Published in final edited form as:

Exp Eye Res. 2013 February ; 107: 52–58. doi:10.1016/j.exer.2012.11.012.

M2/M1 Ratio of Tumor Associated Macrophages and PPAR-gamma Expression in Uveal Melanomas with Class 1 and Class 2 Molecular Profiles

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Abstract

Macrophages have been found to be negative predictors of outcome in patients with uveal melanoma. In particular, recent studies point towards a disease-progressing role of proangiogenic M2 macrophages in melanomas with monosomy 3. Although most studies implicate a protective effect of PPAR-gamma activation in tumors, PPAR-gamma has also been shown to promote the polarization of M1 macrophages towards the M2 phenotype. The purpose of this investigation was first, to characterize the phenotype of tumor infiltrating macrophages and second, to study PPAR-gamma expression in uveal melanomas with molecular gene expression profile as prognostic predictors for patients' outcome. Twenty specimens from patients with uveal melanoma were analyzed for clinical and histologic tumor characteristics. The molecular RNA profile (class 1 or class 2) was commercially determined. Using immunohistochemical techniques, the specimens were dual labeled for CD68 and CD163. CD68+CD163– M1 macrophages and CD68+CD163+ M2 macrophages were analyzed in ten high power fields sparing macrophage-poor areas and a mean value was calculated for each tumor. The tumors were immunostained for von Willebrand factor and the mean vascular density (MVD) was analyzed according to Foss. To assess the proliferative rate of each tumor, Ki67 expression was evaluated in ten high power fields followed by calculation of a mean value. Expression of PPAR-gamma was evaluated using a score from 0 (no staining) to 3 (tumor entirely stained). Statistical analysis and a respective correlation was made between histologic characteristics, molecular profile, type of tumor infiltrating macrophages (M1 versus M2), MVD, proliferative rate, and PPAR-gamma expression. Our results showed a

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Conflict of interest

None

Submission declaration

The work has not been submitted elsewhere and is not under consideration for publication elsewhere.

Role of contributors

Each author materially participated in the research (MCH, CB, JRW, TH, HEG) and/or article preparation (MCH, JRW, HEG). All authors have approved the final article.

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correlation between the ratio of M2/M1 macrophages and the molecular profile with a ratio of approximately 1 corresponding to molecular class 1 and a ratio of approximately 2 corresponding to molecular class 2 ($p=0.01$). The ratio of M2/M1 macrophages was higher in tumors with extraocular extension ($p=0.01$). PPAR-gamma was predominantly expressed in the cytoplasm of tumor cells. Its expression showed no association with the molecular RNA profile ($p=0.83$). This study confirmed that the ratio of M2/M1 macrophages is another prognostic factor in uveal melanoma. Thus, polarization of macrophages plays an important role for patients' outcome. PPAR-gamma is expressed in uveal melanoma tumor cells and further studies are warranted to determine its role in tumor biology.

Keywords

macrophages; uveal melanoma; PPAR-gamma; macrophage polarization

1. Introduction

Uveal melanoma is the most common primary intraocular malignancy in the Western hemisphere. Survival of patients with melanoma is dependent on the presence and extent of liver metastasis. Primary tumor characteristics such as ciliary body involvement, extraocular extension, large basal tumor diameter, and epithelioid cell type have been found to be negative predictors of survival (Affeldt et al., 1980; McLean et al., 2004). Inflammatory cells including macrophages are also associated with an unfavorable outcome (Bronkhorst et al., 2012; de la Cruz et al., 1990; de Waard-Siebinga et al., 1996; Maat et al., 2008; Makitie et al., 2001).

1.1. Tumor associated macrophages in uveal melanoma

Tumor associated macrophages (TAMs) have been found to be associated with uveal melanoma-related mortality and other prognostic factors such as the presence of epithelioid cells and a high tumor microvascular density (MVD) (Makitie et al., 2001). Additionally, there is a correlation between uveal melanomas with a monosomy 3 karyotype and an inflammatory phenotype including the presence of TAMs (Maat et al., 2008). TAMs in uveal melanomas with a monosomy 3 karyotype are predominantly pro-angiogenic M2 polarized macrophages (Bronkhorst et al., 2011). In addition to chromosome 3 analysis, other outcome predictors including gene expression profiling have been utilized for uveal melanoma (Onken et al., 2004). Gene expression RNA profiling allows for classification of uveal melanomas into low grade class 1 or high grade class 2 molecular profiles (Onken et al., 2004).

