Establishment and cidofovir sensitivity of a cell line from a heart transplant recipient with multiple cutaneous tumors

M. Schacke[1], A. Meerbach[1], R. Thust[1], P. Hyckel[2], P. Wutzler[1]

[1]*Institute for Antiviral Chemotherapy, Friedrich Schiller University of Jena, 07745 Jena, Germany, e-mail: Michael.Schacke@med.uni-jena.de;* [2]*Clinic for Maxillofacial Surgery/Plastic Surgery, Friedrich Schiller University of Jena, 07740 Jena, Germany*

Received November 25, 2002

A new cell line, designated as Tuwei00, is described. It originated from an Epstein-Barr virus-positive skin tumor biopsy of a heart transplant recipient, whose numerous cutaneous neoplasms were treated with the antiviral drug cidofovir what caused at least transient remissions. The cell line was established *in vitro* and maintained for more than 70 passages. Cells of early passages were characterized by a slower growth, the inability to form colonies and a higher sensitivity to cidofovir. After overcoming a crisis, the cells grew faster, to a higher density and were able to form adherent colonies from single cells as well as colonies in soft agar. Chromosome analysis showed diploidy/hyperdiploidy at the earlier and hypodiploidy at the later passages. Sensitivity to cidofovir was distinctly higher in early passages of Tuwei00 cells than in later passages and was characterized by distinct decline of cell survival after long term cidofovir exposure. Established normal human keratinocytes, HaCaT cells, which were checked for comparison, showed a low cidofovir sensitivity similar to late passage Tuwei00 cells.

Key words: Preneoplastic keratinocytes, cell line establishment, cidofovir sensitivity.

Organ transplant recipients have a high risk for developing neoplasms [14]. Most frequently seen are squamous cell carcinomas (SCC) and basal cell carcinomas (BCC) of the skin and post-transplant lymphoproliferative diseases (PTLD) [19]. PTLD are Epstein-Barr virus (EBV)-related in a high frequency [23]. In non-melanoma skin cancers as well as in premalignant skin lesions from immunosuppressed patients human papillomavirus (HPV)-DNA is detectable to 75–88% what is more frequent than in immunocompetent patients [9]. Due to the frequent association of post-transplant tumors with various DNA viruses the question arises whether the tumor growth can be influenced or even stopped by treatment with a broad spectrum antiviral drug such as the acyclic nucleoside phosphonate cidofovir. Cell lines isolated from such preneoplastic or neoplastic skin lesions eventually might be used as model systems to elucidate the mode of action of this drug. Here we describe the isolation and cidofovir sensitivity of a cell line from a heart transplant recipient who developed multiple cutaneous neoplasms several years after transplantation. Because of the EBV-positivity of tumor biopsies the patient was repeatedly treated with cidofovir and showed at least transient remissions. The anti-EBV activity of cidofovir in vitro was shown previously by Lin et al [15]. Therefore, it was of interest to study how keratinocytes isolated from the tumors respond to the drug.

Patient, material and methods

Patient. The 63-year old male patient developed multiple cutaneous tumors in the facial region beginning 4 years after heart transplantation [11]. In the following years the patient has been operated 11 times. Histological diagnosis of resected tissue revealed 10 findings of actinic keratosis, 2 findings of keratoacanthomas, 11 findings of basal cell carcinomas, and 15 findings of squamous cell carcinomas. All tissue samples were EBV-PCR positive. With an in situ hybridization technique the presence of EBV-encoded RNA (EBER) was revealed in many tumor infiltrating lymphocytes but not in the tumor cells themselves. Due to the EBV-association of the tumors the patient was treated with

cidofovir. The cidofovir therapy led to a remarkable regression of tumor size, stop of tumor pain and disappearance of small lesions [21]. Samples from histologically different biopsies of the patient were processed in the tissue culture 7 years after heart transplantation.

Reagents. EMEM with HEPES (Bio Whittaker) supplemented with 1% (v/v) L-glutamine (200 mM), 1% (v/v) non-essential amino acids, 1% (v/v) insulin/transferrin/selenium, 100 U/ml penicillin, 100 μg/ml streptomycin and 10–15% FCS was used as culture medium.

Cidofovir (1-[(S)-3-hydroxy-2-(phosphonylmethoxy)-propyl]-cytosine, HPMPC, CDV) was kindly provided by Prof. E. De Clercq (Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium). It was dissolved in complete culture medium.

Primary culture and passaging. Portions of the biopsies were finely minced, placed into T25-flasks and cultured at 37 °C in 5% CO_2 atmosphere. After 5–10 days epithelial cells appeared around the explants. When fibroblasts surrounded the outgrowth, they were removed several times by gentle use of an EDTA (0.02%)/trypsin (1:250; 0.05%) solution in PBS. For passaging of the epithelial cells, a mixture (4:1) of EDTA/trypsin and a collagenase solution (350 U/ml) in PBS was used.

