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CHAPTER 8

Effect of hypoxic stress on induction and plasticity of tumor-induced immune cells in uveal melanoma


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ABSTRACT

Purpose. Highly malignant uveal melanoma contain increased numbers of lymphocytes and macrophages, especially of the M2 phenotype. We wondered whether hypoxia plays a role in the development of this inflammation. Hypoxia is known to activate HIF 1α and NF-κB, and these factors may induce expression in malignant cells that recruits leukocytes (through release of chemokines and cytokines). We analysed whether hypoxia induces uveal melanoma cells to release pro-inflammatory cytokines, and whether tumor supernatant (TSN) affects monocyte migration and differentiation.

Methods. The expression of pro-inflammatory genes in freshly cultured uveal melanoma was studied in an in vitro 24 hour hypoxic culture system using quantitative PCR for expression. Cell lines were cultured under normoxic and hypoxic conditions. Chemotaxis was tested using a transwell system with purified monocytes and TSN. Differentiation was tested by adding TSN to a monocyte-DC culture. CCL2, IL-6 and PGE2 levels in TSN were determined by ELISA.

Results. Exposure of freshly-cultured uveal melanoma cells to hypoxia led to an increased expression of the pro-inflammatory cytokines PLGF, TGFβ, END1, ICAM1 and a lower expression of AIMP1 (EMAP2), CCL2 (MCP-1), and IL1b. TSN from melanoma cells lines, cultured in normoxic as well as hypoxic conditions, was able to attract monocytes. Migration was independent of tumor-produced CCL2. Uveal melanoma supernatant inhibits monocyte differentiation.

Conclusions. Under hypoxic conditions, immune response genes are differentially expressed in cultured primary uveal melanoma cells. TSN from uveal melanoma cell lines is capable of affecting the chemotactic response of monocytes in vitro, irrespectively of the hypoxic or normoxic conditions. Our data suggest that the induction of immune cells is not dependent on intra-tumoral oxygen level, and the uveal melanoma TSN does not skew macrophage polarization phenotype.
INTRODUCTION

Under physiological conditions, tissue is characterized by normoxia. Tissue oxygenation is severely disturbed during pathological conditions such as cancer, which is associated with a local decrease in pO2, i.e. hypoxia. Hypoxia occurs during tumor development. A hypoxic environment influences the formation of metastases. By creating selective pressure and by promoting outgrowth of cells that can circumvent the oxygenation restrictions. For example, hypoxia induces stabilization of hypoxia-inducible factor (HIF) 1α, which upregulates cell-matrix adhesion, invasion and tumor angiogenesis via a variety of mechanisms. In addition, hypoxic changes of the tumor cells themselves may induce expression of specific patterns of genes which confer a survival advantage on cancer cells, allowing tumor growth and spread in this environment. Therefore, tumor hypoxia could function as a key mediator of tumor progression by promoting the expansion of cells with a more aggressive phenotype, and may influence therapy efficiency.

An understanding of the direct role of hypoxia in advanced uveal melanoma is slowly starting to emerge. HIF-1 has been shown to play a critical role in uveal melanoma progression by increasing the expression of a number of target genes involved in invasion. Moreover, HIF-1 has been identified as one of the most critical biomarkers that can predict uveal melanoma metastasis. In uveal melanoma, the genetic progression from 1A-1B-2A to 2B probably represents an adaptation to multiple selective pressures, including hypoxia, immune responses and other factors. When HIF-1α protein is stabilized and translocated to the nucleus, it induces transcription of target genes involved in oxygen delivery and energy metabolism. HIF-1α was shown to be constitutively stabilized and active in at least 50% of the human uveal melanoma. This can happen in hypoxic conditions, but also under influence of other environmental stress, like inflammation.