1.2. PPAR-gamma expression in cancer

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors that regulate transcription of various genes. PPAR-gamma is one of three identified subtypes. In addition to its role in adipose cell differentiation, modulation of inflammatory responses, and cellular apoptosis, it is also involved in the regulation of cellular metabolism (Amato et al., 2012; Kulkarni et al., 2012). Thus, an important role of PPAR activation in cancer, which is characterized as a consuming disease with a high energy demand, has been suggested (Amato et al., 2012). PPAR-gamma expression has been already studied in several tumors such as skin melanoma, breast cancer, prostate cancer, colorectal adenoma, endometrial cancer, medulloblastoma, and lung cancer (Bhatia et al., 2012; Kim et al., 2012; Knapp et al., 2012; Lee et al., 2008; Meyer et al., 2009; Papadaki et al., 2005). Most studies implicate a protective effect of PPAR-gamma activation in tumors (Knapp et al., 2012; Meyer et al., 2009; Nemenoff, 2012). But PPAR-gamma has also been shown to promote

the polarization of M1 macrophages towards the M2 phenotype that is associated with disease progression in cancer (Fujisaka et al., 2009; Nemenoff, 2012). The expression and role of PPAR-gamma in uveal melanoma is still undetermined.

1.3. Purpose of this study

We propose that uveal melanomas with a high risk class 2 gene expression profile have a higher M2/M1 ratio than tumors with a low risk class 1 gene expression profile. We therefore evaluated the polarization of macrophages in a group of twenty uveal melanomas that had been characterized by molecular profile and other prognostic factors. To assess the M2/M1 ratio macrophages were analyzed in ten high power fields excluding macrophage-poor areas from the evaluation.

We further assume that PPAR-gamma is expressed in uveal melanoma since its expression has been described in several tumors as well as in macrophages. Thus, PPAR-gamma expression was analyzed in the same cohort of uveal melanomas.

2. Material and Methods

2.1. Patients and Specimens

Between December 2009 and August 2011, 22 eyes were enucleated for uveal melanoma after careful clinical examination of the patients by the Oncology Service at the Emory Eye Center, Atlanta, GA. The study was conducted after Emory University Institutional Review Board (IRB) approval and with adherence to the Declaration of Helsinki. Fresh tissue biopsies were collected after enucleation of the eye and stored at -80°C in a specimen collection kit (Castle Biosciences). Alternatively, sections of formalin-fixed paraffin-embedded (FFPE) tissue were used for analysis. The specimens were sent for a commercially available analysis (using real-time polymerase chain reaction, RT-PCR) of the molecular gene expression profile to Castle Biosciences, Phoenix, AZ.

Two of the 22 specimens were excluded for insufficient amount of tumor ($n=1$) and extensive necrosis ($n=1$), respectively. The epidemiologic characteristics (age, gender) and clinical parameters (such as previous treatment) of the twenty remaining patients were obtained. The tumors were staged based on histopathological criteria in accordance with the American Joint Committee on Cancer (AJCC) staging system for uveal melanoma (AJCC, 2010).

2.2. Histologic evaluation

The specimens were submitted in 10% formaldehyde and were routinely processed for light microscopic examination and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The histological specimens were analyzed for size (largest basal diameter in millimeters), cell type (spindle type, mixed type, epithelioid type), pigmentation (heavy, moderate, mild), ciliary body invasion (yes/no), extraocular extension (yes/no), and number of mitotic figures per 40 high power fields (HPF). The latter was assessed by light microscopy calculating the average of mitotic figures in the tumor on imaginary 40 high power fields.