Cell growth experiments. Tuwei00 cell growth kinetics was determined in cells from exponentially growing cultures which were seeded into 24-well plates with about $2.5x10^5$ cells in 1 ml medium per well. After 24 hours the medium was changed and the free floating cells were removed. To detect the time- and dose dependent effect of cidofovir, $2.5 x10^5$ cells were seeded in 1 ml medium per well into 24-well plates. 24 hours later, drug exposure was started by a medium change and addition of different cidofovir concentrations. The culture fluid was replaced by fresh medium with the same drug concentration on days 4 and 8. For the antiproliferative assay, cells from a confluent culture were diluted 1:4 and seeded into 24-well plates, thus the cells were able to divide two times during the culture course. After 24 hours, the medium with the unattached cells was removed and replaced by medium containing 32 μg/ml cidofovir or medium without cidofovir as control. The drug concentration had been chosen from findings in pilot experiments in which a significant inhibitory effect was shown in all investigated cell lines. No further medium change was made. After different culture periods, the cells were suspended and counted by means of a Coulter Z-2 particle counter (Beckmann-Coulter). The rates of surviving cells were calculated as the quotient of the cell number in the cultures exposed to cidofovir and in untreated controls, respectively, at a given time. The data are means of at least four parallel determinations. Population doubling times in the logarithmic growth phase were calculated with the formula :

$t_g = \log 2 \times (t - t_0)/\log N - \log N_0$ [16].

HaCaT cells. The HaCaT cell line, which served for comparison, is a spontaneously immortalized cell line from normal skin keratinocytes [5]. The cells were passaged once per week (1:3) in the same medium as described above but without insulin/transferrin/selenium supplement and with 5% FCS.

Plating efficiency. $1x10^3$ and $2x10^3$ cells per well were seeded in 6-well plates containing 3 ml medium per well. The cultures were fixed two weeks later in methanol (100%) and stained with crystal violet (0.5% in 50% methanol). Plating efficiency was calculated as the percentage of colonies in relation to the total number of plated cells.

Anchorage independent cell growth. 2 ml of 0.5% agarose in culture medium with 10% FCS were placed per well in 6-well plates. 30 minutes later, 2 ml of 0.3% agarose in the same medium containing 2 or $3x10^3$ cells/ml, respectively, were added per well. After incubation for 3 weeks the wells were checked for growth of cell colonies.

Immunocytochemistry. For detection of cytokeratins, suspensions of Tuwei00 cells of the 13th passage were dropped on silane-treated slides. Monoclonal mouse anti-human cytokeratin 5/6 and 10/13 antibodies (DAKO) and the ChemMate^{TM} streptavidin-biotin AP/RED detection kit (DAKO) were used according to the manufacturer's protocol.

Chromosome preparation. Exponentially growing cells were supplemented with colcemid, 0.05 μg/ml, three hours prior to cell harvest. After treatment with collagenase-ETDA/trypsin mixture the resulting cell suspension was hypotonized and prepared in the usual manner. Chromosome preparations were stained with Giemsa. 100 well-spread mitoses per sample were evaluated.

Virus detection. DNA was extracted from cell cultures by a DNA extraction kit (QIAamp DNA Blood Mini Kit, Qiagen). The DNA concentration was determined by UV spectrometry.

Detection of EBV sequences. The nested PCR technique for detecting the EBV sequences was carried out as described previously using primers of the virus capsid antigen p23 region of EBV [20].

Agarose gel electrophoresis of viral DNA. The amplified PCR products were detected by direct gel analysis. A 10 μl sample of the amplification product was run on a 0.8% agarose gel electrophoresis. DNA was monitored under UV-light after staining with ethidium bromide.

Detection of HPV sequences. The HPV PCR for detecting numerous HPV types including Type 6, 11, 16, 18, 31 and 33 was performed using two sets of consensus primers as designed by Manos et al [18] and Snijders et al [26].

