhodopsin kinase were neither found at mRNA nor at protein levels (Fig. 1). Immunohistological analysis of paraffin tumor sections confirmed the presence of Tr, Rec and Arr proteins in the histological sections, and demonstrated that not all tumor cells were positive, but instead a rather spot-like staining pattern was observed (Fig. 2). Specificity was proven by omission or preabsorption of the primary antibody. An immunohistological staining with an antibody against Rho did not reveal any Rho-positive cells in the sections. The expression of PDE6 and GC1 in the sections could not be investigated because of the lack of antibodies against PDE6 and GC1 working in immunohistochemistry on paraffin sections. Interestingly, while the staining for Tr and Rec was restricted to melanoma cells, the anti-Arr antibody stained, in addition, some stromal components. The expression of CRA in the tumors of the other 3 patients with MAR could not be assessed because of the lack of tumor material.

The presence or absence of AA against CRA was examined in all 4 sera by Western blotting, using corresponding photoreceptor proteins as antigens. We detected AA (at a serum dilution of 1:100) against Tr in the sera of patients MAR1 and MAR4, and against Arr in sera of patients MAR2 and MAR4. AA against Rho, PDE6 and Rec were not detected in any of the sera. Thus, at least AA against Tr and Arr may play a role in MAR development.

Tumor-bearing ret transgenic mice, but not wild-type mice show signs of retinal degeneration

In order to investigate the potential involvement of CRA in MAR pathogenesis, we used ret transgenic mice spontaneously developing cutaneous malignant melanoma28 as a model system for MAR.

For ophthalmological investigations, we used the following parameters: (i) funduscopy of optic media and of fundus oculi, (ii) ERG, and (iii) histological retina examination. First, we performed funduscopy of 10 C57Bl/6 wild-type mice that did not show any changes in optic media and fundus oculi. Cutoff values for ERG were determined using these mice, as described.25 Histological examination of eye balls obtained from wild-type mice did not show any pathological changes: the retinal structure was intact, all layers were well preserved, and photoreceptor cells, bipolar cells and pigment epithelium were clearly distinguishable (Fig. 3a).

Next, we tested 10 ret transgenic mice without any visible tumors using the same ophthalmological approaches (Table I). Five mice did not show any difference from wild-type mice, neither in funduscopy nor in ERG (Fig. 4b) and retinal histology (Fig. 3b, mouse TgN#02). Four mice (TgN#03, 08, 09 and 10) had slight reduction of the a-wave (mouse TgN#10) and the b-wave (mice TgN#03 and #08) or both (mouse TgN#09) in the ERG, while a histological examination did not reveal any pathological changes in the retinae of these mice (Table I and Fig. 3b). One mouse (TgN#04) showed pathological changes in the ERG (Fig. 4b), which correlated with histological detectable retinal degeneration (Table I and Fig. 3b).

In contrast, tumor-bearing transgenic mice displayed drastic changes in their ophthalmological analysis (Table II and Fig. 4c). Histological analysis of mouse eyes revealed only 2 mice without obvious retinal disturbances (Tg1#01 and Tg1#04), whereas 8 mice had pathological changes in 1 and 5 mice in both eyes (Table
II). Notably, in 11/13 mice both the photoreceptor layer and the bipolar or other layers were histologically altered. These findings correlated well with aberrations in the ERG of the respective eye (Table II).

The observed histological data indicated strong morphological changes in the retinae of tumor-bearing mice. Disorganization of the inner nuclear layer and neuroepithelial degeneration were seen in mice TgT#02, #03, #05, #06, #08 to #10, #12 and #13 to #15, and a fragmentation of the rod and cone layer in mice TgT#06, #09, #10 and #12 to #15 (examples in Fig. 3c). In addition, retinal detachment (mice TgT#06, #09, #10 and #13) and degeneration of the pigment epithelium (mice TgT#06, #08, #09 and #10) were observed in some mice (Fig. 3c).

Since the ERG correlated well with the histological data, we performed only histological examination of eye balls in additional 6 transgenic tumor-bearing mice. All these mice had signs of retinal degeneration in the photoreceptor layer and 4 additionally in other layers (Table III).

Retinal degeneration is not mediated by tumor invasion or the influence of ret transgene

Transgenic mice with pericellular tumor were excluded from the ophthalmological analysis described earlier in order to eliminate any influence by tumor cell invasion. Using microscopical analysis, we found that all investigated mice did not have any melanoma cells in their ocular cups.