2.3. Immunohistochemical Staining

Immunohistochemical stains for CD68 (clone KP1; Novus Biologicals, Littleton, CO; dilution 1: 500), CD163 (clone K20-T; Enzo Life Sciences, Plymouth Meeting, PA; dilution 1:50), von Willebrand factor (Kit ECM590; Millipore, Temecula, CA; dilution 1:200), Ki67 (clone Ki-S5; Millipore; dilution 1:100), and PPAR-gamma (polyclonal antibody [ab19481]; Abcam, Cambridge, MA; dilution 1:100) were performed. 5 μm thick sections were cut

using a manual rotary microtome (Thermo Scientific Shandon Finesse 325 Microtome). After deparaffinization and rehydration, heat-mediated antigen retrieval was performed with 0.01M citrate buffer 2X, 10 minutes each. For CD68-CD163 dual staining and when necessary for the other primary antibodies, bleaching with a hydrogen peroxide solution (3% H₂O₂ in 1% Na₂HPO₄) was performed over 18 hours after deparaffinization and dehydration, followed by rinsing with 1% acetic acid. Washing steps were performed with TBST buffer (Tris buffer [TBS: 50mM, pH 7.6] plus 0.1% Tween 20). After blocking with hydrogen peroxide and Ultra V Block, the specimens were incubated overnight at 4°C with the primary antibody. For the single-staining procedure, the antigen-antibody-binding reaction was visualized with a labeled streptavidin-biotin immunoenzymatic antigen detection system using horseradish peroxidase (HRP) and 3-amino-9-ethylcarbazole (AEC) as the chromogen (Thermo scientific, Fremont, CA; TL-015-HA). Dual staining was conducted with the multivision polymer detection system (Thermo scientific; TL-012-MARH) using HRP-conjugated polymer (red) for the rabbit anti-human antibody (CD163) and alkaline phosphatase-conjugated polymer (blue) for the mouse anti-human antibody (CD68). Appropriate positive and negative controls were employed for each antibody.

2.4. Evaluation of immunohistochemical staining

The number of CD68+CD163⁻ macrophages (M1MΦ) and CD68+CD163⁺ macrophages (M2MΦ) were determined in ten HPFs (at 40X magnification) within a representative section including central and peripheral areas of the tumor sparing macrophage-poor areas. A mean value was calculated for each tumor. The cell bodies (not single dendrites) were counted and cells with only CD68 expression were considered as M1 macrophages and dual labeled cells for CD68 and CD163 were regarded as M2 macrophages (Supplemental Fig. 1 and 2). We decided to exclude macrophage-poor areas from the evaluation as we were more interested in the polarization than the overall number of macrophages. Thus, we focused on the ratio of M2/M1 macrophages.

The MVD was evaluated according to the method described by Foss et al (Foss et al., 1996). Briefly, the most vascular portion in a representative tumor section was identified with low magnification. The number of von Willebrand factor-positive vascular channels per high power field (40X magnification) was determined in three HPFs and the mean value was used for further calculations.

The proliferative rate was assessed counting the Ki-67 positive cells per HPF (40X objective) within a representative tumor section and a mean value was determined for each tumor. PPAR-gamma staining in the tumor was graded as follows: “0” no staining reaction, “1” one third of the tumor stained, “2” two third of the tumor stained, “3” tumor entirely stained. A mean value was calculated for each tumor.

All immunohistochemical evaluations were performed twice, blinded to the molecular profiling results.

2.5. Statistical Analysis

Statistical analysis was performed with SPSS (IBM SPSS Statistics 20; Armonk, NY) using the t-test, Fisher’s exact test and the Pearson correlation coefficient. Comparisons between the molecular classes (class 1 and class 2), macrophage polarization as ratio of M2/M1 MΦ, and several other parameters such as gender, age, cell type, largest basal diameter, pigmentation, mitotic figures/HPF, ciliary body invasion, extraocular extension, MVD, the proliferative rate (Ki-67 expression), and PPAR-gamma expression were conducted. The probability for the alpha error was set to $p < 0.05$ two-sided.

The investigated group of twenty specimens showed a Gaussian distribution for macrophages.