Results

Establishment of the cell line. After 3–6 days, nearly half

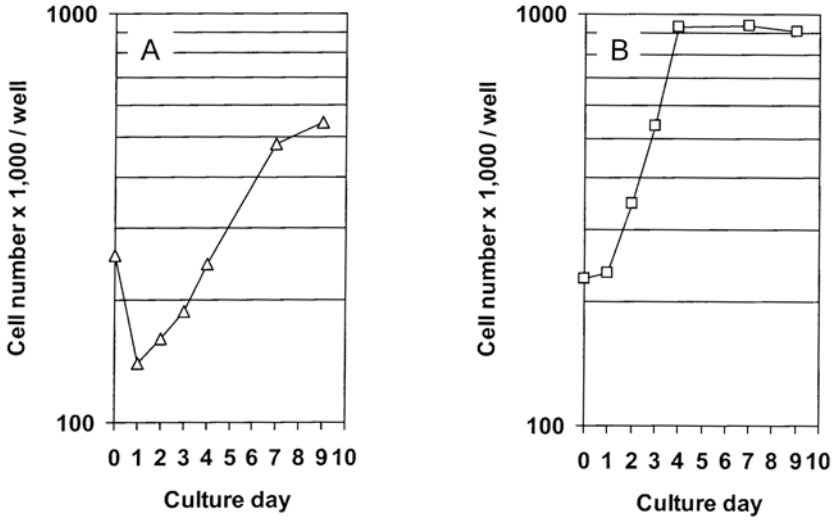

Figure 1. Growth kinetics of Tuwei00 cells at the 15th (A) and the 38th (B) passage. (A) After plating of Tuwei00 cells in early passages a remarkable proportion of the cells did not attach and was removed by medium change after 24 h. (B) In comparison, cells of later passages attached to a higher extent, grew faster and to a higher density.

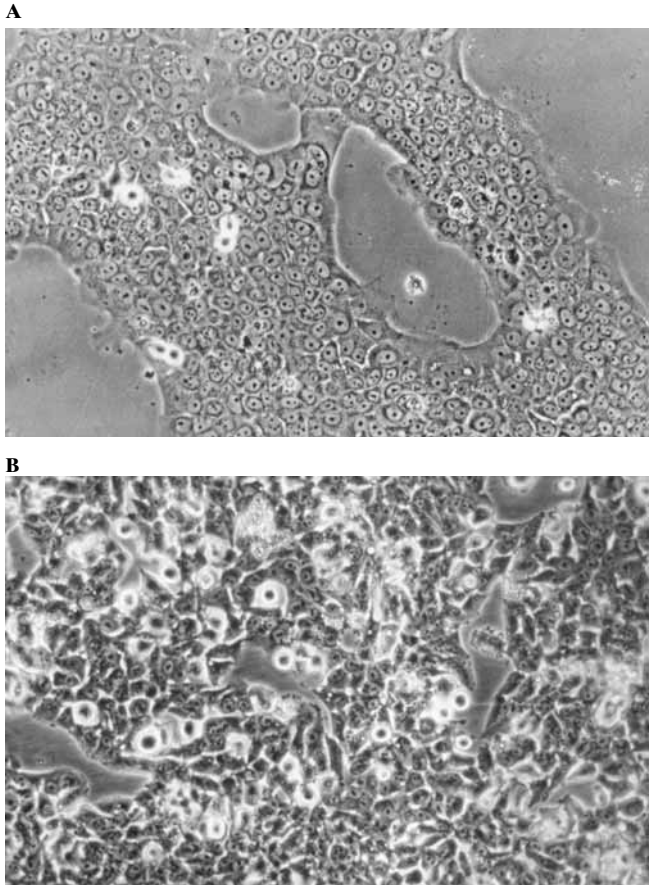

Figure 2. Tuwei00 cells of the 13th passage (A) and of the 65th passage (B) 72 h after seeding. After plating the attached single cells of the early passage assemble and form aggregates before starting proliferation (A). Cells of the later passage do not show this behavior (B). Phase contrast, 180 x.

of the explants were attached to the bottom of the flask and cells with epithelial morphology emigrated. The growth of the epitheloid cells from different tissue samples continued for different time periods. Often the cells started to degenerate and died after several weeks. Usually the outgrowth of epithelial cells was surrounded by fibroblasts which became dominant due to a faster proliferation. Efforts to selectively remove the fibroblasts from the cultures were only transitorily successful. In 2 of the 9 investigated biopsies, however, the epithelial cells did not show senescence and degeneration but overgrew the fibroblasts and, finally, were able to predominate the cultures. The cells referred in this study originated from a premalignant tissue sample diagnosed as acanthosis, hyperkeratosis (dysplasia grade 2) with a chronic inflammation. In the course of about 6 months of *in vitro* culture (comprising 7 passages) it was possible to eliminate the fibroblasts. The resulting epitheloid cell line was designated as Tuwei00. It was continuously cultured and passaged in weekly intervals (at first 1:2 and later 1:5) up to more than 70 passages. Cells of the 13th passage, showed a positive reaction with the antibodies against cytokeratins 5/6 as well as 10/13 what suggests a keratinocyte origin of the cell line.

