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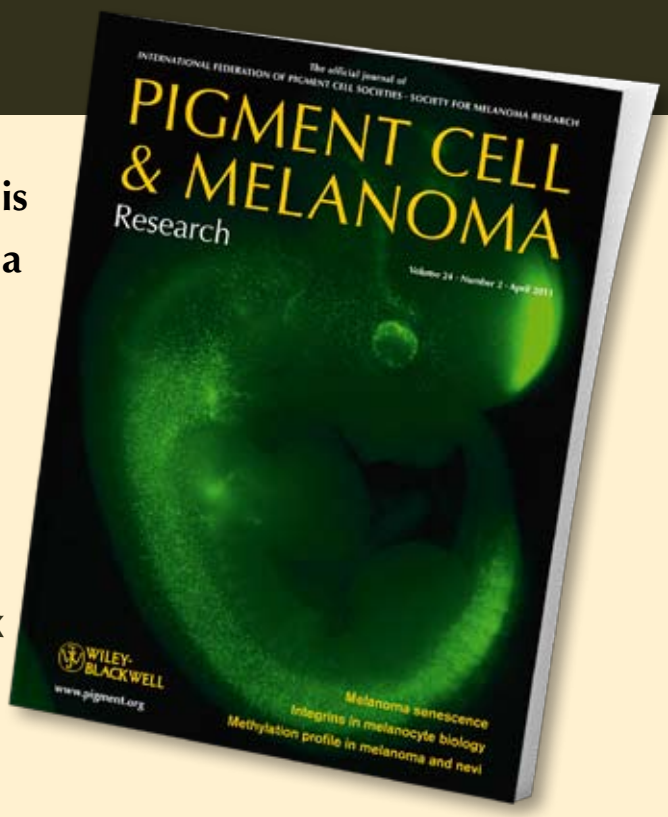

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Oliver Schanab[1], Johannes Humer[2], Andreas Gleiss[3], Mario Mikula[1,4], Sanda Sturlan[2], Susanne Grunt[1,2], Ichiro Okamoto[1], Thomas Muster[1,2], Hubert Pehamberger[1] and Andrea Waltenberger[1,2]

1 Department of Dermatology, Division of General Dermatology, Medical University Vienna, Waehringer Guertel, Vienna, Austria **2** AVIR Green Hills Biotechnology GmbH, Gersthoferstrasse, Vienna, Austria **3** Center for Medical Statistics, Informatics and Intelligent Systems, Medical University Vienna, Spitalgasse, Vienna, Austria **4** Institute of Medical Genetics, Medical University Vienna, Waehringer Strasse, Vienna, Austria

CORRESPONDENCE O. Schanab, e-mail: oliver.schanab@meduniwien.ac.at

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Summary

Human endogenous retroviruses (HERVs) represent a cellular reservoir of potentially pathogenic retroviral genes. A growing body of evidence indicates that the activation of endogenous retroviral sequences might be involved in the transformation of melanocytes. In this study, we investigated the effects of ultraviolet radiation (UVR) on the expression of human endogenous retrovirus type K (HERV-K) in melanoma cells and non-melanoma cells in vitro. Solely in melanoma cell lines, irradiation with UVB (200 mJ/cm^2) resulted in a significant transcriptional activation of the retroviral *pol* gene as well as in an enhanced expression of the retroviral envelope protein (env). In addition, UVB treatment induced the production of retroviral particles in the supernatants of melanoma cell lines. These data indicate that HERV-K expression can be activated by UVB irradiation and suggest an involvement of HERV-K in UVR-related melanoma pathogenesis.

Introduction

The incidence of cutaneous melanoma, by far the most fatal skin cancer, increased by a factor of approximately 15 during the past 60 years (Jemal et al., 2006). The factors responsible for the rapid increase in skin cancer are not yet entirely understood, but behavioral changes such as lifestyle and fashion that lead to an increase in total lifetime exposure to the sun have been implicated as the major cause for acquiring melanoma. In addition to total exposure time to ultraviolet radiation (UVR), several clinical as well as epidemiological indications for intermittent intense UV exposure, especially during childhood, are deemed to be responsible for a large group of melanoma that develop on parts of the skin, which are usually not exposed to the sun (Gilchrest et al., 1999; Whiteman et al., 2001, 2006).There is no doubt that UVR is a potent mutagen. Particularly, the UVB region (280–315 nm) is regarded to be more carcinogenic than the UVA region (315–400 nm) as it directly generates

Significance

The causative relationship between ultraviolet radiation (UVR) and non-melanoma skin cancer is well documented, and important molecular targets have been identified. In contrast, direct molecular evidence for UVR as mutagenic factor in melanoma and consequently a clear mechanistic framework for the role of UVR in the initiation of melanomas is still lacking. In recent years, human endogenous retroviruses (HERVs), remnants of ancestral exogenous retroviral infections fixed in the germ line DNA, have been implicated in melanomagenesis. This study provides evidence that HERV sequences as well as retroviral proteins and particles are not only expressed as a concomitant phenomenon of melanoma development but are specifically activated in melanoma cell lines in response to UVR. Our results suggest an etiological role of HERVs in UVR-related melanoma development.

