Melanoma is a malignant tumor in which UVA (320–400 nm) radiation is considered to be an important risk factor. But the role of UVA in melanoma progression toward an invasive phenotype is still not adequately investigated. For most proliferating tumor cells the preference of aerobic glycolysis has been described as the Warburg effect. Here we investigate the effect of UVA irradiation on changes in the Warburg effect and tumor progression toward invasive potential. On UVA irradiation, melanoma cell lines from initial tumors show an induction of the Warburg effect with increased glucose consumption and lactate production, which is at least partially mediated by reactive oxygen species. Associated with UVA treatment and enhanced lactic acid production, tumor-relevant proteases and in situ invasion is upregulated. Simultaneously, UVA increases intracellular concentrations of progression marker transketolase and activated protein kinase Akt, both involved in metabolic changes that increase with proliferation. Using invasion assays we show that lactic acid, resulting from the UVA enhanced and partially reactive oxygen species-mediated Warburg effect, increases the invasive potential of all melanoma cell lines investigated. Therefore, we demonstrate in melanoma cells that production of lactic acid, induced by UVA irradiation, increases invasiveness of melanoma cells via expression of tumor-relevant proteases.

*INTRODUCTION*

Melanoma is a malignant skin tumor characterized by high morbidity and mortality (Howe et al., 2001; Jemal et al., 2010; Jhappan et al., 2003; Leiter et al., 2014). Melanomas develop from initial tumor cells via radial and vertical growth, ultimately leading to metastasis, and epidemiological evidence indicates that UV radiation is involved in the generation of melanoma (Bald et al., 2014; Fears et al., 2002; Tucker and Goldstein, 2003). Recently it was shown that UVB irradiation enhances perivascular invasion of melanoma cells (Bald et al., 2014). Although this UVB irradiation correlates with sunburn reactions in the skin, solar UV radiation with physiological relevance consists, to the largest extent, of UVA radiation (320–400 nm) (Baczyńska et al., 2013; Parisi and Wong, 2000; Turnbull and Parisi, 2003). The effect of UVA irradiation is partially mediated by reactive oxygen species (ROS), finally leading to intracellular oxidative damage (Beissert and Loser, 2008; Kappes et al., 2006). This is of particular importance because—as opposed to UVB—UVA reaches basal layers of the epidermis containing melanocytes as well as the dermis at pathophysiologically relevant doses. Despite its physiological relevance, the contribution of UVA to solar-induced melanoma is still discussed controversially as studies with a Xiphophorus hybrid fish model could not detect UVA-induced melanomas (Mitchell et al., 2010). In addition to this, epidemiological data report melanoma on usually sun-protected body sites (Levell et al., 2009), but there are also epidemiological data that show a clear correlation between sunburn and melanoma in sun-sensitive patients (Newton-Bishop et al., 2011). Furthermore, a single neonatal UVA irradiation, mimicking sunburn reactions in childhood, in a transgenic mouse model on an albino inbred background did not significantly induce melanomas (De Fabo et al., 2004; Noonan et al., 2001; Zaidi et al., 2011). But interestingly in pigmented transgenic mice, a single neonatal dose of UVA irradiation (Noonan et al., 2012) induces oxidative DNA damage in melanocytes and is sufficient to induce melanotic tumors. Although these studies strongly support the role of a single neonatal high dose of UVA during melanoma pathogenesis, they do not model other modes of UV exposure, such as the repeated exposure of humans to low doses of solar UV irradiation in everyday life. In addition, it is still not clear which role exposure to UVA irradiation plays during early melanoma progression. Two important features during progression of initial melanoma are (i) invasion, needing specific proteases, and (ii) proliferation, needing a specific metabolic setting.
Proliferating cells, including tumor cells, turn to glycolysis for energy production with subsequent lactate fermentation even in the presence of oxygen, a metabolic characteristic described by Warburg (1927), thus called the Warburg effect. Increased glycolysis is associated with the activation of Akt (Elstrom et al., 2004), a protein kinase also involved in anti-apoptotic signaling (Majewski et al., 2004). Interestingly, Akt phosphorylation at serine 473 is present in the majority of immunohistologically investigated melanomas (Dhawan et al., 2002). Another tumor-relevant metabolic change is the activation of the pentose phosphate pathway that provides tumor cells with components for nucleotide synthesis. A key enzyme of this pathway is transketolase like 1 (TKTL1), which is used as a tumor marker for highly proliferative cancers (Diaz-Moralli et al., 2011; Langbein et al., 2006). An important step in melanoma progression is invasion of adjacent tissue, facilitated by proteases such as matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA). Particularly, it was shown that MMP9 and uPA are activated during invasion of melanoma cells (Bianchini et al., 2006; Tang et al., 2013).