In early passages, Tuwei00 cells were characterized by a relatively slow growth (Fig. 1A). Between the 9th and the 16th passage a mean population doubling time of about 73 h in the logarithmic phase of the cultures was observed. Growth kinetics (Fig. 1A) demonstrates that in the early passages a rather large proportion of the seeded cells did not attach and died. The attached cells migrated towards each other (Fig. 2A) before they were able to continue proliferation. In this stage of culture these cells were unable to form colonies when plated as single cell suspensions, neither with $1x10^3$ nor with $2x10^3$ cells per well. Moreover, these cells did not form colonies in soft agar.

A crisis, defined as a period with remarkably reduced proliferation, occurred between the 28th and the 31st passage. Thereafter, the resulting cell population showed an enhanced proliferation (Fig. 1B). The population doubling time in the logarithmic phase decreased to about 39 h, measured at the 38th and the 52nd passage. The cells attached more readily, showed a reduced requirement for close cell contact to continue dividing (Fig. 2B) and reached higher densities. The cells appeared more closely packed, stronger granulated, and they were able to form colonies. The plating efficiency in the 51st passage was 5% by $1x10^3$ cells and 9% by $2x10^3$ cells per well. Furthermore, the cells became able

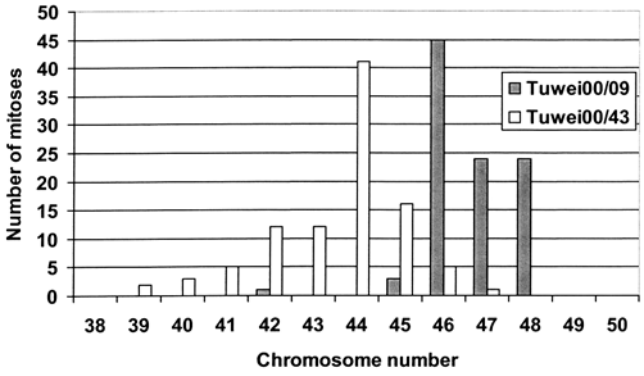

Figure 3. Distribution of chromosome numbers of Tuwei00 cells at the 9th and the 43rd passage. The karyotype of the cells changed from diploid/hyperdiploid in the 9th passage to hypodiploid in the 43rd passage. About 3% of the mitoses of both cultures have chromosome numbers between 78 and 95 (not shown in the figure).

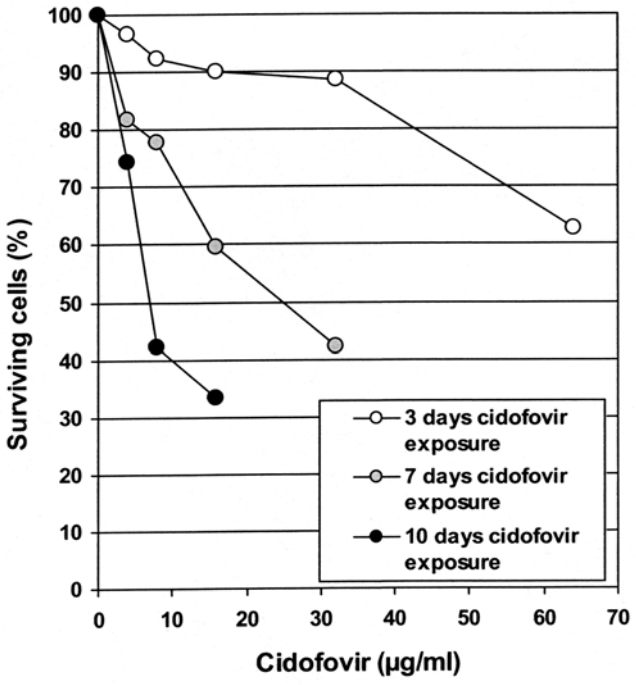

Figure 4. The time- and dose-dependent effect of cidofovir in Tuwei00 cells of the 27th passage. Cell survival rates are quotients of cell number in cultures with different cidofovir concentrations and control cultures without cidofovir at the given time of drug exposure.

to grow in soft agar, as shown in the 63rd passage. A change of the karyotype was obvious in the course of the passages. While in the 9th passage the cells were diploid/hyperdiploid, in the 43rd passage the number of chromosomes was reduced, with a stemline of 44 chromosomes (Fig. 3). The majority of Tuwei00 cells at the 43rd passage had a small marker chromosome.