two classes of DNA photoproducts: cyclobutane pyrimidine dimers (CPDs) and 6-pyrimidine 4-pyrimidone photoproducts (6-4PP). The CPDs are more carcinogenic and if not efficiently repaired, they induce mutations hallmarked by C → T and CC → TT transitions, which are UV specific (also known as UV signature mutations; de Gruijl and Rebel, 2008; Maddodi and Setaluri, 2008). However, the mechanisms by which the UV spectrum of solar radiation initiates melanoma have not yet been sufficiently understood. In melanoma, mutations in the RAS, BRAF, c-Kit oncogenes, losses of PTEN and cyclin-dependent kinase inhibitor 2A (CDKN2A) tumor suppressor genes, and amplifications of cyclin D1 are very common. But almost none of these alterations carry signatures of an UV-induced mutation. Therefore, despite several indications that UVR is important in the pathogenesis of melanoma, the direct molecular evidence for UVR mutations as a causal factor in melanoma is still lacking (Benjamin et al., 2007). This suggests that either UVR is not involved or the UVR effect is caused indirectly via undiscovered mechanisms. Besides the mutagenic effects of UV light, it has been shown that UVR can increase the expression of human immunodeficiency virus type 1 (HIV-1) up to 150-fold in tissue culture. It was further demonstrated that even direct sunlight can activate HIV-1 gene expression and thereby enhance viral replication and development (Valerie et al., 1988). In contrast to the exogenous retrovirus HIV-1, human endogenous retroviruses (HERVs) are footprints of ancient exogenous retroviral germ-cell infections and represent a cellular reservoir of potentially pathogenic genes. The human genome contains about 8% of endogenous retroviral sequences (Bannert and Kurth, 2004; Venter et al., 2001). Human endogenous retrovirus type K (HERV-K) is one of the best-described HERVs, and because of the open reading frames for the structural and enzymatic proteins gag, prt, pol, and env (Lower et al., 1996; Mayer et al., 1999) being conceivably biologically active and potentially oncogenic. Furthermore, the class II HERV-K (HML-2) family is currently the only HERV family that is capable of producing retrovirus-like particles (Bronson et al., 1978; Lower et al., 1984). Several studies demonstrated the potential oncogenic role of the class II HERV-K (HML-2) family in various human malignancies, including breast (Wang-Johanning et al., 2001), germ-cell (Herbst et al., 1996), and ovarian cancer (Wang-Johanning et al., 2007), leukemia (Depil et al., 2002), and more recently melanoma. We discovered retrovirus-like particles produced by human melanoma cells that contain sequences with high homology to the class II HERV-K (HML-2) family (Muster et al., 2003). Expression of retroviral pol, gag, env, and rec proteins was detected specifically in human melanomas and melanoma metastases but not in melanocytes or unaffected lymph nodes, suggesting that the expression of retroviral genes and the production of retroviral particles are activated during melanoma development. This finding in melanoma was largely confirmed and extended by Buscher et al. (2005). Despite well-demonstrated expression of HERV-K mRNA transcripts, proteins and the production of retrovirus-like particles in melanoma, so far a clear mechanistic framework for a causative function of HERVs in melanoma – and other human cancers – is still lacking (Ruprecht et al., 2008; Singh et al., 2009). However, there is a growing body of evidence that indicates a genuine role of the class II HERV-K (HML-2) family in the process of melanoma initiation and/or promotion: Hohenadl et al. (1999) as well as Reiche et al. (2010) demonstrated that external cellular stressors such as UVR can induce the expression of endogenous retroviral mRNA transcripts. Serafino et al. (2009) have shown that in human melanoma cells, stress caused by low-serum cell culture conditions induced their transformation toward a more malignant growth phenotype. Furthermore, this transformation essentially required the expression of HERV-K. In the present study, we analysed the transcriptional activation of HERV-K sequences, expression of retroviral proteins, and retrovirus-like particles in response to UVR in human melanoma and non-melanoma cells. For the first time, we show that the *pol* sequence of the class II HERV-K (HML-2) family, the retroviral envelope protein (env), as well as retrovirus-like particles are not only expressed as a concomitant phenomenon of melanoma development but are specifically activated in melanoma cell lines in response to UVR, the most established environmental risk factor in melanoma cancerogenesis. This finding might substantiate an etiological role of the class II HERV-K (HML-2) family in UVR-related melanoma development.

Results

UVB irradiation increases the expression of HERV-K mRNA in human melanoma cells

To investigate whether the expression of HERV-K mRNA can be activated by UVR in human melanoma cells and normal human melanocytes, 518A2 and NHEM neo cells were irradiated with various doses of UVA and UVB. Irradiation doses starting from 10 to 30 J/cm^2 UVA and from 10 to 100 mJ/cm^2 UVB, respectively, did not induce relevant changes in the retroviral mRNA expression (data not shown). However, irradiation of 518A2 melanoma cells with higher doses of UVB up to 200 mJ/cm^2 revealed a statistically significant ($P = 0.004$) linear, dose-dependent induction of retroviral mRNA expression. Transcriptional activation of the retroviral *pol* sequence reached a maximum induction at an exposure rate of 200 mJ/cm^2 UVB 24 h after irradiation (Figure 1A). Consistent with this finding, we performed further real-time PCR analysis at this UVB dose. Irradiation of 518A2 after 12 and 24 h with 200 mJ/cm^2 UVB led to statistically significant (both $P = 0.001$) transcriptional activation of the *pol* sequence