Inflammation is an important characteristic of malignancy, which includes the presence of tumor-associated macrophages (TAM) and lymphocytes. Tumor cells produce chemotactic cytokines and growth factors that recruit peripheral blood monocytes into the tissue, where they subsequently differentiate into tissue macrophages. Chemokine agonists that accomplish this recruitment, include colony-stimulating factor-1 (CSF-1, also known as M-CSF), monocyte chemotactic protein-1 (MCP-1 also known as CCL2), CCL5 (rantes), CCL7, CCL8, CXCL12 (SDF-1), PDGF and VEGF. Macrophages can differentiate into cells with a pro-inflammatory (M1) phenotype or into the M2 phenotype that is oriented towards tumor growth, angiogenesis, and immune-suppression. M2 macrophages produce anti-inflammatory cytokines including IL-10 and TGF-β as well as molecules associated with increased angiogenesis and metastasis, such as VEGF. In uveal melanoma, an increased density of TAMs correlates with tumor size, microvessel density, and a poor prognosis. Moreover, almost all macrophages in uveal melanoma belong to the M2 subtype.
Monocytes tend to migrate to hypoxic areas, as hypoxia-dependent cytokines are upregulated, such as VEGF, that attract macrophages. In uveal melanoma, larger tumor size is associated with a higher risk of metastases, and we hypothesize that the exponential growth of uveal melanoma will induce ischemia, induce cytokine production and will lead to the influx of inflammatory cells. We already know that when uveal melanoma cells are cultured in a hypoxic environment, production of VEGF is increased through induction of the HIF-1α pathway. We determined whether hypoxia influenced the expression of macrophage-specific chemokines in primary uveal melanoma and uveal melanoma cell lines, and whether hypoxia-derived cytokines help macrophages to acquire a polarized M2 phenotype.

**MATERIALS AND METHODS**

**Primary cell cultures**

Fresh tissue from eight tumors, obtained immediately after enucleation, was placed in Amniochrome Pro Medium (Lonza Group Ltd., Basel, Switzerland) to develop a primary cell culture. Tumor cells were cultured under normoxic or hypoxic conditions for 24 hours, after which time we analyzed expression of selected inflammation- and hypoxia-related genes.

**Chromosome 3 status**

Chromosome 3 status was determined by standard cytogenetic testing, i.e. karyotyping and fluorescence in situ hybridization (FISH) on cultured cells. When either test on cultured cells showed monosomy 3, the tumor was categorized as having monosomy 3.

**Quantitative PCR**

By using an RNeasy Mini Kit (Qiagen, Valencia, CA), RNA was extracted from primary cultures. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR (qPCR) was performed in duplo on selected genes that are associated with inflammation. GAPDH, β-actin, RPL13, and RPS11 were initially included for selecting suitable reference genes. Primers were designed with Beacon Designer (Biosoft, Palo Alto, CA). qPCR was performed according to our standard laboratory protocol, as described previously. By using the CFX-384, it was possible to perform qPCR synchronically on several genes. The PCR reaction settings were 95°C for 3 minutes, 40 cycles containing 96°C for 10 seconds and 60°C for 30 seconds. The results were subsequently validated with the iQ5 Bio-Rad system (Bio-Rad). Calculation of the gene expression was as follows: the Ct value of each sample obtained from qPCR was normalized to the reference genes (genes that are stably expressed in the tissue). Because two genes were
stably expressed (BACT and RPS11, as determined with the geNorm software\textsuperscript{20}), the gene of interest was corrected for the geometric mean of these reference genes, according to the method of Vandesompele et al.\textsuperscript{21} The calculated values were the normalized values of each sample.

Collecting tumor supernatant
Supernatant of uveal melanoma cell lines (OCM8, 92.1, Mel 270, Mel 290 and OMM2.5), primary uveal melanoma cell cultures (10.019 and 12.009), and a lung adenocarcinoma culture (metastasis in the eye; 10.015) was collected. Tumor cell lines were grown in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin and L-glutamine. Uveal melanoma cell lines were grown in flasks at 80–90% confluency and harvested with trypsin/EDTA. The primary cell cultures were grown in T-flasks. For the migration assay, \(1\times10^6\) cells were plated in T75 flasks in 6 ml medium. Supernatant was collected after 24h, 48h, 72h and 96h. Supernatant was stored at \(-20^\circ\text{C}\). For the differentiation assay, 100,000 cells of OCM8, 150,000 cells of 92.1 and 250,000 cells of Mel 270 were plated in 2ml/well of a 6-well culture plate and cultured for 4 days. Supernatant was stored at \(-80^\circ\text{C}\).