Detection of EBV and HPV. EBV DNA could regularly be detected by PCR in explant cultures of the biopsy material. Cultures leading to the Tuwei00 line were positive until the 7th week of culture. In this period, the culture was still composed of different cell types including epitheloid cells and fibroblasts. The presence of surviving lymphocytes could not be excluded. The disappearance of all visible non-epitheloid cells was complete after 6 months or 7 passages, respectively. EBV DNA was detected only by nested

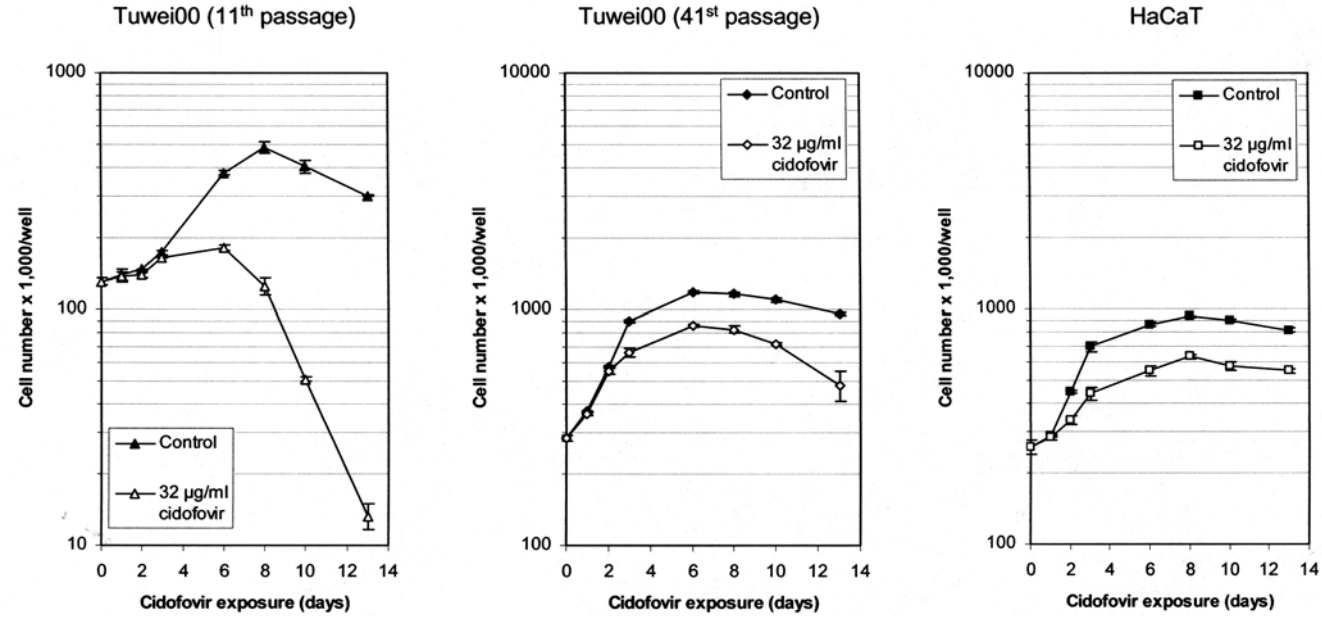

Figure 5. Comparison of the growth kinetics of Tuwei00 (11th and 41st passage) and HaCaT cells treated with 32 μg/ml cidofovir. Exposure started with a medium change 24 h after cell seeding. Data represent mean cell numbers $\pm$ standard deviations (four replicate counts).

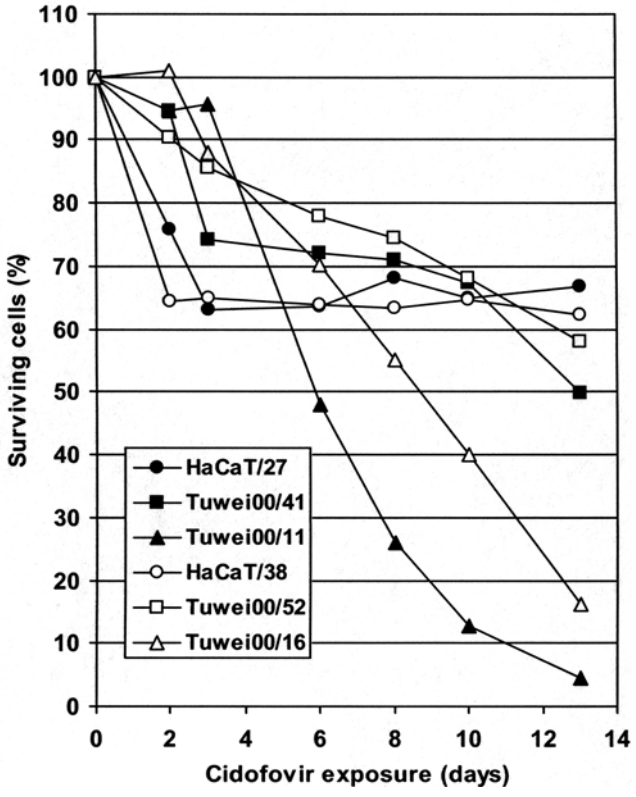

Figure 6. Cell survival rates of early (11th and 16th) and late (41st and 52nd) passages of Tuwei00 cells as well as of HaCaT cells after exposure to 32 μg/ml cidofovir. The rate of surviving cells is the quotient of cell number in cidofovir-exposed culture and control culture. Results of two independent experiments are shown (open and closed symbols).