Since we found some mice with signs of retinal degeneration in the group of transgenic mice without tumor, we wanted to clarify whether the ret transgene could directly induce this retinal degeneration. We performed an immunohistological analysis of Ret expression in the retinae of 5 ret transgenic mice without tumors and 5 tumor-bearing mice. Ret expression was observed in the tumor, but never in the retinae of these mice (Fig. 5).

Transfer of spleen cells and serum from tumor-bearing ret transgenic mice induced retinal degeneration in normal C57Bl/6 recipients

To test the role of the immune system in the observed retinopathy, C57Bl/6 wild-type mice were injected with spleen cells and serum from tumor-bearing transgenic mice. Four out of 16 mice showed obvious signs of retinopathy 3 weeks after the transfer (Fig. 3d): Disorganization of the inner nuclear layer, fragmentation of the rod and cone layers, retinal detachment and neuroepithelial degeneration were observed very prominently in 2 mice. The retina of the third mouse displayed similar but less profound changes. We also found retinal detachment and signs of pigment epithelium degradation in the eyes of the forth animal. In contrast, all 6 wild-type mice, which received the same quantity of spleen cells and serum obtained from wild-type mice, had no visible changes in their retinae.

MAR mice express CRA in their tumors and have AA against CRA in their sera

Next, we wanted to check whether tumor-bearing mice with signs of retinopathy (MAR mice) express CRA in their tumors and can produce AA against these CRA. Using RT-PCR (Table IV), we detected specific mRNA for Rho (3/7 mice), Tr (4/7), PDE6 (6/7), GC1 (6/7), Rec (4/7) and Arr (2/7). Expression at the protein...
level was tested by Western blot analysis for all CRA except GC1 in the same 7 tumor samples. One tumor was positive for Rho, 1 for Tr, 3 for PDE6, 3 for Rec and 2 for Arr. CRA proteins were detected only in those tumor samples, which were tested positive for the corresponding mRNA. In addition, we found AA against Arr in the sera of 2 mice, whereas AA against Rho, Tr and Rec could not be detected (Table IV). None of 10 control mice and 15 transgenic mice without tumor showed any AA in their sera.

Discussion

In this study, we addressed the question, which antigens might be involved in the development of paraneoplastic MAR. While cancer-associated retinopathy is presumably caused by an AA19,20 or CTL response against Rec,21–23 MAR is most likely induced by an immune response against multiple retinal antigens16: (i) diverse ophthalmological symptoms have been described in MAR, (ii) dif-
Figure 3 – Histological analysis of mouse eye balls and retinas. (a) Eye ball and retina obtained from a wild-type mouse without any ophthalmological defects. (b) Two transgenic mice which did not exhibit any visible tumors. The retina of mouse TgN#02 was unchanged, while mouse TgN#04 had slight signs of retinal detachment (open arrows) and a weak degeneration of pigment epithelium (open arrowhead). (c) Eye ball and retinas obtained from 3 transgenic tumor-bearing mice (TgT#15, TgT#10 and TgT#09) as representative examples showing retinal degeneration: disorganization of the inner nuclear layer (arrows), fragmentation of the rod and cone layer (arrowheads), retinal detachment (open arrows) and degeneration of pigment epithelium (open arrowhead). (d) Wild-type mouse after transfer of splenocytes and serum from transgenic tumor-bearing mouse exhibited strong signs of retinal and pigment epithelium degeneration (arrows and arrowheads as in c). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS (inner segments) and OS (outer segments) of photoreceptors; RPE, retinal pigment epithelium.
Different MAR patients have degenerations in distinct retinal cell types, (iii) sera of MAR patients stain different layers of the retina, and (iv) AA against 2 photoreceptor proteins, namely Rho18 and Tr,17 have been described in MAR patients. On the basis of these observations, we hypothesize that CRA may also be paraneoplastic antigens in MAR. We have recently reported that these and other photoreceptor proteins could serve as CRA in melanoma.24,25 On the basis of these observations, we investigated here whether CRA may also play a role as paraneoplastic antigens in MAR.

