Skin carcinomas in organ-transplant recipients: from early oncogenic events to therapy

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CHAPTER 4

UV-induced apoptosis is not diminished in the presence of beta-papillomaviruses in habitually unexposed skin, but does decrease with age

Submitted

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Summary

Background Beta-papillomaviruses (beta-PV) may play a role in early skin cancer development either alone or in combination with UV exposure. Earlier in-vitro studies showed that beta-PVs decreased UV-induced apoptosis.

Objectives To investigate whether beta-PVs decrease UV-induced apoptosis in-vivo and whether UV exposure affects the presence and quantity of beta-PVs.

Methods Thirty organ-transplant recipients (OTR) and 30 immunocompetent individuals (ICI) were exposed to 3 minimal erythema doses (MED) UVB irradiation on unexposed buttock skin. Biopsies were taken from both irradiated and non-irradiated skin. Beta-PV types were detected and identified by PCR and reverse hybridization and quantified by q-PCR. Apoptotic cells were stained with anti-active caspase 3.

Results Beta-PV DNA was detected in 47% of non-irradiated and 53% of UVB-irradiated skin samples from OTR, and in 27% of both non-irradiated and UVB-irradiated skin samples from ICI. The quantity of beta-PV was generally below 1 copy per 1000 cells. There was no effect of a single UV exposure on the detected presence of beta-PV. We did not detect an association between beta-PV presence and UV-induced apoptosis in both groups studied. We did, however, find in both OTR and ICI a decrease in UV-induced apoptosis with age, which was not linked to any significant increase in beta-PV with age.

Conclusions In line with low copy numbers of beta-PV, we did not detect any overall decrease in UV-induced apoptosis in beta-PV positive skin samples, nor any immediate effect of UV irradiation on the beta-PV types detected. In both OTR and ICI, the number of UV-induced apoptotic cells decreased with age.

Introduction

The majority of organ-transplant recipients (OTR) develop multiple skin cancers, especially squamous-cell carcinomas, on sun-exposed skin.[1] In transplant recipients the development of skin cancer is strongly associated with the number of keratotic skin lesions, consisting of viral warts and actinic keratoses.[2;3]

Recently, epidermodysplasia verruci-formis (EV)-related human papillomaviruses were re-classified into beta-papillomaviruses (beta-PV). Infection with beta-PV occurs frequently and may persist for many years.[4] A wide diversity of beta-PV-types can be detected in both pre-malignant skin lesions and skin carcinomas.[2;5-7] Beta-PV-DNA has been detected in up to 90% of squamous-cell carcinomas of OTR.[8] In immunocompetent individuals (ICI) detection rate of beta-PV-DNA in skin carcinomas is lower, namely between 30 and 50%.[8;9] The presence of beta-PV-DNA in plucked eyebrow hairs of ICI is associated with a history of squamous-cell carcinoma.[10] Sero-epidemiological studies also showed an association between sero-response against beta-PV and skin cancer risk.[11;12] The presence of antibodies against HPV8 was particularly associated with the development of squamous-cell carcinomas[11;13-15] and actinic keratoses.[13] All

these findings together provide indirect evidence that beta-PV may play a role in skin cancer development in organ-transplant recipients and immunocompetent individuals either directly or in combination with ultraviolet exposure.[16;17]

Ultraviolet B (UVB) exposure has been recognized as the most important etiological factor in the development of skin cancer. UVB irradiation causes the induction of somatic mutations through the formation of pyrimidine dimers and 6-4 photoproducts. The failure to repair this DNA damage or to remove severely damaged cells by apoptosis may ultimately lead to skin cancer.[18-20]

In-vitro, beta-PV have been reported to hamper DNA repair.[21;22] Recently, it also has been shown that beta-PV E6 proteins inhibit apoptosis in-vitro in response to UV damage.[23;24] The impact on apoptosis has not been studied in-vivo. The aim of the present study was to investigate whether apoptosis was decreased in the presence of beta-PV after an UVB challenge in human skin in-vivo. Furthermore, we investigated whether the presence and diversity of beta-PV in previously non-sunexposed skin were modified by a single UVB exposure. For this purpose we used a novel, highly sensitive beta-PV detection technique.[25]