3. Results

3.1. General Analysis of the Cohort

Our cohort consisted of 20 patients with 10 patients in each molecular class (Tab. 1). Gender was evenly distributed within the subgroups. The patients' age ranged from 24 to 86 years.

3.2. Analysis of M1 and M2 Macrophages

Macrophages were found in all tumors. The ratio of M2/M1 MΦ was approximately 1 (0.89 ± 0.5) in tumors with a class 1 molecular profile and approximately 2 (2.41 ± 1.6) in tumors with a class 2 molecular profile (Fig. 1&2, Tab. 2) ($p = 0.01$). A high ratio of M2/M1 MΦ was associated with the presence of extraocular extension ($p = 0.01$), which is considered a negative prognostic parameter. A larger basal diameter was found in tumors with a high M2/M1 ratio as seen by a regression line (R^2 Linear = 0.191). MVD ($p = 0.98$), Ki67 expression ($p = 0.54$) and other prognostic histologic parameters were not associated with the macrophage ratio.

3.3. Further observations

Two patterns of infiltrating macrophages were observed: tumors that exhibited an even distribution of infiltrating macrophages and tumors in which the macrophages clustered in the marginal areas and in strands (often associated with vascular channels). Additionally, we observed that the large, round, pigment-bearing macrophages were more likely to exhibit an M1 phenotype and dendritic macrophages more likely to exhibit an M2 phenotype (Fig. 3).

3.4. PPAR-gamma expression

PPAR-gamma expression was detected in 19 of 20 tumors. The staining was confined to the cytoplasm of uveal melanoma cells (Fig. 4 a,b). In control sections of placenta, PPAR-gamma expression was regularly located in the nucleus. PPAR-gamma was expressed by spindle-shaped as well as by epithelioid uveal melanoma cells. Only a few of the positively stained cells were identified as macrophages. The staining reaction was unevenly distributed within the tumor and varied within different regions (Fig. 4c). In a few tumors, the staining reaction appeared more intense in the neighborhood of blood vessels. The staining intensity in tumors with a class 1 molecular gene expression profile was 1.78 ± 0.71 versus 1.70 ± 0.81 in class 2 tumors ($p = 0.83$) (Fig. 5). There was no correlation with the ratio of M2/M1 macrophages ($p = 0.2$). The normal choroidal melanocytes did not express PPAR-gamma.

4. Discussion

4.1. Comparison of study results with the literature

Our hypothesis was based on a previous study that detected more M2 macrophages in uveal melanomas with monosomy 3 than in uveal melanomas with disomy 3 (Bronkhorst et al., 2011; Jager et al., 2011) and the finding that the RNA profile can predict prognosis in analogy to chromosome 3 status. We therefore expected a relationship between macrophage polarization (ratio of M2/M1 MΦ) and uveal melanoma molecular gene expression profile (class 1 vs. class 2). Analysis of 20 tumors revealed that a M2/M1 MΦ ratio of 1 was typical for class 1 tumors while a ratio of approximately 2 was more characteristic for uveal melanomas class 2. Thus, our findings support the concept that in addition to macrophage number, polarization of the macrophages is important regarding predicting survival (Bronkhorst et al., 2011; Jager et al., 2011). In recent studies, M2 macrophages were suggested to play the major role in the pathogenesis of uveal melanoma and other

malignancies (Bronkhorst et al., 2011; Mantovani et al., 2010). However, M1MΦ may have a protective role in uveal melanoma. For the interpretation of our data, it has been kept in mind that we did not investigate the total number of macrophages because previous studies had already shown that the number of macrophages is related to an unfavorable prognosis for survival (de Waard-Siebinga et al., 1996; Maat et al., 2008; Makitie et al., 2001). In our study, areas in the tumor that were infiltrated by macrophages were counted to assess their polarization in order to build ratios of M2/M1 MΦ. Furthermore, in a study comparing data with regard to the role of lymphocytes in tumors, ratios have been found to be more informative than quantitative counts (Gooden et al., 2011). Age was not related to macrophage infiltration in our study. However, a previous animal study suggested an association between the number of M2MΦ and the patient age for uveal melanoma (Ly et al., 2010).