To investigate the role of UVA irradiation for melanoma progression and invasion via the Warburg effect, we exposed cell lines of initial melanoma with chronic sublethal doses of UVA. Here we show a UVA-dependent increase in glucose uptake and lactate production, and a pH decrease. This lactate production subsequently led to increased expression of MMPs and uPA, resulting in increased invasiveness of UVA-treated melanoma cells. We provide a functional link between the UVA-induced Warburg effect with enhanced lactic acid production and enhanced expression of MMP and uPA, which finally promotes enhanced invasion.

RESULTS
The UVA-induced Warburg effect mediated by ROS
The Warburg effect is characterized by a preference for glycolysis (enanced glucose consumption) and subsequent lactic acid fermentation. Melanoma cells from initial melanomas (IM) and metastasizing melanomas (MM) (Supplementary Table S1 and Supplementary Figure S1 online) were irradiated with UVA (6 J/cm²) three times daily for 4 consecutive days. Glucose consumption and lactate production, and a pH decrease. This lactate production subsequently led to increased expression of MMPs and uPA, resulting in increased invasiveness of UVA-treated melanoma cells. We provide a functional link between the UVA-induced Warburg effect with enhanced lactic acid production and enhanced expression of MMP and uPA, which finally promotes enhanced invasion.

UVA irradiation enhances transketolase activity
Transketolase isoform TKTL1 is a key enzyme of the pentose phosphate pathway and part of the metabolic network supporting growth of tumor cells (Resendis-Antonio et al., 2010). To investigate the effect of UVA irradiation on TKTL1 expression, melanoma cells (IM and MM) were UVA-irradiated (as described above) and expression of TKTL1 was detected by immunocytochemical staining. All melanoma cell lines (IM and MM) show enhanced TKTL1 expression on UVA irradiation (Supplementary Figure S2d and e). Simultaneously, the same UVA treatment increased total transketolase activity in melanoma cells (IM and MM) in a colorimetric assay (Smith et al., 2006). Increased transketolase activity on UVA treatment (Figure 2a) was visible in every investigated IM and MM, independent from their different genetic background (mean value and SD of three independent experiments). This UVA-enhanced transketolase activity in IM melanoma cells was dependent on UVA-induced ROS, as the addition of Trolox attenuated UVA-induced transketolase activity (Figure 2b and c) (mean value and SD of at least three independent experiments and two-way ANOVA, Bonferroni multiple comparisons post-test shown; asterisk represents P < 0.05). Furthermore, UVA-enhanced transketolase activity showed partially a dose-dependent upregulation of transketolase activity (Supplementary Figure S2c).

Skin reconstructs with different melanoma cells (IM and MM) treated either with or without repetitive UVA doses also showed elevated transketolase activity on UVA irradiation.
Figure 1. UVA induces the Warburg effect. Melanoma cell lines from initial (IM) and metastasizing melanoma (MM) or skin reconstructs with IM and MM were treated repetitively with UVA with 6 J/cm² three times daily for 4 consecutive days. UVA increases glucose consumption and lactate production in IM (a, b) and in MM (c, d, e, f). UVA increases glucose consumption and lactate production in IM and MM. (g, h) UVA decreases the pH value of the medium of IM and MM. (i, j) Treatment of melanoma cells with reactive oxygen species (ROS) quencher Trolox during UVA irradiation attenuates UVA-induced glucose consumption and lactate production. (k, l) UVA-induced glucose consumption and lactate production could be decreased in the presence of 5.5 mM glycolytic inhibitor 2-deoxy-D-glucose (2DG). (m-o) UVA increases lactate production in skin reconstructs with IM and MM melanoma cells.
compared with unirradiated control (Figure 2d–f) (mean value and SD of at least three independent experiments and Student’s t-test shown; asterisk represents $P < 0.05$).