Material and methods

Subjects OTR who were regularly seen at the Department of Dermatology from the Leiden University Medical Center (LUMC) in the period between January 2003 and January 2005 were eligible for the study. To study the impact of beta-PV separately in immunocompetent individuals, volunteers were recruited from the LUMC and the University of Leiden through advertisements. Consequentially, the groups were not matched, but studied independently for the effects of beta-PV. Inclusion criteria among OTR were a functioning graft of 5 years or longer; for both patients and controls inclusion criteria were an age of 18 years or older; skin type I through III and sun-unexposed buttock skin, without pre-existent solar damage. ICI with systemic diseases were excluded. The medical ethical committee of the LUMC approved the study and all participants provided written informed consent. In total, 30 organ-transplant recipients and 30 immuno-competent individuals were included in the study.

MED testing and UVB irradiation For the determination of the minimal erythema dose (MED), which is defined as the smallest dose of UV radiation to result in just detectable erythema, a phototesting device designed by Diffey *et al*. was used.[26;27] The device is made of two flexible metal foils, containing 10 ports with mesh attenuators giving 10 irradiance levels with an 8 : 1 ratio from the highest to the lowest exposure. The device was slightly modified to maintain a constant distance of 3 mm to the skin.

At the day-1 visit, the MED test was performed on the previously non-sunexposed buttock skin of each individual. The phototesting device was placed on the skin and irradiated with broadband UVB TL-12 tubes (Philips, Eindhoven, The Netherlands) for 4 minutes, giving an irradiance ranging from 30 to 240 mJ cm^{-2} UV. The MED was assessed visually 24 hours later. No difference between the MED values of the OTR and the ICI was observed. The average MED values [95% CI] were 125 [117-133] and 135 [120-150] mJ cm^{-2}, respectively. Subsequently, an area of 1.5 cm^2 of the opposite unexposed buttock skin was exposed to 3 MED UVB irradiation. On the third day, 24 hours after irradiation, one 4-mm punch biopsy was taken from the 3-MED area, together with a control biopsy from adjacent (a maximum distance of 1 cm) non-irradiated buttock skin of OTR and ICI. All biopsies were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), snap frozen in liquid nitrogen and stored at −80 °C.

To investigate chronically sunexposed skin, we also took paired biopsies of UV-irradiated and non-irradiated dorsal forearm skin. MED determination was, however,

very difficult in this heavily sun-damaged skin with high numbers of solar keratoses and other hyperkeratotic skin lesions. The MED readings were considered very unreliable. Therefore, these forearm specimens were not suitable for comparative measurements of apoptosis. After testing 9 OTR on the forearm, this part of the experiment was discontinued, and only the presence of beta-PV types was ascertained in the acquired forearm skin biopsies.

Processing of the samples For all samples, first 5 µm cryosections for immuno-histochemistry were cut. Six serial cryosections were used for DNA isolation. DNA was extracted for both the PM-PCR RHA method (see below) and the quantitative PCR using the Genomic Tip kit (Qiagen, Hilden, Germany).
To prevent contamination, after each sample strict cleaning of the blade was performed. Furthermore, a control (blank) sample, consisting solely of Tissue-Tec, was cut after each pair of biopsies of one individual. All control samples were processed identically to the skin samples. During analysis seven out of 60 blank control samples were beta-PV-positive, each for a different type of beta-PV. Since these types showed no correspondence with the ones found in samples cut directly preceding or following the control sample, they were considered false positives from minor random contamination. An airborn infection cannot be excluded and appears to be the most likely explanation. This false positive rate constituted a low background noise, which was substantially and significantly lower than the positive rates in the skin samples of both OTR and ICI ($p<0.001$).