4.2. PPAR-gamma expression in cancer

PPAR-gamma was found in the cytoplasm of predominantly tumor cells in 19 of 20 uveal melanomas. For PPAR-gamma, a nuclear localization has been described. In our control tissue (placenta), the expected nuclear staining reaction was found. Nuclear staining for PPAR-gamma was also reported in skin melanoma (Lee et al., 2008; Meyer et al., 2009) and colorectal adenoma (Kim et al., 2012). Perinuclear staining was found in endometrial cancer (Knapp et al., 2012). However, in breast cancer, medulloblastoma, skin melanoma cells, and lung cancer, cytoplasmic staining was reported and/or illustrated (Bhatia et al., 2012; Freudlsperger et al., 2006; Giaginis et al., 2012; Papadaki et al., 2005). Upon mitogenic stimulation, a MAPK/ERK kinase-dependent shuttle may be involved in nuclear export of PPAR-gamma (Burgermeister et al., 2007). Activation of the MAPK pathway, that is itself activated by GNAQ mutations (Harbour, 2012), has been found to be commonly involved in the development of uveal melanoma (Zuidervaat et al., 2005).

In general, expression of PPAR-gamma in primary skin melanoma was - in accordance with our results - not associated with the overall survival (Meyer et al., 2009). With regard to the pathways involved in the PPAR-gamma export from the nucleus into the cytoplasm, a difference of PPAR-gamma localization between class 1 and class 2 tumors is not expected. Metastases from skin melanoma with a high PPAR-gamma expression were associated with a longer progression-free survival and a better response to biomodulatory therapy compared to metastases with a lower PPAR-gamma expression (Meyer et al., 2009). Upregulation of PPAR-gamma via ligands was found to inhibit cancer cell proliferation (Kulkarni et al., 2012; Nemenoff, 2012). In addition, pigment epithelium-derived factor (PEDF), whose overexpression inhibits uveal melanoma growth and metastasis (Yang et al., 2010), upregulates PPAR-gamma expression (Hirsch et al., 2011; Ho et al., 2008). In contrast, PPAR-gamma agonists such as pioglitazone stimulates the polarization of M1 macrophages towards the M2 phenotype (Fujisaka et al., 2009; Nemenoff, 2012). At first glance, this looks contradictory. However, Nemenoff pointed out that PPAR-gamma activation plays a dual and opposing role in cancer (Nemenoff, 2012). In macrophages, PPAR-gamma leads to an alternatively activated phenotype resulting in tumor growth and metastasis. In cancer cells, it inhibits proliferation and promotes differentiation (Nemenoff, 2012). He concluded that the effect of PPAR-gamma activators “depends on which cell type is playing a dominant role” (Nemenoff, 2012). Although macrophages seem to play a significant role in uveal melanoma, our study indicates a predominant PPAR-gamma expression in tumor cells. However, other scenarios with loss of PPAR-gamma activation resulting in upregulation of NF-κB in an inflammatory phenotype also seem possible (Maat et al., 2008).

This may suggest a potential therapeutic role for PPAR-gamma activators for this tumor, but further studies are warranted to investigate the role of PPAR-gamma in uveal melanoma before a final statement is made. Further information is needed regarding the role of PPAR-

gamma for different phases of uveal melanoma progression and for the development of metastases.

4.3. Shortcomings of this study

Shortcomings of our study are the small sample size and the dual labeling of the macrophages. Although the dual labeling of the M2MΦ was clearly visible after immunohistochemical staining, RNA isolation and quantitative PCR measurements for CXCL11 (for M1MΦ) and CCL22 (for M2MΦ) may enhance the analysis (Cao et al., 2011). However, the RNA yield from formalin-fixed paraffin-embedded tissue is limited and localization of macrophages within the tumor is not feasible.

In conclusion, our study supports the concept that macrophage polarization influences patient outcome since a high ratio of M2/M1 macrophages is associated with a class 2 molecular gene expression profile. While previous studies have shown that proangiogenic M2 macrophages lead to the development of metastasis, there is also some evidence from this study that tumoricidal M1 macrophages may have a protective role.

