HSV-1 Infection Modulates the Radioresponse of a HPV16-positive Head and Neck Cancer Cell Line

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Abstract. *Background: The combined effects of Human papillomavirus (HPV) and Herpes simplex type 1 (HSV-1) infections and their effects on cancer cell radioresistance are unexplored. Materials and Methods: An HPV16-positive hypopharyngeal carcinoma cell line (UD-SCC-2) was infected with wt-HSV-1 at low multiplicity of infection (MOI) and irradiated with 2 Gy at 24 h postinfection. Viability assays and quantitative reverse-transcriptase PCR for HPV16 E6, E7, nuclear factor kappa B1, B-cell CLL/lymphoma 2 (BCL2), and caspases 3, 8 and 9 at 24, and 72 h, as well as immunocytochemistry for BCL2, caspase 3, cyclin E, mouse double minute 2 homolog (MDM2), HSV-1 and Ki-67 were performed at 144 h postirradiation. Results: At 144 h, cell viability was significantly lowered by irradiation only in uninfected cells. Infection combined with irradiation resulted in increased expression of E6, E7, BCL2 and NF-κB1 at 144 h. Simultaneously, E6 and E7 were down-regulated in non-irradiated infected cells. Irradiation and infection with 0.00001 MOI separately up-regulated caspase 3 but infection with 0.0001 MOI halved its expression in irradiated cells. Conclusion: HSV-1 infection modulates radioresistance of HPV16-positive hypopharyngeal carcinoma cells.*

Oral and lip cancer are the 15th most common malignant tumors worldwide (1). Oral cancer is mainly a disease of the patients in their 50s, but an increasing trend among younger people has recently been reported for oral and oropharyngeal cancer (2, 3). Tobacco and alcohol have traditionally been regarded as the main causes of oral and oropharyngeal cancer, explaining some 75% of all oral squamous cell carcinomas (SCC). However, there is sufficient evidence that *Human papillomavirus* (HPV) infection is associated with a sub-group of these (4, 5), particularly type 16 (HPV16).

HPV16 -induced malignant transformation *in vitro* is well-characterized (6, 7). The key viral oncogenes are E6 and E7, permanently expressed in transformed cells (8, 9). E6 and E7 oncoproteins bind to several host proteins resulting in loss of cell-cycle control and failure to undergo programmed cell death, apoptosis, when sensing DNA damage that could lead to carcinogenesis (10). These effects are mediated through degradation of p53 and the retinoblastoma protein (pRb), key proteins involved in apoptosis and DNA damage recovery (10, 11). Treatment of oral and oropharyngeal cancer consists of surgical resection, radiation or chemoradiation alone or in combination. Patients with HPV-associated head and neck squamous cell carcinomas (HNSCC) have better survival than those with HPV-negative carcinomas (5, 12). This is why deintensified treatment of HPV-positive HNSCC has been suggested, using curative irradiation only (13). However, it was recently shown that patients with oral cancer with HPV16 infection were at higher risk of distant metastasis and poor survival while undergoing radiation-based adjuvant therapy (14). Furthermore, the same research group also found that HPV infections with low-risk HPVs predicted poor 2-year disease-free survival in HNSCC. In our series of HNSCC, the poorest survival was found among patients with co-infection by HPV16 and HPV6/11, or HPV16 and *Herpes simplex virus type 1* (HSV-1) (4). Reactivation of HSV-1 infection is frequent and often asymptomatic in patients treated for HNSCC (15-17). Similarly patients, regardless of their underlying disease, display a high prevalence of HSV-1 in their saliva when attending a clinic for oral surgery (18). Stress associated with the impending procedure or healing trauma postoperatively might explain this predisposition to HSV-1 infection in these patients. HSV-1 can prevent apoptosis of infected cells *via* its ICP-0, Us3 and Us5 proteins. These can affect many cellular apoptotic pathways such as p53, in a

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down-regulating manner, providing resistance to cell death until the infection has progressed to high viral copy numbers (19-21). HSV-1 also interacts with DNA repair mechanisms, particularly the MRN protein complex of the ataxia telangiectasia mutated (ATM) pathway essential in cellular responses to radiation. These functions, along with the fact that herpesviruses cause chromosomal instability (22, 23), indicate that HSV-1 might affect the radiation response of infected cells. As there is a possibility that HSV-1 might co-infect HPV-infected premalignant or malignant cells, it is important to characterize whether HSV-1 infection can affect the survival of HPV-infected cells after irradiation.