Hyperactivation of the protein kinase Akt by phosphorylation at the serine residue 473 is associated with increased glucose metabolism and significantly correlates with TKTL1 expression in tumors (Volker et al., 2008). Melanoma cells (IM and MM) were exposed to UVA, and after treatment, the levels of phosphorylated AKT were determined by western blot. Repetitive UVA irradiation significantly (Student’s t-test; $P < 0.05$) enhances the level of phosphorylated Akt in IM cells and it was also increased in MM cells (Figure 2g) (mean value and SD of at least three independent experiments and Student’s t-test shown; asterisk represents $P < 0.05$).

Persistence of the UVA-induced Warburg effect after UVA irradiation
To test whether the UVA-induced Warburg effect persists beyond cessation of UVA treatment, IM cells were UVA-irradiated with 6 J/cm$^2$ for 4 days and afterward seeded for growth on a new plate for another 5 days without irradiation. Subsequently, glucose consumption, lactate production, and transketolase activity (Figure 3a) were measured. When compared with unirradiated controls, UVA-mediated induction of glucose consumption and lactate production continued (Student’s t-test; $P < 0.01$) 5 days after cessation of the last UVA irradiation (Figure 3b and c) (mean value and SD of at least three independent experiments and two-way ANOVA, Bonferroni multiple comparisons post-test shown; asterisks represent $P < 0.01$). Again, this UVA-induced long-term persistence of elevated glucose consumption and lactate production was ROS mediated, similar to the immediate UVA-induced Warburg effect, as the addition of Trolox during UVA treatment again decreased this long-term persistence (Figure 3b and c). Similar to the UVA-induced Warburg effect, UVA-induced transketolase activity also persisted, as transketolase activity 5 days after irradiation was still significantly higher when compared with unirradiated controls.

Figure 2. Repetitive UVA irradiation enhances transketolase activity. (a) UVA increases transketolase activity in IM and MM cells, and the addition of ROS quencher Trolox attenuates this effect in (b) IM and (c) MM. (d–f) UVA irradiation increases transketolase activity in skin reconstructs with IM and MM melanoma cells. (g) UVA irradiation enhances activation of protein kinase Akt. Melanoma cell lines (IM and MM) were UVA-irradiated with subsequent detection of phosphorylated (Ser 473) Akt and β actin and the level of phosphorylated (Ser 473) Akt relative to β actin was quantified densitometrically. IM, initial melanoma; MM, metastasizing melanoma; ROS, reactive oxygen species.
Expression of MMPs and uPA is important for melanoma invasion. Lactate concentrations of 11 mM are described in tumors in vivo (Serganova et al., 2011), and lactate levels of approximately 16 mM were measured on UVA irradiation of melanoma cells in vitro (Figure 1b and d). To determine whether lactic acid alone, a major derivate of the UVA-induced Warburg effect, is able to induce expression of MMPs and uPA in melanoma cells, IM cells were treated with 20 mM lactic acid for 12 hours. After lactic acid treatment, we analyzed transcription of MMP1, MMP2, MMP3, MMP9, MMP13, MMP15, tissue inhibitor of metalloproteinase (TIMP1), and uPA. Lactic acid increased the transcription of all investigated proteases (MMPs and uPA) relevant for tumor progression (Figure 4a and Supplementary Figure S3a and b online). In addition, we found a slight induction of TIMP1 in IM melanoma cells visible, but compared with the induction of transcription levels of most MMPs and uPA, these effects of lactate on TIMP1 transcription were negligible (Figure 4a) (transcriptional level of MMP, uPA, TIMP1 relative to housekeeping gene beta actin shown in 2^{-ΔΔCt} in logarithmic scale; data presented as the mean value with SD of at least four independent experiments). To show that either UVA irradiation or UVA irradiation-associated lactic acid is capable of inducing secretion of active tumor, relevant MMP2 and MMP9 zymography was performed. UVA irradiation induced secretion of active MMP2 and MMP9 in IM and MM cells and UVA-induced activity of MMP2 and MMP9 was slightly reduced in the presence of ROS quencher, indicating a partially ROS-mediated mechanism of UVA-induced MMP2 and MMP9 induction (Figure 4b and c, Supplementary Figure S3c and d) (IM and MM treated with and without UVA or 20 mM lactic acid; Student’s t-test; asterisk represents P < 0.05; data shown as the mean value with SD of at least three independent experiments). Furthermore, treatment of lactic acid alone was also capable of facilitating secretion of MMP2 and MMP9 when compared with control (Figure 4b and c, Supplementary Figure S3c and d). Additionally, UVA irradiation enhanced secretion of uPA in IM and MM cells (Supplementary Figure S4c and d online).