PCR in small quantities (10 copies in 10^5 cells) in the resulting epitheloid cell line Tuwei00 at the 9th and the 12th passage. Thereafter, also the nested PCR was negative.

All biopsy samples as well as the Tuwei00 cells tested in the 20th passage were negative for HPV DNA.

Antiproliferative activity of cidofovir. Considering the cytostatic response of skin tumors to cidofovir administration in vivo [11, 21], it was of interest to check the cidofovir sensitivity of the Tuwei00 line. For this purpose, cultures exposed to different cidofovir concentrations were observed for 3, 7 and 10 day periods and compared with controls. The data (Fig. 4) show a dose- and time-dependent effect of cidofovir of Tuwei00 cells.

Another question was whether there are differences to other keratinocyte cell lines with respect to cidofovir sensitivity. For this purpose the growth of Tuwei00 cells under cidofovir administration was compared with that of HaCaT cells, a spontaneously immortalized keratinocyte cell line from normal skin [5]. Moreover, the sensitivity of early and late passages of Tuwei00 cells was compared. The effect of cidofovir presumably depends on drug incorporation into DNA (see Discussion). The cells of the considered lines and passages grew with different rates. To generate comparable conditions for drug uptake, the cells were plated at densities permitting them to pass two population doublings until confluence. Thus, the cells should be able to incorporate similar amounts of cidofovir during the experiments, and the resulting inhibition effects should be comparable. Figure 5 shows how proliferation and survival of Tuwei00 cells at the 11th and 41st passage and of HaCaT cells are influenced by 32 μg/ml cidofovir. The calculated rates of surviving cells are shown in Figure 6 together with the results of another similar experiment. Under administration of cidofovir, the population doubling time was 83 ± 17 h in Tuwei00 cells of early passages, but 44 ± 3 h and 50 ± 11 h in Tuwei00 cells of late passages and HaCaT cells, respectively. Thus, there was no striking difference regarding the growth inhibitory effect of cidofovir in Tuwei00 cells in early passages and HaCaT cells in the first growth phase, if the different population doubling times were taken into consideration. But during the further growth and especially in stationary cultures cidofovir showed a remarkably stronger effect on Tuwei00 cells of early passages (Fig. 5). Therefore, a single addition of cidofovir resulted in a stronger reduction of the number of surviving cells in long term cultures of Tuwei00 cells of early passages than of HaCaT cells. Late passages of Tuwei00 cells showed a much lower sensitivity to cidofovir. A 32 μg/ml cidofovir exposure for 13 days led to 5% and 16% survival of Tuwei00 cells of the 11th and 16th passage, respectively, and to 50% survival of Tuwei00 cells of the 41st passage compared with 67% in HaCaT cells (Fig. 6).

Discussion

Successful establishment of keratinocyte cell lines mostly from skin cancers [10, 12, 29, 30], less from premalignant skin tissue [24] and normal skin [1, 4, 5, 25] has been reported repeatedly. Most of these lines were used in studies on cell differentiation *in vitro* or in order to compare different stages of tumor development. We tried to isolate cell lines from malignant or premalignant skin tissue with the aim to get data regarding cidofovir sensitivity. To our knowledge, this is the second description of a spontaneous keratinocyte cell line derived from dysplastic face skin of a transplant recipient [24]. Isolated normal keratinocytes under the described culture conditions *in vitro* (culture medium with a physiological Ca^{2+}-concentration and without EGF addition) would show a terminal differentiation connected with a proliferation stop after a limited number of passages [17]. The fact that it was possible to isolate and keep the cells proliferating with the utilized culture medium indicates that the original cells of the Tuwei00 line were different from normal keratinocytes. This is supported by

the partly abnormal karyotype even in early passages (Fig. 3). Moreover, the Tuwei00 cells were subject to changes in the course of passages and entered a crisis, i. e. a period of reduced or lacking proliferation, around the 30th passage. Thereafter, the cells showed a remarkably altered growth behavior and a different karyotype. The cell line establishment seems to be associated with an immortalization in two steps. Such a behavior is known for the *in vitro* immortalization of cells by DNA tumor viruses or chemicals [6]. Proby et al [24] also described this for the establishment of two spontaneously immortalized keratinocyte cell lines isolated from dysplastic forehead skin of an immunosuppressed renal transplant recipient.