PPAR-gamma was expressed in uveal melanoma but further studies are warranted to shed more light on a possible protective role of PPAR-gamma in this tumor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study was presented in part at the Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO, 2012) and the Biennial Meeting of the International Society for Eye Research (ISER, 2012).

Financial support

Supported in part by the German Research Foundation (DFG), Bonn, Germany (MCH: HE 5775/3-1) and an unrestricted departmental grant (HEG: R01CA126557, Departmental Core Grant, NIH P30EY06360). Dr. Grossniklaus is a Research to Prevent Blindness Senior Scientific Investigator (RPB Inc., New York, NY).

The sponsor or funding organization had no role in the design and conduct of this research.

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Highlights

- Macrophage (MΦ) polarization is a prognostic factor in uveal melanoma.
- The ratio of M2/M1 MΦ correlates with the molecular gene expression profile.
- PPAR-gamma is expressed in the cytoplasm of tumor cells.

Fig. 1. Immunohistochemical pattern of class 1 tumors (A) and class 2 tumors (B). Class 1 tumors showed more blue-only M1 macrophages (M1M Φ , arrow) indicating CD68 staining (A; CD68-CD163 dual labeling, 40x). In class 2 tumors, more M2 M Φ (arrow) were found that were blue (CD68 expression) and red (CD163 expression) (B; CD68-CD163 dual labeling, 40x).

Fig. 2.

A high ratio of M2/M1 MΦ was found in tumors with a class 2 gene expression profile while a ratio of 1 was characteristic for class 1 tumors ($p = 0.01$). The bottom and the top of the boxplot represent the 25th and 75th percentile, respectively. The line within the box is the 50th percentile (median). The whiskers represent the highest and lowest values that lie within 1.5 times the interquartile range.

Fig. 3. The round pigmented macrophages (insert; H&E, 40x) showed more often an M1 phenotype (A, arrow; CD68-CD168 dual labeling, 40x). In contrast, dendritic macrophages (arrow) were more likely to exhibit the M2 phenotype (B; CD68-CD163 dual labeling, 40x).

Fig. 4. PPAR-gamma was predominantly expressed in the cytoplasm of uveal melanoma cells (A, PPAR-gamma staining, 20x; B, PPAR-gamma staining, 40x). In some tumors an uneven distribution of PPAR-gamma expression was observed (C, PPAR-gamma staining, 10x).

Fig. 5. PPAR-gamma expression was expressed in a similar intensity in tumors with a class 1 and class 2 gene expression profile ($p = 0.83$).

Tab. 1

Baseline Characteristics of our Cohort. [HPF = high power field]

	Class 1	Class 2	Significance
Gender			
Male	n = 5	n = 5	p = 1.0
Female	n = 5	n = 5	
Age	56 ± 13 years	68 ± 20 years	p = 0.126
Cell type			
Spindle cell type	n = 1	n = 0	p = 0.089
Mixed cell type	n = 9	n = 8	
Epithelioid cell type	n = 0	n = 2	
Largest basal diameter	14 ± 5.8 mm	17.3 ± 1.6 mm	p = 0.082
Ciliary body invasion	n = 3	n = 7	p = 0.081
Pigmentation			
minimally	n = 6	n = 7	p = 1.0
moderately	n = 3	n = 1	
heavily	n = 1	n = 2	
Mitotic figures [per HPF]	2.5 ± 1.6	3 ± 1.4	p = 0.22

Tab. 2

Statistical Analysis of M2/M1 ratio with the analyzed tumor characteristics

	Pearson correlation coefficient	Significance two-sided
Molecular Class	,571	,009 **
Age [years]	,194	,412
Type of UM [spindle, mixed, epithelioid]	,129	,588
Largest basal diameter	,436	,054
Pigmentation of UM	-,199	,401
Mitotic figures [per 40 HPF]	,386	,093
Ciliary body extension	,300	,198
Extrasccleral extension	,561	,010 *
M1 macrophages	-,679	,001 **
M2 macrophages	,494	,027 *
MVD	,162	,496
Ki67 expression	,048	,842

* Significance (two-sided) of 0.05.

** Significance (two-sided) of 0.01.