The PM-PCR RHA method and quantitative PCR Beta-papillomavirus detection and genotyping was carried out with the PM PCR Reverse Hybridization Assay (PM-PCR RHA) method (Skin (beta) HPV prototype research assay; Diassay BV, The Netherlands). We have applied strict guidelines adapted from Kwok et al.[28] to prevent contamination. The method was designed for the identification of 25 established beta-PV types, namely beta-PV genotypes 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, cand92, 93 and cand96. This method was described in detail earlier.[25] In short, a broad-spectrum consensus PCR primer set was used for amplification of a 117 bp fragment from the beta-PV E1 gene. Generated amplimers were subsequently analysed in the RHA on nitrocellulose membrane strips containing oligo nucleotide probes for 25 beta-PV types. The RHA includes several hybridisation and (stringent) washing steps followed by an enzymatic colouring reaction that visualises the presence of biotinylated amplimer bound to the probes. The analytical sensitivity of the PM-PCR RHA method was reported to be 10 to 100 viral genomes. Intra- and interlaboratory variability experiments showed that the reproducibility of the assay was very high. Furthermore, no aspecific results were reported as assessed by theoretical alignments, challenging the genus specificity of the PCR and analysis of high concentrations of amplimers derived from the 25 beta-PV types.[25]
For a subset of positive buttock skin samples a quantitative PCR for beta-PV types 5, 8, 15, 20, 23, 24, 36, 38 was performed, as described previously.[29] The quantity of beta-PV was expressed as 1 beta-PV copy per n cells.

Activated caspase 3 immunohistochemistry and scoring apoptosis Frozen skin sections (5 µm) on 3-amino-propyltriaethoxysilane-coated glass slides were fixed for 10 min. in acetone at room temperature. Before and between incubation steps, the slides were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 for 5 min. After blocking with 2% normal human serum diluted in PBS /1% BSA for 20 min, the slides were incubated overnight with the primary antibody; polyclonal rabbit anti-active caspase 3 (Cat. No. 557035, BD Pharmingen) diluted 1:30 in PBS/1% BSA. After washing, skin sections were incubated

with the second antibody for 60 min; biotinylated goat anti-rabbit (IgG) (1:200; Vector, Burlingame, CA, USA). Subsequently, the slides were incubated with a horseradish peroxidase (HRP)-labelled avidin-biotin complex (DAKO, diluted 1:100 in PBS/1% BSA) for 60 min. Antibody binding was visualized by incubating the sections in 0.1 M acetate buffer (pH 5) containing 20 mg of 3-amino-ethyl-carbazole (Sigma, St Louis, MO, USA) and 100 µl of 30% hydrogen peroxide per 100 ml during 7 min. The skin sections were counterstained with haematoxylin and rinsed with running tap water for 10 min. All slides were mounted with Kaiser's glycerin and kept at room temperature. Anti-active caspase 3 positive cells in the epidermis were quantified per high-power field (original magnification x 400). The number of positive cells in all fields (mean 17; range 4-36) was assessed. Subsequently, the average number of positive epidermal cells per high-power field was calculated.

Statistical analyses The sample size of this study was calculated according to the criterion that a difference of 2 apoptotic cells/high-power field between beta-PV-positive and -negative skin still should be detectable. Beta-PVs have been detected more frequently in normal skin samples of organ-transplant recipients (17-87% positivity) than in immunocompetent individuals (16-35% positivity).[4;6;30;31] It was expected that in 80% of the OTR and 30% of immunocompetent individuals beta-PV would be present in healthy skin. With a power of 80% and a significance level (alpha) of 0.05, we calculated that 30 OTR would be sufficient to distinguish significantly between beta-PV-positive and -negative skin in level of apoptosis. We also included 30 ICI and studied both groups separately.

For the statistical analyses, we used SPSS version 12.0.1 for Windows. To compare the mean levels of apoptosis between beta-PV-infected and non-infected skin in and between OTR and ICI the Student's T-Test was used. The association between age and apoptosis was tested by linear regression analysis. All analyses were performed for the OTR and ICI, separately.