Regarding the detection of EBV DNA in the course of establishment of the Tuwei00 cells, it seems likely to be due to residual lymphocytes from the original biopsy material in the stage when the cell population was still mixed in the first passages. Until the 12th passage of the Tuwei00 cells, there were only traces of EBV DNA detectable by nested PCR, which later became negative. There is no strong indication that EBV is critical for maintenance of the immortal phenotype of these cells, but it is conceivable that EBV is in some manner involved in the first step of immortalization. This would be an explanation of the effect of cidofovir on the tumor cells *in vivo*, but it seems less likely that the effect is just a direct antiviral effect against EBV. In spite of the fact that there is no indication for the existence of HPV in Tuwei00 cell line, cells in early passages show a behavior similar to HPV-positive cells with regard to cidofovir sensitivity. Andrei et al [2] treated several HPV-positive cell lines with cidofovir and also demonstrated an increase of the sensitivity after long term cidofovir exposure.

Eventually, it should be taken into consideration that cidofovir acts as a cytostatic drug, in a way independent of its antiviral properties. Such an effect may also be involved in the recently reported successful treatment of a squamous cell carcinoma of an eyelid with cidofovir [7]. The mode of cytostatic action of cidofovir, however, is still unclear. To our knowledge, as yet it has not been unequivocally proven, whether cidofovir is internally incorporated into the genomic DNA of mammalian cells, but it seems obvious [22]. It might be assumed that incorporated cidofovir is processed by repair systems in a way similar to that recently reported with respect to ganciclovir, thus causing cell killing by apoptosis [28]. Induction of apoptosis by cidofovir has been shown *in vitro* in HPV-transformed keratinocytes [13] and various other HPV-positive cells [3, 27], but also in several HPV-negative tumor cell lines cidofovir seems to induce cell death via apoptosis [3].

Taken together, our studies have shown that keratinocytes isolated from premalignant tissue of a transplant recipient with cutaneous tumors are inhibited by cidofovir *in vitro* in a time- and dose-dependent manner. A comparison with a spontaneously immortalized keratinocyte cell line of non-malignant origin (HaCaT) in cell growth experiments to check their cidofovir sensitivity indicates that no striking difference exists in the early stage of drug exposure to growing cells. There is, however, a remarkably stronger effect of Tuwei00 cells after prolonged cidofovir exposure. This higher sensitivity disappeared in cells of later passages. Clearly, the therapeutic effects of cidofovir seen in virus-associated and virus-independent human tumors deserve more detailed investigations concerning the mode of action of this drug.

References

[1] Allen-Hoffmann BL, Schlosser SJ, Ivarie C, Sattler CA, Meister LF, O'Connor SL. Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. J Invest Dermatol 2000; 114: 444–455.

[2] Andrei G, Snoeck R, Piette J, Delvenne P, De Clercq E. Antiproliferative effects of acyclic nucleoside phosphonates on human papillomavirus (HPV)-harbouring cell lines compared with HPV-negative cell lines. Oncol Res 1998; 10: 523–531.

[3] Andrei G, Snoeck R, Schols D, De Clercq E. Induction of apoptosis by cidofovir in human papillomavirus (HPV)-positive cells. Oncol Res 2001; 12: 397–408.

[4] Baden HP, Kubilus J, Kvedar JC, Steinberg ML, Wolman SR. Isolation and characterization of a spontaneously arising long-lived line of human keratinocytes (NM1). In Vitro Cell Dev Biol 1987; 23: 205–213.

[5] Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988; 106: 761–771.

[6] Bryan TM, Reddel RR. Telomere dynamics and telomerase activity in in vitro immortalized human cells. Eur J Cancer 1997; 33: 767–773.

[7] Calista D, Riccioni S, Coccia L. Succesful treatment of squamous cell carcinoma of the lower eyelid with intralesional cidofovir. Br J Ophthalmol 2002; 86: 932–933.

[8] De Clercq E, Andrei G, Balzarini J, Hatse S, Liekens S, Naesens L, Neyts J, Snoeck R. Antitumor potential of acyclic nucleoside phosphonates. Nucleosides Nucleotides 1999; 18: 759–771.

[9] Harwood CA, Surentheran T, Mc Gregor JM, Spink PJ, Leigh IM, Breuer J, Proby CM. Human papillomavirus infection and non-melanoma skin cancer in immunosuppressed and immunocompetent individuals. J Med Virol 2000; 61: 289–297.

[10] Hozumi Y, Kondo S, Shimoura T, Aso K. Human squamous cell carcinoma from skin: establishment and characterization of a new cell line (HSC-5). J Dermatol 1990; 17: 143–148.