Materials and Methods

Cell culture. UD-SCC-2 cells were derived from a hypopharyngeal carcinoma (24) and were a kind gift from professor Kunz-Schughart LA. Institute of Pathology, University of Regensburg, Germany. These cells are HPV16-positive, harboring around 600 integrated copies of the genome per cell. The cells were thawed from liquid nitrogen at passage 68 and grown in 80 cm^2 Nunclon flasks (Sigma-Aldrich, St. Louis, MO, USA) for three passages before trypsinization and seeding into 24-well plates (Nunc, Roskilde, Denmark). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) with 10% inactivated fetal bovine serum (FBS). The infections were performed in RPMI-1640 medium with bovine serum albumin (BSA) (Gibco).

HSV-1 infection. The cells were seeded into 24-well plates at 65,000 cells/well. After 2 days of culture when a confluence of 75% was reached, the cells were infected with low viral loads of wild-type (wt) HSV-1 (strain 17+): 0.0001, 0.00005 or 0.00001 multiplicity of infection (MOI). The HSV-1 titers were confirmed by plaque titration in Vero cells as per standard protocol. Uninfected cells were considered as controls. The cells were infected by replacing the medium with 300 μl of RPMI with 0.1% BSA with HSV-1 at the MOI required for 1 h. Then, the medium was replaced with 1 ml of DMEM with 10% FBS and replaced every 3 days. The effect of irradiation on HSV-1 replication was examined at 144 h by plaque titration from medium samples derived from the cultures to be analyzed by immunocytochemistry.

Irradiation. The cultures were irradiated at the Turku University Hospital (Department of Oncology and Radiotherapy) one day post-infection. A total dose of 2 Gy of 6 MV x-ray irradiation at a dose rate of 3 Gy/min was selected using a linear accelerator (Clinac 2100C/D; Varian Medical Systems, Palo Alto, CA, USA). Non-irradiated cultures were mock-irradiated by placement on the accelerator table for the same duration without exposure.

Viability assays. The viability of the cells was determined with an ATP assay (CellTiter-Glo®, Luminescent Cell Viability Assay; Promega, Madison, WI, USA), which detects viable cells as extracellular ATP is rapidly degraded, at 24, 72 and 144 h after irradiation as previously described (25). 24-Well plates were used to standardize the conditions between cell cultures, according to the following protocol suggested by the manufacturer: A 50/50 medium/reagent rate was achieved by replacing half of the medium with the CellTiter-Glo reagent. Using an orbital shaker for 2 min at low speed, the cells were then lysed and the reaction continued in the dark at room temperature for 10 min. Two hundred microliters of every sample was then transferred into white 96-microplate wells (Culturplate 96 White; Perkin Elmer, Waltham, MA, USA) for analysis in a luminometer (Wallac Victor 3 1420; Perkin Elmer) according to the manufacturer's instructions. Triplicate medium samples and empty wells were used as controls for background luminescence. The experimental assays were carried out in quadruplicates for each MOI and the uninfected controls of that time point with or without irradiation.

RNA extraction. Cells from the 24-well plates were suspended in Trizol reagent (Invitrogen, Paisley, UK) at 24, 72 and 144 h after irradiation. RNA was then extracted according to the instructions of the manufacturer.