Results

Baseline characteristics

The baseline characteristics of the organ-transplant recipients and immunocompetent individuals are listed in Table 1.

Beta-PV DNA was frequently detected in low quantities and not affected by UV exposure

Tables 2 and 3 show the results of beta-PV DNA typing in skin biopsies of 30 OTR and 30 ICI, respectively. In the OTR beta-PV DNA was detected in 14 (47%) non-irradiated and 16 (53%) irradiated buttock skin biopsies (Table 2). In the 30 ICI beta-PV DNA was detected in 8 (27%) of both non-irradiated and irradiated buttock skin biopsies (Table 3). In the forearm skin of 9 OTR the beta-PV positive percentages were 89% and 63%, respectively (Table 2). In both groups, the most prevalent beta-PV types in buttock skin were beta-PV 8, 23, and 38. In OTR vs. ICI beta-PV 8 was present in 12% (7/60) and 7% (4/60) of the samples respectively, beta-PV 23 was found in 17% (10/60) and 7% (4/60), and beta-PV 38 in 15% (9/60) and 3% (2/60) of the samples, respectively (Tables 2 and 3).

Age was not associated with the presence of beta-PV (Figure 1). The mean age (SD) of the OTR with 1 or more beta-PV types in any biopsy was 55 (11) compared with an age of 54 (14) years in OTR without beta-PV. In ICI with and without beta-PV present the mean age was 33 (12) and 32 (12) respectively. Time after transplantation did not influence beta-PV positivity either. The beta-PV positive patients were tested at a mean of 19 years after transplantation, while the beta-PV negative patients were 16 years post transplantation. Also, gender was not significantly associated with presence of beta-PV DNA in the OTR and ICI. There was a large inter- and intraindividual variation in the number and types of beta-PV

Table 1. Characteristics

	Organ-transplant recipients	Immunocompetent individuals
No. of individuals	30	30
Sex		
Male	15	15
Female	15	15
Age (years)		
Mean ± SD	55 ± 12	32 ± 12
Range	32 – 77	19 – 57
Time after transplantation (years)		
Mean ± SD	18 ± 9	
Range	5 – 36	
History of skin cancer		
No. of patients with skin cancer (%)	19 (63%)	0 (0%)
Mean no. of skin cancers ± SD	6 ± 9	
Range	1-38	
Medication type (No. of patients; %)		
P + A	14 (47%)	
P + C	8 (27%)	
P + C + A	2 (7%)	
P + M	2 (7%)	
P + C + M	2 (7%)	
P	1 (3%)	
Unknown (clinical trial)	1 (3%)	

Abbreviations: SD=standard deviation; P=prednisone; A=azathioprine; C=cyclosporine; M=mycophenolate mofetil

Table 2. Beta-PV types in buttocks and forearms and apoptosis in buttock skin of organ-transplant recipients, presented with increasing levels of apoptosis.