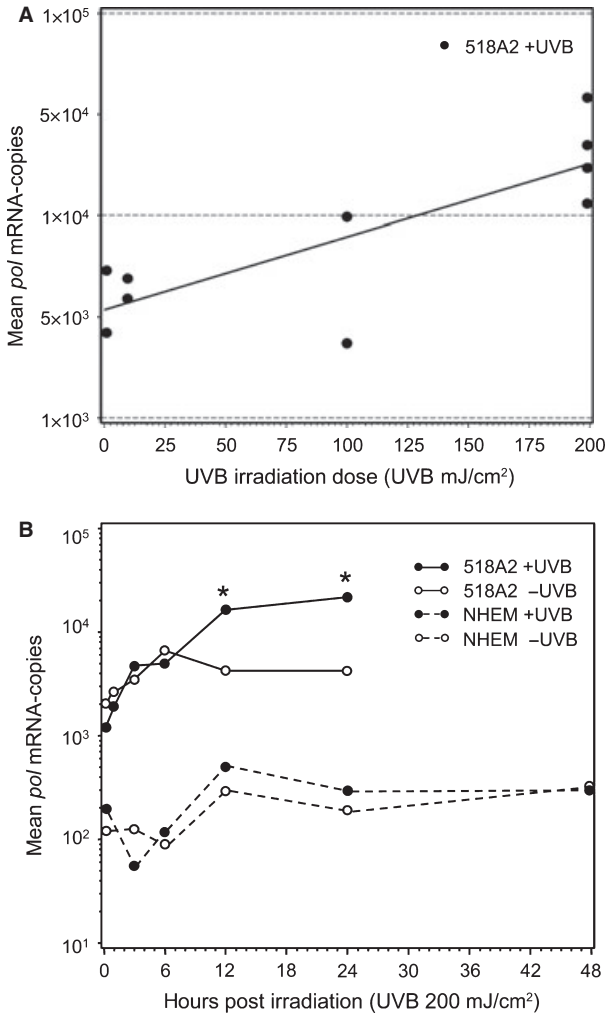

Figure 1. UVB radiation stimulates human endogenous retrovirus type K *pol* mRNA expression in human melanoma cell line 518A2. (A) UVB dose–response curve (log-linear) for the induction of retroviral *pol* mRNA expression in melanoma cell line 518A2 24 h after exposure. Transcriptional activation of *pol* was determined by real-time PCR and increased dose dependently (0, 10, 100, and 200 mJ/cm^2 UVB) in a linear manner (P = 0.004). Each data point represents mean *pol* expression of one biological replicate used to calculate the linear regression. (B) Melanoma cells 518A2 and normal human neonatal melanocytes (NHEM neo) were irradiated with 200 mJ/cm^2 UVB (+UVB) in Dulbecco's phosphate-buffered saline (DPBS). Non-irradiated control cells (-UVB) were kept in DPBS in a separate tissue culture hood and were treated identically except that irradiation was omitted. At the indicated time points, cells were harvested and *pol* mRNA expression levels were determined by real-time PCR. Points represent means of 2–4 biological replicates for each time point and treatment modality (+/– UVB). Asterisks indicate statistical significance for the difference between irradiated cells and corresponding non-irradiated control cells (*P = 0.001).

as compared to the non-irradiated controls. This activation reached a maximum level of 5.2-fold induction of the *pol* gene after 24 h. Basal expression of *pol* in 518A2 cells after 12 and 24 h was between 4226 copies and 4235 copies, respectively. In UVB-treated 518A2 cells, copy numbers of *pol* increased to levels between 16469 (12 h) and 21779 (24 h) post-exposure (Figure 1B). Importantly, UVB exposure of NHEM neo to the same dose (200 mJ/cm^2) did not lead to a statistically significant transcriptional activation of the HERV-K *pol* sequence at any analysed time point. Similar to 518A2, UVB exposure had no relevant influence on the level of the *pol* sequence expressed by NHEM neo cells between the initial level (0 h) and 6 h post-irradiation. After 12 and 24 h, UVB treatment induced a slight but not significant transcriptional activation of the *pol* sequence in NHEM neo cells (P = 0.746 and P = 0.622, respectively) as compared to the non-irradiated controls. Even 48 h after irradiation, the results revealed an expression pattern of the retroviral *pol* sequence similar to the initial level, in both treated (305 copies) and untreated (318 copies) NHEM neo cells. Untreated 518A2 cells had a 14.6-times higher expression of the HERV-K *pol* sequence (P < 0.001) than UVB-treated NHEM neo cells 24 h post-irradiation.

Transcriptional activation of the HERV-K *pol* sequence by UVB light is specific for melanoma cells

To determine whether the induction of HERV-K *pol* transcription after UVB exposure was specific for melanoma cells, three additional melanoma cell lines, namely MELJUSO, 6F, and SKMEL28, and cells representative of normal human skin, i.e. primary human epidermal keratinocytes (NHEK), human skin fibroblasts, and normal human melanocytes (NHEM neo), were analysed. We focused on the 24-hour time point to be consistent with our finding in 518A2 (Figure 1B) and with recent discoveries by Enk et al. (2006). As demonstrated in Figure 2A, transcriptional activation of HERV-K sequences by UVB light is a specific feature of melanoma cells. Twenty-four hours after UVB exposure (200 mJ/cm^2), real-time PCR analysis of the melanoma cell lines 518A2, MELJUSO, 6F, and SKMEL28 revealed a 4.1-fold upregulation of the HERV-K *pol* sequence. This upregulation reached statistical significance in 518A2, MELJUSO (both P = 0.001), and 6F (P = 0.008), but not in SKMEL28 cells (P = 0.145). Basal expression of the retroviral *pol* sequence was between 1541 copies (SKMEL28) and 4538 copies (518A2). After UVB treatment, *pol* expression increased in all melanoma cell lines to levels between 6395 copies (SKMEL28) and 18828 copies (518A2). In contrast, our data revealed no detectable UVB response in non-melanoma cells (NHEK, NHEM, fibroblasts). At the 24-h time point, only basal-level expression of the HERV-K *pol* sequence was measurable in UVB-treated or untreated non-melanoma cells, reflecting the established constitutive and ubiquitous HERV expression (Medstrand and Blomberg, 1993; Seifarth et al., 2005; Stauffer et al., 2004). In all analysed non-melanoma cells, the *pol* sequence was less

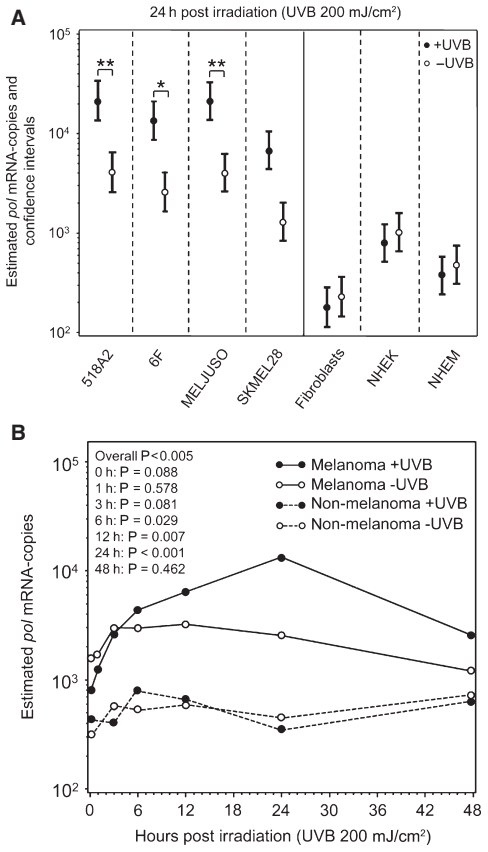

Figure 2. Transcriptional activation of the human endogenous retrovirus type K (HERV-K) *pol* sequence by UVB light is specific for melanoma cells. (A) Real-time PCR analysis of UVB-treated (+UVB) and untreated (–UVB) melanoma cell lines (518A2, SKMEL28, MELJUSO, 6F) compared with non-melanoma cells (fibroblasts, NHEM, NHEK) resulted in a different expression pattern of the HERV-K *pol* sequence 24 h after exposure. Points and vertical bars represent mean *pol* mRNA copies and confidence intervals, respectively, as estimated from the model (see statistical analysis) of 2–4 biological replicates for each time point and treatment modality (+/– UVB; *P = 0.008; **P = 0.001). (B) Pooled real-time PCR data, tested simultaneously (see statistical analysis), of all analysed melanoma cell lines and non-melanoma cells. Points represent estimated mean *pol* mRNA copies of 6–12 biological replicates for each time point and treatment modality (+/– UVB). P-values refer to testing for a potential difference of the irradiation effect between melanoma and non-melanoma cells.