Migration assay
The protocol for the migration assay was kindly provided by A.M. van der Does (Departments of Physiology and Pharmacology and Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden). Monocyte migration was measured with a modification of the method of Boyden, by using a 96-well microchemotaxis transwell system with a membrane of 8µm pore size (Corning® HTS Transwell® 96 well permeable support). CD14 purified monocytes were added in the insert and in the lower chamber, we added the tumor supernatant (TSN) of interest. CD14 purified monocytes were resuspended in medium and placed in the upper compartment (\(1\times10^5\) cells in 100µl). TSN, medium (negative control), and medium with MCP1/LPS (positive control) (100ul; 100 ng/ml CCL2 and 200 ng/ml LPS) were added to the lower compartments, and subsequently incubated for 16h at 37 °C. The next day, we removed the 96 well filter rack, and pipetted the liquid from the lower wells into small FACS tubes. The wells were rinsed with 100 µl 5 mM EDTA and this was added to the FACS tubes. Migratory responses were quantified by counting monocytes obtained from the wells with flow cytometry (30 seconds).

Differentiation assay
The influence of tumor-produced cytokines on macrophage differentiation was determined as described previously.\textsuperscript{22} Using this in vitro test, it is possible to show a differentiation into M2 macrophages. In short, to establish a mo-DC (monocyte derived dendritic cells) culture, peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy donors. CD14+ monocytes (95% purity) were isolated using MACS cell
separation and stored in liquid nitrogen until further use. Monocytes were thawed and cultured in 24-well plates in a density of 0.5 million cells/well in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml) (mo-DC) and 20% TSN. 20% TSN of the cervical cancer cell line CC-8, which is known to induce M2 macrophages, was used as a positive control. After 3 days, fresh medium with cytokines and TSN was added. At day 6, the cells were stained and analyzed for differentiation by flow cytometry.

Flow cytometry
Mouse mAbs to human CD14 (PE), CD1a (APC), CD206 (FITC) and CD163 (APC) were used. Cells were recorded (20,000/live gate) using a BD FACSCalibur with Cell Quest software (BD Biosciences) and analyzed by FlowJo software.

Cytokine protein analysis
CCL2, IL6, and PGE2 production in the TSN was measured with a commercially available ELISA kit from eBioscience.

RESULTS

Effect of hypoxia on macrophage-attraction molecules
Using qPCR, we found that hypoxia induced a differential cytokine response in all eight primary uveal melanoma cell cultures, with upregulation of VEGF, our control GLUT1, and the other pro-inflammatory genes PLGF, TGFβ, endothelin 1, and ICAM1. In contrast, AIMP1 (EMAP2), CCL2 (MCP-1), and IL1b were significantly downregulated (paired t-tests) (Fig. 1).

Effect of chromosome 3 status
When comparing the inflammatory gene expression of four disomy and four monosomy 3 tumors, CCL2 and IL6 (both \( P = .11 \)) expression tended to be higher in monosomy 3 tumors, whereas all others, for example VEGF (\( P = .88 \)), show no difference (Mann Whitney test) (Fig. 2). Of the two other primary uveal melanoma cultures of which TSN was used in the further experiments, 10-019 was the disomy tumor and 12-009 the monosomy 3 tumor.