The UVA-enhanced Warburg effect increases invasion via production of lactic acid

To test whether production of lactic acid, which can be induced by UVA-mediated ROS, is functionally capable of enhancing invasion of IM and MM cells, we performed in vitro invasion assays. In these assays, IM and MM cells were grown on an artificial collagen matrix where they invade this matrix according to their invasive potential. Melanoma cells were treated with and without UVA irradiation (6 J/cm^2 for 4 days), ROS quencher Trolox, glycolytic inhibitor 2DG, or lactic acid (Figure 4d and e) (in vitro invasion assay of melanoma cells [IM and MM] treated with and without UVA, 20 mM lactic acid, ROS quencher Trolox, and 2DG; one-way ANOVA with Newman Keuls multiple comparison post-test; asterisk represents P < 0.05). Data are presented as the mean value with SD of at least three independent experiments. In the invasion assay, UVA irradiation significantly increased invasion of melanoma cells, and this effect was partially ROS mediated as Trolox significantly attenuated UVA-induced invasion (Figure 4d and e). Similarly, the inhibitor of glycolysis (2DG) significantly attenuated UVA-induced invasion in IM and MM cells (Figure 4d and e). Treatment of melanoma cells with lactic acid significantly increased invasion to an extent comparable to UVA irradiation (Figure 4d and e). These data show that the addition of lactic acid can mimic the UVA-induced increase of the
Figure 4. Increased UVA-associated lactic acid enhances invasion. (a) Lactic acid enhances transcription of MMP and uPA in melanoma cell lines (IM). (b, c) UVA irradiation or lactic acid alone increases MMP2 and MMP9 secretion. (d, e) UVA irradiation or lactic acid alone increased invasion, whereas the addition of ROS quencher Trolox or inhibitor of glycolysis 2DG previous to irradiation inhibited UVA-induced invasive potential. (f) In human skin reconstructs with epidermal melanoma cells (IM and MM), UVA induces more initial invasion of melanoma cells in the dermis than in control, detected with melanoma-specific S100 staining and counted per field of vision. Scale bar represents 70 μm. (g) Proposed model for UVA-enhanced invasion via production of ROS, lactic acid, and expression of MMP and uPA. 2DG, 2-deoxy-D-glucose; IM, initial melanoma; MM, metastasizing melanoma; MMP, matrix metalloproteinase; ROS, reactive oxygen species; uPA, urokinase-type plasminogen activator.
invasive potential in vitro. To investigate to which extent the UVA- or lactic acid-induced invasion is facilitated either by MMP proteases or by uPA proteases we tested the invasive potential of UVA-treated cells with and without inhibitors of MMP and uPA. The presence of MMP inhibitor (GM6001) as well as the presence of uPA inhibitor (amiloride) partially abrogated UVA-induced invasive potential (Supplementary Figure S4a and b), indicating that MMP and uPA are functionally needed for UVA-induced in vitro invasion. Furthermore, UVA irradiation of skin reconstructs with melanoma cells (IM and MM) enhanced expression of MMP9 and uPA (Supplementary Figure S4e and f). In addition to this, UVA irradiation increased initial dermal invasion of melanoma cells (IM and MM) in skin reconstructs (Figure 4i) (mean value with SD of at least three independent experiments shown; Student’s t-test; asterisk represents \( P < 0.05 \)), which was verified by a histopathologist.