First, we tested CRA expression in the cell line and tumor tissue obtained from a melanoma patient suffering from MAR. We found CRA expression both in the cell line and in the tumor at mRNA and protein levels. In addition, AA against Tr were found in the serum of this patient. AA against Tr have previously been shown to be present in the serum of a patient with MAR.17 Furthermore, we succeeded in finding AA against Arr in the serum of 2 additional MAR patients; one of them also had AA against Tr. To our knowledge, this is the first description of AA against Arr in MAR patients. Unfortunately, we did not have the possibility to check

### TABLE I – OPHTHALMOLOGICAL EXAMINATION OF Ret-TRANSGENIC MICE WITHOUT TUMORS

<table>
<thead>
<tr>
<th>Mouse Tg#</th>
<th>Funduscopy</th>
<th>ERG</th>
<th>Retina histology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
<td>a-Wave 500 cd/m² (&gt;-2.5)</td>
</tr>
<tr>
<td>03</td>
<td>w.p.</td>
<td>w.p.</td>
<td>–3</td>
</tr>
<tr>
<td>06</td>
<td>w.p.</td>
<td>w.p.</td>
<td>–6</td>
</tr>
<tr>
<td>07</td>
<td>w.p.</td>
<td>w.p.</td>
<td>–4</td>
</tr>
<tr>
<td>08</td>
<td>w.p.</td>
<td>w.p.</td>
<td>–15</td>
</tr>
<tr>
<td>09</td>
<td>w.p.</td>
<td>w.p.</td>
<td>–2</td>
</tr>
<tr>
<td>10</td>
<td>Bright spots</td>
<td>–1</td>
<td>149</td>
</tr>
</tbody>
</table>

Transgenic mice without tumors (TgN#01 to 10) were analyzed by funduscopy, ERG and histology. Values and observations differing from normal are bold face. n.d., not done; w.p., without pathology.

1ERG cutoff values are given in brackets.2Photoreceptor layer: 1–3 gives slight to drastic changes.

**Figure 4** – Representative ERGs from a wild-type mouse (a), 2 transgenic mice without (b) and 3 with tumors (c). Mouse TgN#04 showed a reduced b-wave in contrast to the normal response in mouse TgN#02. The tumor-bearing mice showed a reduced b-wave. In addition, mice TgT#10 and TgT#09 showed no a-waves.
CANCER-RETINA ANTIGENS AND MAR

While MAR is a very rare syndrome in melanoma patients, the question arises, why we found retinal degenerations in almost all tumor-bearing animals investigated. It has recently been published that subclinical manifestations of MAR may appear more often than previously suspected in melanoma patients. Authors have investigated about 30 patients with malignant melanoma by ERG, static and kinetic perimetry, and nyctometry. Subclinical MAR symptoms were found in 90% of the patients. This frequency correlates well with our data obtained in melanoma-bearing ret transgenic mice. Thus, retinal degeneration may be a frequent symptom in melanoma patients remaining, however, at a subclinical level. Pföhler et al. have shown that these subclinical manifestations of MAR are more frequent in advance stages of melanoma. Therefore, an ophthalmological examination may not be suitable for an early melanoma diagnosis, although this needs to be tested by further clinical studies in melanoma patients and/or in mice during melanoma development.

Retinal degeneration in melanoma-bearing mice was found to be associated with the expression of several CRA at mRNA and protein levels in their tumors and the presence of serum AA against Arr. These data confirmed our previous observations of CRA expression and circulation of serum AA against Arr, Tr and Rec in some ret transgenic mice and HGF transgenic Ink4a knockout mice bearing melanoma. In the present study, we also detected AA against Arr and Tr in the sera of MAR patients. In addition, we found neither tumor invasion nor ret overexpression in the mouse retina.

All these findings allowed us to propose that immune cells and/or AA could be responsible for the observed retina degeneration similar to other paraneoplastic syndromes. To prove this hypothesis, we isolated splenocytes (as a source of both T and B cells) and serum (as a source of immunoglobulins) from tumor-bearing mice and adoptively transferred them into healthy mice. One quarter of the recipients showed retinal degenerations. The observed effect and adoptively transferred cells may be suitable for an early melanoma diagnosis, although this needs to be tested by further clinical studies in melanoma patients and/or in mice during melanoma development.