expressed compared to melanoma cells, but detectable. The results in untreated non-melanoma cells revealed a slightly higher expression of *pol*, reaching levels between 240 copies (fibroblasts) and 987 copies (NHEK). In conclusion, our data verified that the effect of UVB exposure on the transcriptional activation of HERV-K led to a statistically significant ($P < 0.005$) upregulation of the *pol* sequence in melanoma cell lines as compared to non-melanoma cells over all analysed time points (Figure 2B). Additionally, this upregulation in melanoma cell lines showed a time-dependent increase, which peaked 24 h post-irradiation. *Pol* activation in UVB-treated melanoma cells was detectable even after 48 h, but on a lower level. Non-melanoma cells revealed a different expression pattern without a significant *pol* UVB response during the whole analysed time frame of 48 h.

UVB irradiation stimulates the expression of retroviral proteins in melanoma cells

Whereas HERV-K full-length mRNA is detectable in many tissues, the expression of retroviral proteins and the formation of virus-like particles have only been demonstrated in cell lines established from human teratocarcinoma (Boller et al., 1993), seminoma (Sauter et al., 1995), breast cancer (Seifarth et al., 1998), and melanoma (Buscher et al., 2005; Muster et al., 2003). To determine whether UVB irradiation leads to transcriptional activation of HERV-K and also increases retroviral protein expression in melanoma, the expression of the HERV-K-specific envelope protein (env) in response to UVB was analysed in immunofluorescence studies using a specifically designed rabbit anti-HERV-K-env antibody. Immunostaining of irradiated (UVB 200 mJ/cm^2) and non-irradiated melanoma cells revealed similar cytoplasmatic expression patterns of the HERV-K-env protein 12 and 24 h after treatment (data not shown). Additionally, no expression of the env protein was detected in non-melanoma cells 12 and 24 h post-irradiation (data not shown). However, 48 h after UVB exposure, increased expression of the HERV-K-env protein was evident in response to UVB treatment in melanoma cells. A representative experiment is shown in Figure 3. Upon UVB irradiation, enhanced expression of the retroviral env protein was detected by rabbit polyclonal anti-HERV-K-env antibody in the melanoma cell lines 518A2, SKMEL28, and MELJUSO, whereas no expression was detected in NHEM neo and fibroblasts.

UVB irradiation stimulates HERV-K particle production in melanoma cells

We used a product-enhanced reverse transcriptase (PERT) assay to investigate whether UVB light was capable of both activating retroviral mRNA and protein expression and producing and secreting retrovirus-like particles in melanoma cell lines. The PERT assay, a test for detecting extremely low levels of reverse transcriptase (RT) activity, is 10E+06 to 10E+07 times more sensitive than a conventional RT test and detects retroviral particles (Arnold et al., 1998). We found a statistically significant ($P < 0.001$) linear dependence of induction of RT activity in melanoma cell lines on exposure to UVB. Induction of RT activity in the melanoma cell supernatants of, for example, 518A2 revealed a dose-dependent increase with a maximum induction at an exposure rate of 200 mJ/cm^2 UVB 24 h after irradiation (Figure 4A). Consistent with this finding, we performed further analysis at this UVB dose. A PERT analysis carried out 24 h after treatment revealed induction of RT activity solely

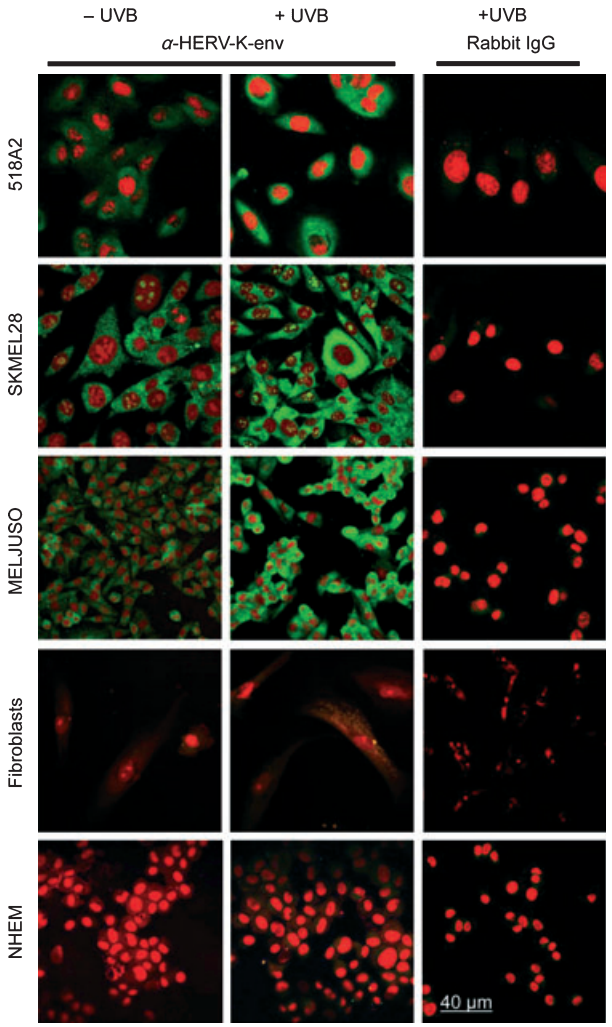

Figure 3. UVB radiation stimulates human endogenous retrovirus type K ((HERV-K)-env protein expression. Irradiated and non-irradiated cells were grown on chamber slides and fixed, and immunofluorescence analysis was performed with rabbit polyclonal anti-HERV-K-env antibody. 48 h post-UVB exposure (200 mJ/cm^2), melanoma cell lines 518A2, SKMEL28, and MELJUSO elucidated increased expression of the retroviral env protein (merged images: env, *green fluorescence*; PI nuclear staining, *red fluorescence*). In human dermal fibroblasts as well as in cultured normal human melanocytes (NHEM), expression of the HERV-K-env protein was not detectable. Isotype control antibody (rabbit IgG) did not reveal any unspecific staining under all experimental conditions. This figure shows one representative experiment of a total of three experiments.