No.	Gender and age (yrs)	Buttocks		Forearms		Apoptosis after UV in buttock skin (no. of pos. cells/ field)
		Control biopsies (not irradiated)	Biopsies irradiated skin	Control biopsies (not-irradiated)	Biopsies irradiated skin	
1	M 56	-	-	76	-	0.1
2	F 53	19	23,37	-	-	0.4
3	M 53	20,23	8,17,38	22,23,80,92	22,23,80,92,93	0.6
4	M 65	22,24,76	24,36	N.d.	N.d.	0.9
5	F 52	-	5,23,80	17,24	-	1.0
6	M 44	-	-	20,37	93	1.2
7	F 42	20,23	9	8,12,20,23,75	23,24	1.4
8	F 69	8,25	25,38	17,23,25,37,38,96	5,15,17,23,36,38	1.4
9	M 57	-	-	N.d.	N.d.	1.6
10	F 45	96	-	N.d.	N.d.	1.6
11	F 50	-	38,76	12,22,24,80	12,22,37	1.6
12	F 63	8,36,38,92	8,36	N.d.	N.d.	2.0
13	M 77	-	-	N.d.	N.d.	2.9
14	M 74	8,23,38	-	N.d.	N.d.	2.9
15	F 57	-	15,92	N.d.	N.d.	3.0
16	M 70	-	-	N.d.	N.d.	3.2
17	M 61	-	-	N.d.	N.d.	3.4
18	F 65	75	-	N.d.	N.d.	3.5
19	F 64	38	38	N.d.	N.d.	3.6
20	M 72	19,23,93	23,37,80,93	N.d.	N.d.	4.5
21	F 36	17	-	N.d.	N.d.	4.9
22	F 60	-	22	38	-	6.0
23	M 39	-	8,23	N.d.	N.d.	6.7
24	M 68	-	-	N.d.	N.d.	7.0
25	M 44	-	-	N.d.	N.d.	7.3
26	M 44	22,38,92	23	N.d.	N.d.	7.4
27	M 32	-	-	N.d.	N.d.	8.0
28	F 42	-	38	N.d.	N.d.	12.4
29	F 57	8,23,80,92,93	24,92	N.d.	N.d.	12.9
30	F 40	-	-	N.d.	N.d.	24.5

Abbreviations: N.d.=not done; M=male; F=female; - =no beta-PV present

Table 3. Beta-PV-types and apoptosis in buttock skin of immunocompetent individuals, presented with increasing levels of apoptosis.

No.	Gender and age (yrs)	Buttocks		
		Control biopsies (not irradiated)	Biopsies irradiated skin	Apoptosis after UV (no. of pos. cells/ field)
1	M 56	-	-	1.0
2	F 57	-	-	2.4
3	F 30	15	-	2.6
4	M 24	-	-	3.3
5	M 57	-	8,38	3.7
6	M 25	-	-	3.7
7	M 42	-	-	3.8
8	F 51	-	24	4.3
9	F 36	-	23	4.3
10	M 32	-	-	5.5
11	F 26	-	-	6.3
12	M 46	-	-	6.4
13	M 21	-	38	6.5
14	F 21	-	-	7.9
15	M 40	8	-	8.6
16	F 39	-	-	9.3
17	F 25	80	15	9.9
18	M 44	-	-	10.1
19	F 20	-	-	10.2
20	F 25	9	-	10.6
21	F 23	-	-	10.7
22	M 27	-	25	11.6
23	M 23	8	-	12.0
24	M 19	-	-	12.0
25	M 24	-	-	12.2
26	M 23	14	-	13.9
27	F 20	-	-	15.7
28	F 32	23	23	16.9
29	F 49	8	-	20.0
30	F 22	-	5,22,23	37.0

Abbreviations: M=male; F=female; - = no beta-PV present

detected in both OTR and ICI (Tables 2 and 3). In the OTR, the number of types detected in irradiated skin varied from 1 to 4 (mean 1.9), and in the non-irradiated skin from 1 to 5 (mean 2.3). In the ICI the number of types in irradiated skin varied from 1 to 3 (mean 1.4), and in the non-irradiated biopsies all beta-PV positive samples showed only 1 beta-PV type. UV-irradiation did not affect the presence or number of beta-PV in buttock skin nor in forearm skin (Tables 2 and 3).

The quantity of beta-PV was determined in 26 beta-PV positive samples, of which only two samples yielded a positive signal in the quantitative PCR (for beta-PV 23 and 38). One copy of beta-PV DNA was found in less than around 1000 cells in both samples. In the remaining 24 samples beta-PV-DNA loads were apparently below the detection limit (< 10 copies of beta-PV per reaction) of the quantitative PCR (data not shown).[29]