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While MAR is a very rare syndrome in melanoma patients, the question arises, why we found retinal degenerations in almost all tumor-bearing animals investigated. It has recently been published that subclinical manifestations of MAR may appear more often than previously suspected in melanoma patients. Authors have investigated about 30 patients with malignant melanoma by ERG, static and kinetic perimetry, and nyctometry. Subclinical MAR symptoms were found in 90% of the patients. This frequency correlates well with our data obtained in melanoma-bearing ret transgenic mice. Thus, retinal degeneration may be a frequent symptom in melanoma patients remaining, however, at a subclinical level. Pföhler et al. have shown that these subclinical manifestations of MAR are more frequent in advance stages of melanoma. Therefore, an ophthalmological examination may not be suitable for an early melanoma diagnosis, although this needs to be tested by further clinical studies in melanoma patients and/or in mice during melanoma development.

Retinal degeneration in melanoma-bearing mice was found to be associated with the expression of several CRA at mRNA and protein levels in their tumors and the presence of serum AA against Arr. These data confirmed our previous observations of CRA expression and circulation of serum AA against Arr, Tr and Rec in some ret transgenic mice and HGF transgenic Ink4a knockout mice bearing melanoma. In the present study, we also detected AA against Arr and Tr in the sera of MAR patients. In addition, we found neither tumor invasion nor ret overexpression in the mouse retina.

All these findings allowed us to propose that immune cells and/or AA could be responsible for the observed retina degeneration similar to other paraneoplastic syndromes. To prove this hypothesis, we isolated splenocytes (as a source of both T and B cells) and serum (as a source of immunoglobulins) from tumor-bearing mice and adoptively transferred them into healthy mice. One quarter of the recipients showed retinal degenerations. The observed effect was specific, since the transfer of splenocytes and serum from wild-type mice induced no changes in the recipients’ retina. In addition to circulating AA, melanoma-bearing mice may possess both CD8 and CD4 T cells specific for CRA. It was demonstrated that the peripheral blood of CAR patients frequently contained Rec-specific CTLs.

It is interesting to note that 1 ret transgenic mouse without clinically visible tumor also showed moderate signs of retina degeneration. Immunohistological analysis of such tumor-free mice revealed microscopic melanoma lesions in their lymph nodes.
suggesting thereby that even small tumor load could already stimulate immunological reactions leading to pathological changes in the retina. We are currently investigating which cellular and/or humoral components of immune systems play a major role in the observed MAR.

Another important question is related with the role of the blood–ocular barrier in the development of this paraneoplastic syndrome. It is still poorly understood how AA and/or T cells can penetrate the ocular capillary endothelium forming the blood–ocular barrier. We suppose that occasional dysfunction of this barrier in tumor-bearing mice may allow the internalization of cellular and/or humoral components of the immune system that may finally result in the development of MAR. A similar dysfunction of the blood–ocular barrier could also occur in MAR patients.16 Moreover, the difference in the level of this dysfunction could explain why retinal degenerations are not always found in both eyes of MAR patients.18,19 Using our mouse melanoma model, we also found that only 62% of affected animals had retinopathy in both eyes.

Even when the first hurdle (blood–retina barrier) is overcome, AA have to enter the retinal cells, as many CRA are located intracellularly. This issue was precisely investigated on Rec antibodies in the works of Adamus’ group.19,20 The authors have shown an AA internalization by the retinal cells and a destruction of photoreceptor and bipolar cells by apoptosis.

In summary, we suppose that—similar to cancer-associated retinopathy—MAR is most likely induced by an immune response originally mediated against the tumor, but targeting retinal components. We propose that a broad range of antigens, most prominently CRA, are the proteins against which the specific immune response is directed, which is supported by our transfer experiments. Future experiments should clarify, whether AA or T cells or both are the cause of MAR, which epitopes are targeted in MAR and in antitumor responses, and which molecular mechanisms are responsible for MAR.

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Figure 5 – Histological analysis of Ret protein expression in the tumor (a) and retina (b) of ret transgenic mice. (a) Ret-positive membrane staining of a tumor (cryosection), obtained from a tumor-bearing transgenic mouse (positive control). (b) Retina obtained from a tumor-bearing mouse is negative for Ret. All scale bars: 100 µm. INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS (inner segments) and OS (outer segments) of photoreceptors; RPE, retinal pigment epithelium.

Table IV – Expression of CRA in the tumor and AA against CRA in sera of tumor-bearing mice with retina degeneration

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<th>Mouse TgT#</th>
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<th>Protein AA</th>
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Mouse number (TgT#) corresponds to the numbers in Table II. n.d., not done.
References