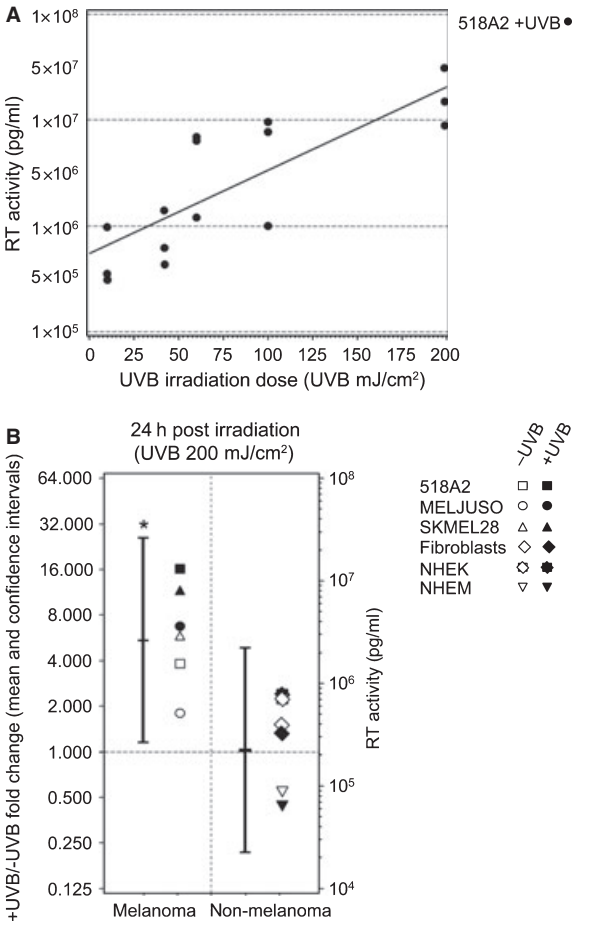

Figure 4. UVB exposure stimulates human endogenous retrovirus type K particle production in melanoma cells. Melanoma cell lines (518A2, SKMEL28, MELJUSO) and non-melanoma cells (NHEM, fibroblasts, NHEK) were UVB-irradiated (200 mJ/cm^2) in Dulbecco's phosphate-buffered saline (DPBS). Non-irradiated control cells were kept in DPBS in a separate tissue culture hood and were treated identically except that irradiation was omitted. 24 h after UVB exposure, supernatants were harvested and ultracentrifuged. The resulting pellets were resuspended and used as enzyme source for reverse transcriptase (RT) activity determined by product-enhanced reverse transcriptase (PERT) assay. (A) UVB dose–response curve for (log-linear) RT activity induction in melanoma cell line 518A2 24 h after exposure. Reverse transcriptase activity increased dose dependently (10, 42, 60, 100, and 200 mJ/cm^2 UVB) in a linear manner ($P < 0.001$). Each data point represents mean RT activity of one biological replicate used to calculate the linear regression. (B) Pooled PERT data of all tested melanoma cell lines and non-melanoma cells. For each group, the horizontal bars give the mean fold changes (according to the left vertical axis); additionally, the 95% confidence interval limits are shown. Fold changes >1 ($P = 0.037$) are marked with an asterisk (*). Each single plot symbol represents two biological replicates and shows the absolute RT activity (according to the right vertical axis) for each cell line and treatment modality (+/– UVB).

in melanoma cell lines as compared to non-melanoma cells. As shown in Figure 4B, UVB treatment resulted in a statistically significant 5.5-fold ($P = 0.037$) induction of RT activity in the supernatants of melanoma cells, while no induction of RT activity in response to UVB exposure was detectable in non-melanoma cell supernatants. These data suggest the increased dose-dependent release of viral particles from melanoma cells in response to UVB exposure. The particle production after UVB treatment (200 mJ/cm^2) reached a maximum level of 8.5-fold induction in melanoma cell line 518A2 and a minimum level of 2.8-fold induction in melanoma cell line SKMEL28.