DISCUSSION

In many patients, initial small melanomas are not discovered and patients continue solar exposure and many patients even increase their sun exposure after diagnosis of initial melanoma (Idorn et al., 2014). The importance of single neonatal high doses of UVB or UVA radiation for initiation of melanoma was shown previously in mice (Noonan et al., 2003, 2012). But despite its physiological relevance, it is still not clear how UVA irradiation influences progression of human initial melanoma cells. The dose of UVA irradiation we used is within the physiological range to which an individual can readily be exposed to during a summer day in Europe (http://www.soda-is.com/eng/map/maps_for_free.html#uv). Here we demonstrate that UVA induces the tumor-associated Warburg effect. The literature indicates that tumor cells benefit from this Warburg effect as part of the glycolysis-derived pyruvate can be used for further anabolic pathways such as amino acid synthesis or fatty acid synthesis (Vander Heiden et al., 2009). Although UVA irradiation decreased survival of the tumor cells in a dose-dependent way (Supplementary Figure S1a and b), the remaining viable cells have a higher rate of glucose consumption and lactate production per cell than unirradiated controls (Figure 1a–f, Supplementary Figure S2a and b). These metabolic changes seem to be partially mediated by ROS because the addition of lactic acid in physiological concentrations. In contrast to this, no significant upregulation of the inhibitors of MMPs, TIMP1, can be found. It has been shown that induction of MMP 2, MMP3, MMP9, MMP13, and uPA proteases is important during melanoma progression (Bianchini et al., 2006; Corte et al., 2005; Girouard et al., 2012; Ji et al., 2013; Orimoto et al., 2008; Rotte et al., 2012; Tang et al., 2013; Tas et al., 2005, 2008; Zigrino et al., 2009). So far it has not been reported that MMP15 transcription is induced in melanoma. Lactic acid can also be produced in hypoxic conditions (Pasteur effect), which is often observed in melanoma (Scott et al., 2011) and many other tumors. Interestingly, lactic acid-induced expression of MMPs and invasion in distant areas could be one efficient strategy of melanoma cells to escape from unfavorable areas with hypoxia, high lactic acid concentrations, and senescence inducing conditions (Mo et al., 2013). Finally, the finding that in matrigel-based assays, UVA irradiation-induced lactic acid increases invasion of melanoma cells provides a functional link between melanoma invasion and the UVA-induced Warburg effect (Figure 4d and e, Supplementary Figure S3e). Furthermore, we could show that uPA and MMP activity are functionally important for UVA-induced invasion (Supplementary Figure S4a and b). UVA also induced the Warburg effect and invasion in melanoma cells of metastasizing melanomas, although to a smaller extent than in melanoma cells of initial melanoma. This is in line with the notion that UVA irradiation is of particular relevance at an early stage of metastasis (IM cells) rather than for cells that derive from a tumor that already did metastasize. Furthermore, UVA irradiation may be more relevant to IM cells that are in the upper layers of the skin and are usually in the penetration range of physiological UVA.
irradiation but not distant lymph nodes or other organs, which are targets for metastasizing melanoma cells.

With regard to the role of oxidative stress, it is important to note that in our system, ROS quenchers cannot completely abrogate UVA-mediated effects, indicating that either cumulative ROS production during UVA treatment exceeds the ROS detoxification capacity of Trolox or other mechanisms such as activation of mitogen-activated protein kinase, NF-κB, β-selectin, or chemokine signaling may also be relevant.

Taken together, at least partially, UVA irradiation enhances the Warburg effect in an ROS-dependent fashion with increased lactate production, which induces MMP and uPA transcription, finally leading to invasion of tumor cells, which is also visible in skin reconstructs. This indicates that UVA irradiation is not only important in initiation of melanoma, but also during progression. These findings support therapeutic strategies of using MMP inhibitors together with antioxidants and inhibitors of glycolysis. Furthermore, our data support the therapeutic recommendation of continuous sun protection.

MATERIALS AND METHODS

Cell culture

Human melanoma cells of different progression levels (vertical growth phase, radial growth phase, and MM) (Supplementary Table S1) were cultured in DMEM as described previously (Berneburg et al., 2005; Kamenisch et al., 2010; Koch et al., 2001) and in Supplementary Materials and Methods online. Patient consent for experiments was not required for experiments with established cell lines.