cDNA synthesis and quantitative reverse-transcriptase PCR (qRT-PCR). First-strand cDNA was synthesized using First-strand cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and using total RNA as a template. Real-time RT-PCR (Taq-Man) reactions were performed in a reaction volume of 20 μl containing 25-100 ng of cDNA with TaqMan Universal PCR MasterMix and TaqMan® Gene Expression assays (Applied Biosystems) for nuclear factor kappa-B, subunit 1 (*NFKB1*) (manufacturer's identification number Hs00765730_m1), B-cell cell/lymphoma 2 (*BCL2*) (Hs00608023_m1), caspase 3 (Hs00154261_m1), caspase 8 (Hs01018151_m1), caspase 9 (Hs00154261_m1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Hs02758991_g1). Applied Biosystems custom TaqMan primers and probes for HPV16 E6 and E7 were used as previously described (26). Triplicate reactions were performed from triplicate samples, using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The reaction conditions were 2 min at 50˚C, 10 min at 95˚C, and a two-step cycle of 95˚C for 15 s and 60˚C for 60 s, for a total of 40 cycles. Standard curves were calculated from a dilution series of 400 ng to 12.5 ng of cDNA from UD-SCC-2 and SiHa controls, included in each run. Three no-template control reaction mixtures were also present. The amplification curves and standard curves were drawn and analyzed using the manufacturer's software SDS2.3 and Microsoft Excel 2010. The results were normalized against the mRNA level of GAPDH housekeeping gene using averages calculated from triplicate analyses.

Immunocytochemistry. From a repeat of the experiments described above, cells from 24-well plates at 144 h post-irradiation were gently scraped into 250 μl PBS per well using a sterile technique. Cells either uninfected or infected with 0.0001 MOI were used for these assays. The cell suspension was diluted to achieve an estimated 20,000 cells/250 μl PBS. Two hundred and fifty microliters per sample was pipetted onto glass slides, allowed to dry and fixed using 10% formalin for 10 min. After fixation, the slides were washed twice with PBS for 3 min and after drying, stored at −20˚C. For immunocytochemistry, primary antibodies and their dilutions were as follows: BCL2 (#M0887, 1:100; Dako, Glostrup, Denmark), cleaved caspase 3 (#CP229B, 1:200; Biocare Medical, Concord, CA, USA), cyclin E (#NCL-CYCLIN E, 1:20; Leica Novocastra, Newcastle upon Tyne, UK), HSV-1 (#PU084-UP, 1:100; Biogenex, Fremont, CA, USA), MDM2 (#NCL-MDM2, 1:100; Novocastra) and Ki67 (#M7240, 1:150; Dako) using a Dako TekMate automatic tissue stainer. Manufacturer-recommended controls were included for each stain. For pretreatment, the slides were brought to room

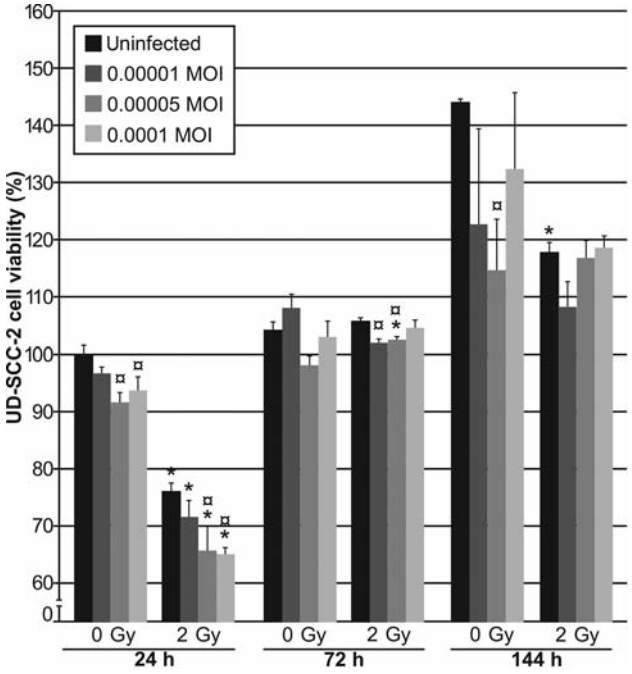

Figure 1. *UD-SCC-2 cell viability as a percentage when compared to uninfected, non-irradiated cells at 24 h time point measured using the CellTiter-Glo viability assay. The chart shows the different multiplicities of infection (MOI) of HSV-1 used to infect the cells irradiated (2 Gy) or not (0 Gy) at the time points indicated. Bars are the means±SEM. All values are calculated from quadruplicate cultures. p-Values were calculated using the Mann–Whitney U-test. *p<0.05 comparing irradiated and non-irradiated cultures at the same time point and MOI; ¤p<0.05 when infected cultures are compared to uninfected cultures at the same time point. Please note that the cells proliferated during the experiment and therefore viability ratings of over 100% are seen when compared to the 24-h time point.*