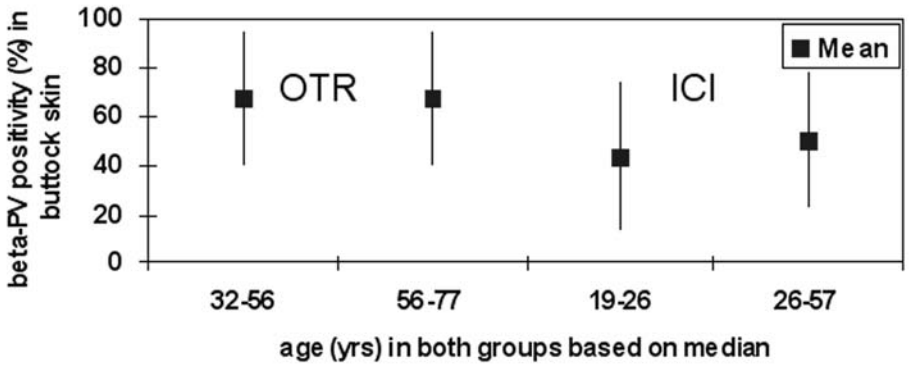

Figure 1. Beta-PV positivity (%) with 95% confidence intervals in different age groups based on median age in organ-transplant recipients and immunocompetent individuals. The findings were based on the cumulative test results obtained from two buttock skin biopsies per individual

Apoptosis was not reduced in beta-PV-infected skin but decreased with age

As expected no apoptotic cells were detected in non-UVB irradiated skin. In UVB-exposed buttock skin of organ-transplant recipients the number of apoptotic cells/high-power field varied from 0.1 to 24.5 (median 3.1). In the immunocompetent individuals the number of apoptotic cells varied from 1.0 to 37.0 (median 9.0) (Figure 2) (Tables 2 and 3).

Interestingly, in the UVB-irradiated biopsies of both the OTR and ICI, the number of apoptotic cells decreased with age (p=0.05 and 0.06 respectively) (Figure 3). Gender was not associated with apoptosis.

In both groups there was no difference in apoptosis between beta-PV positive and negative buttock skin (Figure 4). Also, when examining the effect of the most prevalent beta-PV-types, beta-PV 8, 23, 38, separately, no effect on apoptosis could be detected. Additionally, there was no difference in apoptosis levels between organ-transplant recipients with and without a history of skin cancer. The type of medication (cyclosporine or azathioprine) was not associated with the level of apoptosis either (data not shown). Adjusting our analyses for age and sex of the OTR and ICI did not change these findings.

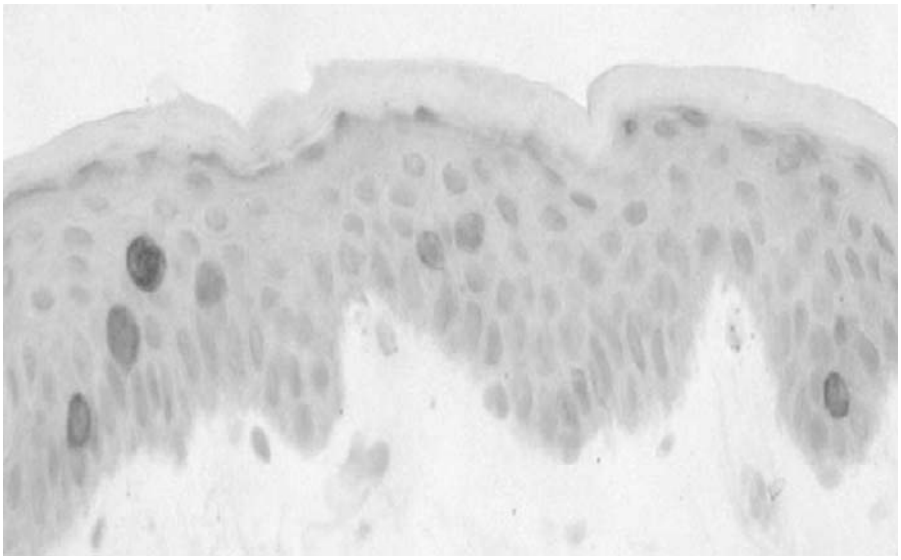

Figure 2. Anti-active caspase 3 positive cells in the epidermis (original magnification x 400).