Discussion

Melanoma is the most serious form of skin cancer and responsible for most skin cancer deaths. Despite the large body of mutational data available for melanoma and epidemiological studies linking this cancer to UVR, the fundamental carcinogenic mechanisms involved in melanoma remain largely unknown. In the present study, the effects of UVR, particularly the UVB region, on the class II HERV-K (HML-2) family were analysed. It should be pointed out that exposure to 120–180 min of midday summer sun corresponds to approximately 200 mJ/cm^2 of UVB. Hence, the UVB exposure used in this study is environmentally relevant. Furthermore, recent conclusions from the HGF/SF-transgenic mouse model study (De Fabo et al., 2004) as model system for melanoma initiation in response to UVR provide compelling evidence that natural sunlight UVB (280–315 nm) and not UVA wavelengths (315–400 nm) initiate mammalian melanoma. Transcriptional activation of the HERV-K *pol* sequence in response to 200 mJ/cm^2 UVB was exclusively detectable in all analysed human melanoma cell lines 24 h after irradiation, suggesting that in melanoma the retroviral *pol* gene is a UVB-regulated gene. In both UVB-treated and untreated non-melanoma cells (NHEK, NHEM, fibroblasts), only a small number of *pol* copies was detectable, reflecting the established constitutive and ubiquitous HERV expression without any signs of response to environmental hazards such as UVB. Taken together, these findings suggest that transcriptional activation of HERV-K does not occur randomly in some melanoma cells but constitutes a specific response to UVB in melanoma. We demonstrated by immunofluorescence analysis that the expression of the HERV-K-specific envelope protein (env) in melanoma cell lines is triggered by exposure to UVB (200 mJ/cm^2). This finding is of special interest as it indicates the increased expression of a HERV-K-encoded protein in response to UVB, which may directly promote tumor progression by subverting tumor immunosurveillance (Mangeney et al., 2005). In contrast to melanoma cell lines, immunofluorescence analysis revealed no HERV-K-env protein expression in UVB-treated or untreated non-melanoma cells. Additionally, when measured by PERT assay, UVB treatment (200 mJ/cm^2) led to the production of up to 5.5 times more retroviral particles in the supernatants of melanoma cell lines as compared to the non-irradiated controls. On the contrary, no production of retroviral particles in response to UVB exposure was detectable in non-melanoma cell supernatants. It should be noted that increased expression of the HERV-K-env protein was detectable 48 h after UVB exposure in our immunofluorescence analysis, while UVB-induced RT activity was detectable earlier, i.e. after 24 h measured by PERT assay. Earlier detection of stimulated particle production in response to UVB exposure might be attributed to the higher sensitivity of the PERT assay. However, both immunofluorescence and the PERT analyses suggest that solely in melanoma cells the expression of HERV-K proteins and retroviral particles dramatically increases after exposure to 200 mJ/cm^2 UVB. To the best of our knowledge, we have, for the first time, demonstrated the increased production of retroviral particles in melanoma in response to UVB irradiation. Despite the growing epidemiologic evidence for intermittent, and in particular repeated, intense sun exposure, as a causative factor of melanoma (Elwood and Jopson, 1997), we did not observe a statistically significant transcriptional activation of the HERV-K *pol* sequence in cultured melanocytes (NHEM neo) even after repeated (data not shown) UVB exposure. In addition, we neither observed cytoplasmatic expression of the HERV-K-env protein nor induction of RT activity in response to UVB exposure in NHEM neo or any other analysed non-melanoma cell line. In contrast to our results, a study by Reiche et al. (2010) on the same topic revealed a contrary result. Interestingly the authors found in response to ultraviolet C radiation (UVC; 100–280 nm), the induction of HERV-K *rec* and *np9* transcripts in cultured melanocytes but not in the four different analysed melanoma cell lines. There are several possible reasons for this discrepancy: First, the application of a different UVR spectrum, where we used the physiological wavebands UVA and UVB, whereas Reiche et al. used UVC wavelengths. The importance of the UVR wavelength was emphasized by our experiments where the activation of HERV-K in melanoma cells was detectable only after UVB but not after UVA irradiation. Second, the expression pattern of HERV-K depends on the analysed melanoma cell lines (Buscher et al., 2005; Hirschl et al., 2007). Each melanoma cell line produces HERVs with a particular pattern. The third reason for the discrepancy concerns the selection of the studied genes, i.e. *rec* and *np9* versus *pol* gene. In comparison with the *pol* activation in response to UVB in melanoma cells, the analysis from Reiche et al. demonstrated an inconsistency in the dose response and activation time between genes; *rec* transcription was activated 24 h after exposure to 10 mJ/cm^2 but not after irradiation of the cells with 30 mJ/cm^2, whereas the activation of *np9* was induced with both doses after 12 h; however, it was transient and not detectable 24 h after exposure. This substantial variation in the retroviral gene expression profile in melanoma and non-melanoma cells in response to UVR as observed in different studies suggests that HERV-K could have different biological functions in the initiation and/or progression of melanoma. Earlier, it was also shown that UVB irradiation induces the *pol* gene activation in a spontaneously immortalized keratinocyte cell line as well as in primary epidermal keratinocytes (Hohenadl et al., 1999). This study revealed the activation of *pol* sequences belonging to different HERV families, namely ERV 9, HERV-L, HERV-H families, and HERV-K-T47D, a member of the HERV-K (HML-4) subfamily. Although the authors used degenerate oligonucleotide primers and despite the fact that HERV-K