Migration assay
Peripheral-blood derived monocytes migrated towards the TSN derived from uveal melanoma cell lines and the primary cultures. However, we did not observe a difference in effect of TSN from cells grown under normoxia or hypoxia conditions (Fig. 3). The TSN from the three primary cultures were also able to induce migration, of which the control lung adenocarcinoma cell culture 10.015 attracted most monocytes (data not shown).
CCL2 expression

We specifically analyzed CCL2 (MCP-1), which is one of the best known recruiters of monocytes. Cultured uveal melanoma cell lines OCM8, OMM2.5 and Mel 290 expressed CCL2 in a time-dependent manner with a maximum peak after 96 hours in culture, but 92.1 and Mel270 did not. The concentration of CCL2 was lower when the cells were grown under hypoxia than normoxia (Fig. 4A). The primary cultures expressed CCL2 (Fig. 4B).
Figure 3. Migration assay. Monocytes were added in an insert and tumor supernatant (TSN) was added underneath the insert. After 16h, the number of migrated cells was counted with flow cytometry. Mixture of CCL2 and LPS served as a positive control. The TSN from all five cell lines had migration-inducing capacity, which was independent of normoxic or hypoxic conditions.

Figure 4. CCL2 expression. A.) Supernatants (TSN) from different uveal melanoma cell lines grown under hypoxia and normoxia were tested after 24h, 48h, 72h and 96h for CCL2 expression. CCL2 concentrations in the TSN of cell line 92.1 and Mel270 was below 5pg/ml. The concentration in Mel290 TSN could only be analyzed and detected at a low level after 96 hours. B.) Primary uveal melanoma cell cultures 10-019, 12-009 and the lung adenocarcinoma cell culture 10-015 express CCL2.
**Differentiation assay**

GM-CSF- and IL4-differentiated mo-DCs are characterized by a high expression of CD1a and a low expression of CD14. Addition of 20% TSN of three different uveal melanoma cell lines exposed to either hypoxia or normoxia conditions, and the two primary uveal melanoma cell cultures were not able to differentiate monocytes into CD14+ macrophages, while TSN from cell line OCM8 inhibited the formation of mo-DC (very low expression of CD1) (Fig. 5). Only cell line CC-8 (positive control) and lung adenocarcinoma cell culture 10.015 had the potential to differentiate mo-DC into M2 macrophages, showing

![Image of differentiation assay](image)

**FIGURE 5.** Differentiation assay. Monocytes were cultured for 6 days with GM-CSF and IL-4 in 20% control medium, or in culture medium with 20% tumor supernatant (TSN) of the indicated cell lines. Cells were analyzed for the expression of the mo-DC marker CD1, macrophage marker CD14, and M2 marker CD163. TSN from CC-8 cells served as a positive control as it is known to induce M2 macrophages (high expression of CD163). Soluble factors derived from uveal melanoma cell lines (whether in normoxic or hypoxic conditions) and primary uveal melanoma cell cultures do not skew the differentiation towards M2 macrophages, while the lung adenocarcinoma cell line 10.015 has the capacity to skew mo-DC towards CD14+ macrophages with a high CD163 expression.
low CD1, high CD14 and high CD163 expression. Thus, mo-DCs do not differentiate into M2 macrophages by TSN of uveal melanoma cells.

**IL6 and PGE2 expression**

Our tested cell lines do not produce IL6 or PGE2. The primary uveal melanoma cell culture 10.019 did not express IL6 or PGE2 but 12.009 and the lung adenocarcinoma 10.015 did (Fig. 6). This could be verified on mRNA level, as IL6 showed extremely low expression in all cell lines tested (data not shown), but is detectable in primary cultures (Fig. 2).

![Figure 6. PGE2 and IL6 expression. Uveal melanoma cell line 92.1, OCM8, Mel 290, OMM2.5 and the primary uveal melanoma cell culture 10.019 do not express PGE2 or IL6. However, 12.009 and the lung adenocarcinoma culture 10.015 express IL6 and PGE2.](image)

**DISCUSSION**

Part of the inflammatory response involves the massive migration of immune cells from the circulation to the site of the tumor. One of the signals that guides the cells is the hypoxia that arises when the blood vessels at tumor site are disrupted. Hypoxia can also lead to an inflammatory response. By negatively regulating the local adaptive immunity, hypoxia may prevent excessive activation of the host’s immune defense, which might otherwise lead to an anti-tumor response. We hypothesize that, under hypoxic conditions, uveal melanoma acquire an inflammatory phenotype, and activate immune gene expression by its tumor cells. This can have an effect on immature inflammatory cells, thereby undergoing differentiation. If this would be indeed the case, it will be interesting to see whether targeting hypoxia-dependent signaling pathways\textsuperscript{23} will enable a clinically-significant reduction in the pro-tumoral inflammatory response in uveal melanoma.