[11] Hyckel P, Berndt A, Wutzler P, Kosmehl H. Tumoren des Gesichts bei organtransplantierten Patienten. Mund Kiefer Gesichts Chir 2001; 5: 94–97.

[12] Jaffe DR, Montero-Puerner Y, Beckett MA, Cowan JM,

Weichselbaum RR, Diamond AM. Differential retention of tumour- and differentiation-suppressor functions in cell derived from a human squamous cell carcinoma. Mol Carcinog 1992; 5: 278–285.

[13] Johnson JA, Gangemi JD. Selective inhibition of human papillomavirus-induced cell proliferation by (S)-1-3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine. Antimicrob Agents and Chemother 1999; 5: 1198–1205.

[14] Lampros TD, Cobanoglu A, Parker F, Ratkovec R, Norman DJ, Hershberger R. Squamous and basal cell carcinoma in heart transplant recipients. J Heart Lung Transplant 1998; 17: 586–591.

[15] Lin J-C, De Clercq E, Pagano JS. Inhibitory effects of acyclic nucleoside phosphonate analogues including (S)-1-(3-hydroxy-2-phosphonomethylmethoxypropyl)cytosine on Epstein-Barr-virus replication. Antimicrob Agents and Chemother 1991; 35: 2440–2443.

[16] Lindl T. Zell- und Gewebekultur: Einführung in die Grundlagen sowie ausgewählte Methoden und Anwendungen. Heidelberg, Berlin: Spectrum Akademischer Verlag, 2000: 272.

[17] Linge C. Establishment and maintenance of normal human keratinocyte cultures. In: Jones GE, editor. Methods in molecular medicine. Human cell culture protocols. Totowa: Humana Press Inc., 1996: 1–8.

[18] Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. In: Greaves M, Furth M, editors. Molecular diagnostics of human cancer. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 1989: 209–214.

[19] Mc Geown MG, Douglas JF, Middleton D. One thousand renal transplants at Belfast City Hospital: post-graft neoplasia 1968-1999, comparing azathioprine only with cyclosporin-based regimes in a single centre. Clin Transpl 2000; 193–202.

[20] Meerbach A, Gruhn B, Egerer R, Reischl U, Zintl F, Wutzler P. Semiquantitative PCR analysis of Epstein-Barr virus DNA in clinical samples of patients with EBV-associated diseases. J Med Virol 2001; 65: 348–357.

[21] Meerbach A, Schacke M, Hyckel P, Kosmehl H, Wutzler P. Successful treatment of Epstein-Barr virus-associated tumors in a heart transplant patient with cidofovir . Case report. Antiviral Res 2002; 53: A 62.

[22] Naesens L, Snoeck R, Andrei G, Balzarini J, Neyts J, De Clercq E. HPMPC (cidofovir), PMEA (adefovir) and related acyclic nucleoside phosphonate analogues: A review of their pharmacology and clinical potential in the treatment of viral infections. Antiviral Chem Chemother 1997; 8: 1–23.

[23] Penn I. Post-transplant malignancy: the role of immunosuppression. Drug Saf 2000; 23: 101–113.

[24] Proby CM, Prudie KJ, Sexton CJ, Purkis P, Navsaria HH, Stables JN. Spontaneous keratinocyte cell lines representing early and advanced stages of malignant transformation of the epidermis. Exp Dermatol 2000; 9: 104–117.

[25] Rice RH, Steinmann KE, De Graffeenreid LA, Qin Q, Taylor N, Schlegel R. Elevation of cell cycle control proteins during spontaneous immortalization of human keratinocytes. Mol Biol Cell 1993; 4: 185–194.

[26] Snijders PJF, van den Brule AJC, Schrijnemakers HFJ, Snow G, Meijer CJLM, Walboomers JMM. The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J Gen Virol 1990; 71: 173–181.

[27] Snoeck R, Andrei G, DE Clercq E. Cidofovir in the treatment of HPV-associated lesions. Verh K Acad Geneeskd Belg 2001; 63: 93-120, discussion 120–122.

[28] Tomicic MT, Thust R, Sobol RW, Kaina B. DNA polymerase β mediates protection of mammalian cells against ganciclovir-induced cytotoxicity and DNA breakage. Cancer Res 2001; 61: 7399–7403.

[29] Tsukamoto T. Establishment and characterization of a cell line (Ku-8) from squamous cell carcinoma of the penis. Keio J Med 1989; 38: 277–293.

[30] Watanabe F, Kojima T, Morita E, Kobayashi M, Fujita M. Establishment of a human skin squamous cell carcinoma cell line (SCC-1CB) in vitro and its characteristics. Nippon Hifuka Gakkai Zasshi 1989; 99: 793–800.