Skin reconstructs

Preparation of skin reconstructs and S100 staining was performed as described previously (Meier et al., 2000). Briefly, layers of collagen, fibroblasts, keratinocytes, and different melanoma cells were cultivated and treated with and without UVA irradiation as described in Supplementary Materials and Methods.

UVA irradiation

Irradiation was carried out as described (Berneburg et al., 2005; Kamenisch et al., 2010; Koch et al., 2001). Briefly, cells were irradiated with a sellasol (SELLAS, Ennepetal, Germany) UVA irradiation device as described in Supplementary Materials and Methods.

Statistical analysis

Unless mentioned otherwise, data are shown as the mean with SD of at least three independent experiments, and statistical significance was tested with paired and unpaired Student’s t-test, two-way ANOVA with Bonferroni multiple comparisons after test analysis, one-way ANOVA with post-test for a linear trend and one-way ANOVA with Newman Keuls multiple comparison post-test analysis, and a P-value < 0.05 was considered significant.

Chemical treatment

Cells were treated as described in Supplementary Materials and Methods.

Lactate and glucose measurement

The same number of melanoma cells were treated with and without 4 days of repetitive UVA irradiation and aliquots of the supernatant of the treated cells were used for lactate and glucose measurement and normalized to the number of cells after treatment as described in Supplementary Materials and Methods.

Protein measurement

Protein measurements were performed using the BCA Protein Assay Reagent A and B (Pierce, Rockford, IL).

Transketolase measurement

Transketolase activity was measured colorimetrically (Smith et al., 2006) as described in Supplementary Materials and Methods.

Quantitative analysis of MMP and uPA transcription via real-time quantitative reverse transcription-PCR

After treatment with lactic acid, RNA was isolated out of the cells according to protocol (Nucleospin RNA protocol) and cDNA was made by reverse transcriptase PCR according to protocol (Bio-RAD, Germany). Real-time PCR was performed on a Roche LightCycler using SYBR Green Supermix (Bio-RAD, Germany). Primers are listed in Supplementary Table S2 online. Subsequent to real-time PCR, products were verified on gel by gel electrophoresis with marker (PEQ gold Ultra Low Range DNA Ladder I).

Zymography

For zymography the supernatant of IM and MM cells treated with UVA, Trolox, or lactic acid was used and zymography was performed as described in Supplementary Materials and Methods.

Assay of uPA

For quantitative detection of human uPA, the uPA assay according to the protocol of uPA (URK) Human ELISA Kit (abcam, Germany) was performed as described in Supplementary Materials and Methods.

In vitro cell invasion assays

Cell invasion assays were performed according to protocols of Milipore Cell invasion Assay protocol (Millipore, Germany). Briefly, 250,000 melanoma cells were incubated per insert for 24 hours with and without treatment, and the fraction of invaded cells was determined fluorometrically with a fluorescence plate reader using a 480/520 nm filter set.

CONFLICT OF INTEREST

The authors state no conflict of interest except the conflict of interest of MR Rocken. He has received grants (or other support as listed) from AB Science (Study), Abbott Laboratories (Study), Abbott Pharmaceuticals (Study), Almirall Hermal (Grant/Honorarium; Study and consulting), AstraZeneca (Study), Bayer (Study) Biogen Idec (Grant/Honorarium; Study, consulting and speaker), Bristol-Myers Squibb (Study), Bundesministerium für Bildung und Forschung (Research project), Celgene (Study), Deutsche Dermatologische Gesellschaft (Officer), Deutsche Forschungsgemeinschaft (Research project), Deutsche Krebshilfe (Research project), European Union (Research project), European Academy of Dermatology and Venereology (Officer), Galdema (Grant/ Honorarium; Study and consulting), GSK (Study), Hokusai (Study), Janssen- Cilag (Grant/Honorarium; Study and consulting), Johnson & Johnson (Grant/ Honorarium; Study and consulting), Lilly (Study), Merck (Study), MSD Sharp & Dohme (Study), Novartis (Study), Pfizer (Study), Philogen (Study), Regeneron (Honorarium; Consulting), Roche (Study), sterna biologicals (Honorarium; Speaker), Wilhelm Sander-Stiftung (Research project).

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