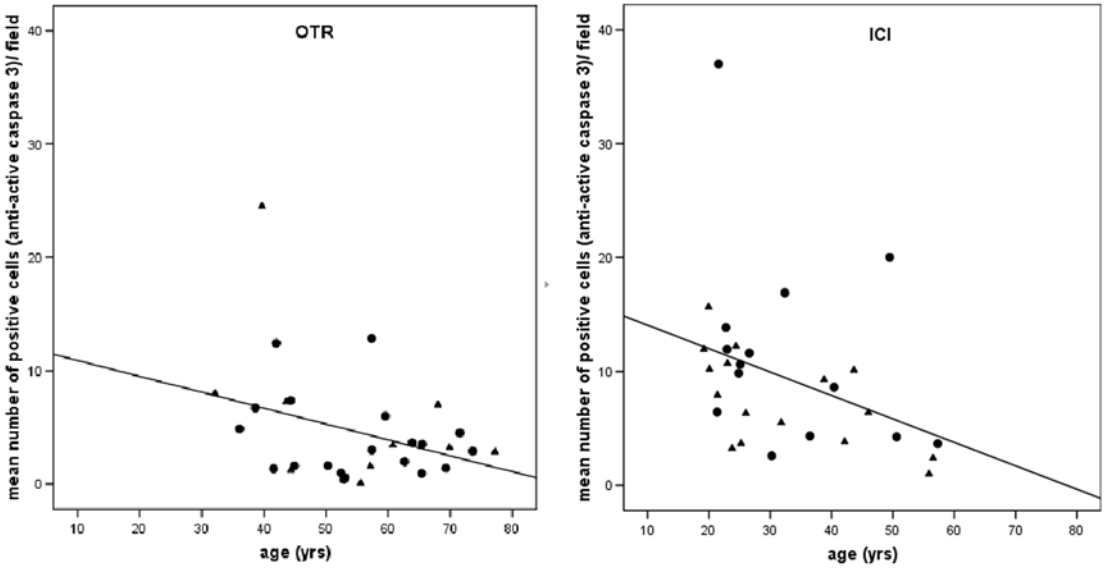

Figure 3. Relation between mean number of anti-active caspase 3 cells per high-power field and age in organ-transplant recipients and immunocompetent individuals.●: beta-PV positive, ▲: beta-PV negative

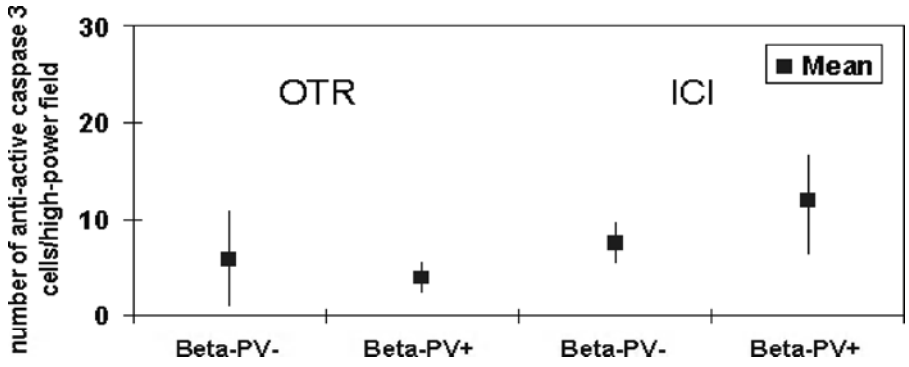

Figure 4. Mean number of anti-active caspase 3 cells per high-power field with 95% confidence intervals in beta-PV-positive and -negative skin samples in organ-transplant recipients and immunocompetent individuals