Eltzschig and Carmeliet discuss the cross-talk between hypoxia and inflammation, and how this is implicated in cancer.\textsuperscript{24} The link between hypoxia and inflammation is
regulated by HIF, which might interact with the nuclear factor kappa-B (NF-kB) transcription factor that regulates inflammation. We hypothesised that hypoxia is essential for myeloid cell infiltration and activation in vivo. The master transcription factor HIF1α binds to HREs (hypoxia response elements) in the promoter of its target genes to activate their expression.23 One of these targets is VEGF, which is increased under influence of hypoxia in vitro18 and overexpressed in 20/60 (33%) cases of primary uveal melanomas.9 Besides affecting VEGF, hypoxia-induced changes in gene expression in uveal melanoma cells may help to increase the production of inflammatory signaling molecules. When we look at the effect of hypoxia on the inflammatory gene expression of the primary uveal melanoma cultures, we observe upregulations as well as downregulations of mRNAs as a consequence of hypoxia. As VEGF was most differentially expressed molecule. Approach- ing this as a cell autonomous process, we compared the expression with chromosome 3 status. As von Hippel-Lindau (VHL) is located on chromosome 3, loss of this factor will result in loss of inhibition of HIF1α. Only IL6 and CCL2 differed in those groups.

The combination of all factors may be sufficient to induce macrophages, and therefore we wondered what the overall effect of TSN on monocyte migration would be. We performed a migration assay where we tested the different TSN and showed that these, confirming the observations in situ earlier, are able to attract monocytes. Assuming that the migration of monocytes is dependent on CCL2, we could show on a protein level that the uveal melanoma cell line OCM8, Mel 290 and OMM 2,5, as well as the primary uveal melanoma cell cultures expressed CCL2. However, there is no correlation between expression of CCL2 and the number of migrated cells. To our surprise, we did not see a difference between TSN from cells cultured under hypoxic or normoxic conditions.

Macrophage phenotype can vary in different areas of a tumor. In a mammary adenocarcinoma model, TAMs with high expression of major histocompatibility complex (MHC) class II can localize to normoxic tumor tissues and express M1 markers as well as antiangiogenic chemokines, whereas TAMs with low expression of MHC class II were found in hypoxic tumor tissues, preferentially expressed M2 markers and had greater pro-angiogenic functions.26 The majority of macrophages in uveal melanoma are of the M2 type. It was shown that TSN of cervical cancer cell lines can screw mo-DC into a M2 phenotype and that this process is dependent on IL6 and PGE2.22 When performing the differentiation assay, none of the tested uveal melanoma cell lines grown under normoxia and hypoxia, and the primary cell cultures, could differentiate mo-DC into macrophages. Therefore, there must be other soluble factors that play a role in uveal melanomas than in cervical cancer cell lines and (lung) adenocarcinomas.27 Another study showed that uveal melanoma cell lines did not express IL6 or PGE2,28 this correlates with the fact that we could not observe any M2 differentiation. Although, we did observe IL6 expression by uveal melanoma primary cultures, especially in the monosomy 3 group. Another negative factor is IFN-gamma, as it has been shown that IFN-gamma may hamper the differentia-
tion of monocytes into M2 macrophages as it induces M1 macrophages. Therefore, it could be that in our TSN the presence of IFN-gamma overrules the effect of IL6 and PGE2.

In conclusion, culturing primary uveal melanoma under hypoxia resulted in an altered balance in cytokine expression. CD163 M2 macrophages are detected in situ, but TSN - whether from normoxic or hypoxic cell lines - did not differentiate monocytes toward M2-like macrophages in vitro. However, while uveal melanoma cells may not influence differentiation, we show that all TSN stimulated macrophage kinesis. It seems that the generation of an inflammatory environment around the tumor, which corresponds to a worse prognosis, it is not oxygenation dependent.
REFERENCES