(HML-2) and HERV-K (HML-4) express 40–60% sequence homology, *pol* gene activation of the HERV-K (HML-2) family was not detected in response to UVB in primary keratinocytes and in an immortalized keratinocyte cell line. This substantiates our finding that transcriptional activation of the *pol* sequence of class II HERV-K (HML-2) constitutes a specific response to UVB in melanoma cells but not in non-melanoma cells. The primers used in our study were highly specific for the *pol* gene of the class II HERV-K (HML-2) subfamily. For this reason, an additional appearance of different HERV family RNA transcripts in response to UVB irradiation in the analysed melanoma and non-melanoma cells as described by Hohenadl et al. (1999) in primary keratinocytes and in an immortalized keratinocyte cell line cannot be ruled out. Similar to our findings, Hohenadl et al. observed no additional induction of HERV families in response to UVA exposure. This emphasizes the importance of UVB wavelengths for the activation of the retroviral *pol* sequence. Although the role of HERVs in human tumorigenesis remains controversial, their potential involvement in carcinogenesis via the activation of proto-oncogenes or the expression of functional proteins, immunosuppressive proteins, and virus-like particles has been suggested (Canaani et al., 1983; Ruprecht et al., 2008; Ryan, 2009). Furthermore, important characteristics of replicating retroviruses are the de novo insertion by retrotransposition. De novo insertion into exons frequently interrupts open reading frames and may result in the loss of gene function (Kazazian, 1998; Kazazian et al., 1988). Destruction of tumor suppressor genes by insertional mutagenesis, in particular by insertionally polymorphic HERV-K family members, may contribute to the multistep process required for melanoma carcinogenesis triggered by a higher replication of retroviruses (Miki et al., 1992; Morse et al., 1988; Moyes et al., 2007). The functional proteins rec and np9, which are encoded by HERV-K, have been shown to interfere with the germ-cell development in mice and may cause germ-cell tumors by derepressing proto-oncogene c-myc (Boese et al., 2000; Denne et al., 2007; Galli et al., 2005). Considering the UV-inducible transcriptional activation of *rec* and *np9* in melanocytes as demonstrated by Reiche et al. (2010), the possibility of HERV-K involvement in the initiation of melanoma is conceivable. In contrast, our data so far do not suggest the involvement of HERV-K in the initiation of melanoma but rather in the progression of melanoma, which is in accordance with the observations recently published by Serafino et al. (2009). This study revealed that the transition from an adherent to a more malignant non-adherent melanoma growth phenotype essentially requires the expression of HERV-K. Both, the published stress-induced transition by Serafino et al. (2009) and the UVB-induced activation of HERV-K in our study, are associated with the increased expression of the HERV-K-env protein and the increased production of retrovirus-like particles in melanoma. The additional importance of the HERV-K-encoded env protein in the tumor–host interaction was demonstrated by Mangeney et al. (2005) who proved that the env protein has immunosuppressive properties that favor tumor progression by reducing the immunosurveillance of the host. Oricchio et al. (2007) substantiated the role of HERV-K in melanoma progression by showing that the inactivation of HERV-K by RNAi in melanoma cells reduced their tumorigenic potential. Our study demonstrates the increased transcriptional activation of HERV-K and the augmented expression of the immunosuppressive retroviral env protein in melanoma cells in response to UVB. The lack of this phenomenon in non-melanoma cells suggests that UVB might be involved in the progression of melanoma via activation of HERV-K. In conclusion, there are at least two conceivable rationales for the involvement of UVB-induced HERV-K expression in melanoma progression: First, the increased expression of the immunosuppressive retroviral env protein in melanoma may subvert the tumor immunosurveillance and hence encompasses an important role in promoting melanoma development. Second, UVB as an external cellular stressor generates the acquisition of an increased melanoma cell malignancy. Nevertheless, it is also conceivable that our results are rather an epiphenomena or markers for different transformational stages. Further studies are required to clarify the functional consequences of UVB-induced HERV-K activation and its impact on melanoma genesis. However, the driving force behind melanoma development is the acquisition of somatic mutations in key regulatory genes, but almost none of the established alterations carries signatures of an UVR-induced mutation. Only the *INK4a-ARF* (CDKN2A) locus exhibits UV signature mutations, suggesting that this locus might be a direct target for UVR (Hocker and Tsao, 2007; Pollock et al., 1995). As hardly any mutational signatures of UVR exposure were found in the established functional alterations of melanoma, the increased expression of HERV-K in response to UVB exposure as well as the potential role played by endogenous retroviruses in human tumorigenetic processes might open up a new perspective in the complex relationship between melanoma and its most established risk factor: UVR.

Methods

Cell culture

The human melanoma cell line SKMEL28 was obtained from the American Type Culture Collection (ATCC). Melanoma cell line 518A2 was provided by Peter Schrier from the University of Leiden (Leiden, the Netherlands), and cell line MELJUSO by Jürgen Lehman from the Institute of Immunology (University of Munich, Germany). Primary melanoma cells 6F were derived from a metastasis of a nodular melanoma. To confirm that 6F cells are derived from melanoma cells, expression of melanoma-specific antigens such as gp 100, MART1, MIA, and tyrosinase (Carlson et al., 2005) was demonstrated. All human melanoma cell lines were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10%

heat-inactivated fetal bovine serum (Gibco, Paisley, UK) and 1% penicillin/streptomycin (Invitrogen). Normal human neonatal melanocytes (NHEM neo) from two donors were obtained from Cell Systems (Vienna, Austria) and cultured according to the manufacturer's protocol. Human dermal fibroblasts were obtained from the ATCC and grown in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 15% heat-inactivated fetal bovine serum (Gibco). Normal human keratinocytes (NHEK) were isolated from human skin samples of healthy adult volunteers after cutaneous surgery and grown in keratinocyte growth medium (Clonetics, San Diego, CA, USA). Donors of all analysed skin and tumor samples gave informed consent. The protocol was approved by the Institutional Review Board.

UV irradiation

Cells were grown in 100-mm dishes (TPP, Switzerland) until 90% confluency. Before irradiation, cells were washed with Dulbecco's phosphate-buffered saline (DPBS; BioWhittaker Europe, Verviers, Belgium). During irradiation, cells were kept in DPBS in order to avoid potential hazardous effects of medium components. Non-irradiated control cells were kept in DPBS in a separate tissue culture hood. UV irradiation was performed with two different light sources for UVB and UVA (UV 208T, UVA 236T; Waldmann Medizintechnik, Schwenningen, Germany) emitting a continuous spectrum between 290 and 330 nm (UVB) and 340 and 390 nm (UVA), respectively. UV lamps were covered with a 2-mm-thick transparent polyacryl screen filtering out short-wave UVR (<290 nm). The fluence rate of the lamps at the site of irradiation was measured with an UV meter (Waldmann Medizintechnik). Doses between 10 and 200 mJ/cm^2 UVB and 10 and 30 J/cm^2 UVA were applied by varying the exposure time. After irradiation, DPBS was removed, fresh standard culture medium was added, and cells were further incubated in a humidified atmosphere of 5% CO_2 and 95% ambient air at 37°C until the time point of analysis.

Preparation of particles and PERT

Cell supernatants were centrifuged at 853 ***g*** at 4°C and sterile-filtered through a 0.22-μm low-protein binding membrane (Nalge Nunc, Naperville, IL, USA) to remove cellular debris. Clarified supernatants were ultracentrifuged for 1 h 20 min at 287 000 ***g*** at 4°C using a Beckman SW55Ti rotor. Pellets were washed with ice-cold PBS and ultracentrifuged once more for 1 h 20 min at 287 000 ***g***. The resulting pellets were resuspended and used as enzyme source for reverse transcriptase (RT) activity determined by a product-enhanced reverse transcriptase assay (PERT) based on real-time TaqMan PCR as previously described (Arnold et al.,1998), with modifications. First, pellets were resuspended in lysis buffer (50 mM Tris, pH 8.3, 50 mM KCl, 7.5 mM MgCl, 1% Triton X-100). Second, RT was performed using 0.3 g/l MS2-RNA (Roche, Indianapolis, IN, USA) as a template to define reverse transcriptase activity and the MS2-RNA-specific primer 3′-A10 (5′-CACAGGTCAAACCGCCTAGGAATG-3′). To limit unspecific RT activity, 1 g/l of Activated Calf-Thymus DNA (Sigma-Aldrich, St Louis, MO, USA) was added. After incubation at 42°C for 1 h, a 5-μl aliquot of this reaction was amplified by real-time PCR by adding 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 μl of the primers 3′-A10 and 5′-A11 (5′TCCTGCTCAACTTCCTGTCGAG-3′) at a concentration of 10 pmol/μl each, 0.5 μl of fluorescent probe-based product-enhanced reverse transcriptase probe (genXpress, 10 μM; 5′ FAM-TCTTTAGCGAGACGCTACCATGGCTA-TAMRA 3′), and 6 μl of sterile water. The resulting mixture was amplified using the ABIPRISM® 7700 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Samples were analysed in triplicates. For quantification, a calibration curve was generated by plotting the RT activity of serial dilutions of Moloney murine leukemia virus reverse transcriptase (Superscript II Life Technologies, Inc., Invitrogen, Carlsbad, CA, USA).