Discussion

In our study, we could not confirm in-vivo the hypothesis that beta-PVs may decrease UV-induced apoptosis. Based on our study, however, we cannot immediately conclude, that beta-PVs do not decrease UV-induced apoptosis. The low quantity of beta-PV DNA in the skin biopsies, with generally less than one copy of beta-PV per 1000 cells, would explain that we did not find any appreciable decrease in the number of apoptotic cells in beta-PV positive biopsies. In-situ hybridization with beta-PV types of individual apoptotic and non-apoptotic cells is a more precise method to study the effect of beta-PV infection on UV-induced apoptosis in individual cells. Unfortunately, this technique is not available yet and would be not conclusive in our biopsies because of the low viral DNA loads.

It cannot be entirely excluded that part of the beta-PV DNA is present on the surface of the skin. But this DNA probably originated from local, viral replication as can be inferred from the recent findings that beta-PV DNA is persistently present in plucked hairs[32] and, importantly, also in swaps of skin surface.[33] These data strongly suggest that the presence of this viral DNA is due to persistent beta-PV infections and not to contamination.

It is likely that not all beta-PV types exert similar effects on UV-induced apoptosis,

which may be an alternative explanation of the non-detectable effect of beta-PV infection on UV-induced apoptosis. To study the effect of all 25 known individual beta-PV types, separately, much larger series of skin biopsies would be needed. To discriminate better between the effects of the different beta-PV genotypes and to overcome the problem of low viral loads, the impact on UV-induced apoptosis could be studied in cultures of beta-PV transfected keratinocytes.

The dynamics of beta-PV infection in reaction to UV-irradiation is also not known yet. We observed no appreciable effect, but a single UV exposure may not be sufficient to measure an effect of beta-PV infection on UV-induced apoptosis in-vivo. Multiple exposures may lead to a more pronounced effect. We did find a tendency of a higher prevalence of beta-PV in the forearms than in the buttocks of OTR, which supports the notion that beta-PV is more prevalent in chronically sun-exposed, lesional skin. A recent study by Harwood et al. did not show such a difference between chronically sun-exposed and unexposed skin.[31] An earlier study by de Jong-Tieben et al. showed a higher prevalence of beta-PV-DNA in benign skin lesions from chronically sun-exposed sites compared with unexposed skin.[2] This latter result is in line with our supposition that chronic sun-exposure may induce beta-PV replication. It is known that at least some beta-PVs have UV responsive elements [34;35]

In our samples numerous beta-PV genotypes were detected and the mean number of beta-PV types in buttock skin was clearly higher in skin from OTR than from

ICI. However, the OTR and ICI were not age and sex-matched, and therefore not directly comparable. But in the overlapping age range from around 30 to 57 years the beta-

PV positive rate was higher in OTR than ICI (75 vs. 50 %, Figure 1), although not significantly.

Beta-PV effects were meant to be assessed in each group separately. The viral loads were indeed found to be low, and most frequently below the detection limits of the quantitative PCR, but in the similar range as reported before.[29] Corresponding to a sparseness of the viruses in the biopsies, there was a large inter- and intraindividual variation in the number and types of beta-PV.

Interestingly, our study showed that the level of UV-induced apoptosis decreased with increasing age in both OTR and ICI without a link to any appreciable age dependence of beta-PV or of MED values (the overall difference in apoptosis between OTR and ICI is fully attributable to age differences).

A relation between decreased apoptosis and skin cancer development has been established in animal experiments.[36] A study in which UV-induced apoptosis was measured in peripheral blood lymphocytes, showed a significant decrease in apoptosis with increasing age in melanoma patients.[37]

In conclusion, our data show that normal unexposed skin frequently contained viral DNA of various beta-PV types, but in very low quantities. We observed a decreased level of apoptosis with increasing age, not linked to any increase in beta-PV. However, we did not find a relation between beta-PV presence and overall UV-induced apoptosis. To ascertain the effect of beta-PV infection on UV-induced apoptosis in-vivo studies need to focus specifically on beta-PV-carrying cells.

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