Table 1. Primers and Taqman probes used for real-time PCR analysis

pol forward primer: 5′-CCACTGTAGAGCCTCCTAAACCC-3′
pol reverse primer: 5′-TTGGTAGCGGCCACTGATTT-3′
pol probe: 5′-FAM-CCCACACCGGTTTTTCTGTTTTCCAAGTTAA-TAMRA-3′
β-actin forward primer: 5′-GCGAGAAGATGACCCAGATC-3′
β-actin reverse primer: 5′-CCAGTGGTACGGCCAGAGG-3′
β-actin probe: 5′-FAM-CCAGCCATGTACGTTGCTATCCAGGC-TAMRA-3′

Real-Time PCR

Cells were washed in DPBS, scraped from the bottom of tissue dishes, pelleted, and washed with DPBS by centrifugation at 213 ***g*** for 5 min. Total RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA was subjected to DNase digestion by using the RNase-free DNase Set (Qiagen). The resultant DNA-free RNA was quantified using Genova MK3 Life Science Analyzer (Jenway, Essex, UK). Of total RNA, 0.5 μg was used to generate random primed hexamer cDNA by using the Superscript II reverse transcription kit (Invitrogen). To avoid DNA contaminations, which give false-positive results, an endogenous control without reverse transcriptase was added. Primers and Taqman® probes (purchased from genXpress, Wiener Neudorf, Austria) for the retroviral *pol* fragment were designed using PrimerExpress Software (PE Applied Biosystems, Weiterstadt, Germany; see Table 1) based on genomic regions in various subtypes and genotypes of the class II HERV-K (HML-2) family. The resulting cDNA was used in subsequent real-time PCR. Real-time PCR was performed using the Taqman® Universal PCR Master Mix and the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems). Five microliters of cDNA solution was used as the PCR template in the presence of 12.5 μl of TaqMan Universal Mastermix, 0.5 μl (10 pmol/μl) of *pol*-specific forward and reverse primers, 0.25 μl (10 pmol/μl) of *pol*-specific TaqMan probe, and 6.375 μl of water. As endogenous control, cDNA was also amplified in the presence of 12.5 μl of TaqMan Universal Mastermix, 0.5 μl (10 pmol/μl) of *β-actin*-specific forward and reverse primers (PE Applied Biosystems), 0.5 μl (10 pmol/μl) of *β-actin*-specific TaqMan probe (PE Applied Biosystems), and 6 μl of water. The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Samples were analysed in triplicates. For absolute quantification, the *pol* fragment cloned into the vector pCR4®-TOPO was used to establish a standard curve.

Antibodies

Rabbit polyclonal anti-HERV-K-env (envelope) protein antibody was used for immunofluorescence staining. The sequence of melanoma-associated endogenous retrovirus (MERV/National Center for Biotechnology Information accession no. AX743231) was screened for potential B-cell epitopes based on B-cell epitope prediction by routine E-Score (Emergentec Biodevelopment, Vienna, Austria), resulting in a MERV-env 39–55 sequence stretch used as antigen for antisera generation in rabbits (BioGenes, Berlin, Germany). The selected MERV peptide is fully conserved on protein level in HERV-K_3q21.2, HERV-K_8p23.1, HERV-K_19q12, HERV-K108, and HERV-K109. As negative control, rabbit IgG isotype control (Sigma-Aldrich, Saint Louis, USA) was used.

Immunofluorescence

Cells grown on chamber slides were fixed in 4% paraformaldehyde (Sigma-Aldrich). Immunofluorescence staining was carried out by incubating chamber slides with rabbit polyclonal anti-HERV-K-env sera at a dilution of 1:200 and subsequent incubation with Alexa Fluor-488-Goat anti-Rabbit (Invitrogen) secondary antibody at a dilution of 1:500. Counterstaining was performed by mounting in Vectashield containing propidium iodide (Vector Lab Inc., Burlingame, CA, USA). Preparations were analysed using confocal laser scan microscopy (LSM, Zeiss Axiovert 100, Jena, Germany).

Statistical analysis

In order to investigate the potential difference of the effect caused by UVB irradiation at various time points on log-transformed *pol* mRNA copy numbers between melanoma and non-melanoma cells, a mixed model was set up. In this model, an indicator of malignancy, a cell line variable nested in this indicator, time (as a class variable), and an UVB indicator were the main fixed effects. For the first three variables, all two-way terms and the three-way interaction term were included in the model. In addition, a random factor indicating the various experiments for each cell line was nested in the malignancy time's cell line interaction. Comparisons at 24 h were tested using appropriate contrasts, while the overall difference of the irradiation effect between melanoma and non-melanoma cells was tested using the three-way interaction. In order to compare log-transformed fold changes (irradiated versus non-irradiated values) for PERT assay data between melanoma and non-melanoma cells, a simpler model was used, because there was only one time point (24 h) and irradiated and non-irradiated values could be paired within each experiment. A potential UVB dose dependence of (log-transformed) retroviral *pol* mRNA expression and (log-transformed) RT activity induction was investigated using linear regression, where higher-order dose terms were not significant and thus dropped from the model. The reported P-values are the results of two-sided tests. P-values ≤0.05 were considered to be statistically significant. All computations were performed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA, 2008).

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