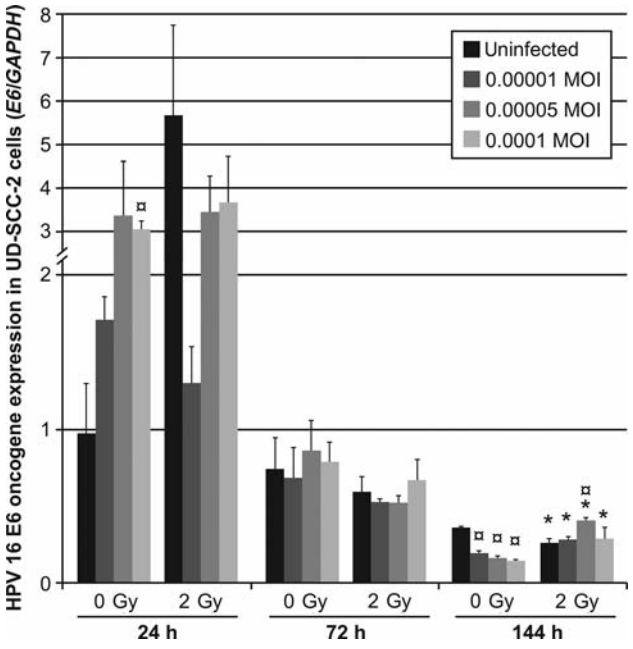

Figure 2. *HPV16 E6 mRNA expression. HPV16 E6 mRNA expression in UD-SCC-2 cells measured using quantitative reverse-transcriptase PCR. The level is a normalized value calculated from E6/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression from triplicate cultures. The chart shows the different multiplicities of infection (MOI) of HSV-1 used to infect the cells irradiated (2 Gy) or not (0 Gy) at the time points indicated. Bars are the means±SEM. p-Values were calculated using the Mann–Whitney U-test. *p<0.05 comparing irradiated and non-irradiated cultures at the same time point and MOI; ¤p<0.05 when comparing infected cultures to uninfected cultures at the same time point. Note the y-axis scale change.*

temperature, microwaved for 5 min in a citrate buffer (pH 6; Ki67, caspase 3, cyclin E, MDM2, HSV-1) or 10 mM Tris EDTA (pH 9; p16, BCL2), and brought to room temperature. Blocking was carried out using hydrogen peroxide. For MDM2, serum blocking was necessary. Visualization was achieved *via* the LSAB-kit (Dako REAL Detection System Peroxidase/DAB+ Rabbit/Mouse, #K5001) using the manufacturer's protocol. p16 staining was performed as per manufacturer's instructions from a ready-to-use kit (CINtec p16 INK4A Histology Kit #9511; Roche, Heidelberg, Germany). The slides were examined by one author (AT) using a cell counter. The percentage positivity (nuclear, cytoplasmic or both) was assessed under a light microscope from five random high-power fields (×400) in two blinded measurements on different days. On average, 950 cells were counted per treatment group per measurement. Intraobserver agreement was then calculated.

Statistical analysis. SPSS 19 software with SPSS advanced statistical package IBM SPSS Statistics for Windows, Version 19.0.0.1 (IBM Corp., Armonk, NY, USA) was used for statistical evaluations. The Mann–Whitney U-test was chosen for significance calculations. *p*-Values of less than 0.05 were considered statistically significant. For immunocytochemistry results, intraobserver agreement was determined using Cronbach's Alpha, two-way mixed effects model to calculate the intraclass correlation coefficient (ICC) (0.953, $p=0.0001$).

Results

Cell viability. HSV-1 infections at 0.00005- or 0.0001 MOI significantly lowered the viability at 24 h of both irradiated and non-irradiated cells ($p=0.021$ and 0.043, respectively). Irradiation reduced UD-SCC-2 cell viability at 24 h (Figure 1, $p=0.05$). Reduction of viability by 0.00001 MOI continued until 72 h in non-irradiated cells ($p=0.021$). At 144 h postirradiation, viability was lowered in uninfected cells ($p=0.021$), and 0.00005 MOI HSV-1 lowered the viability of non-irradiated cells. At 144 h, irradiation did not significantly affect the viability of infected cultures.

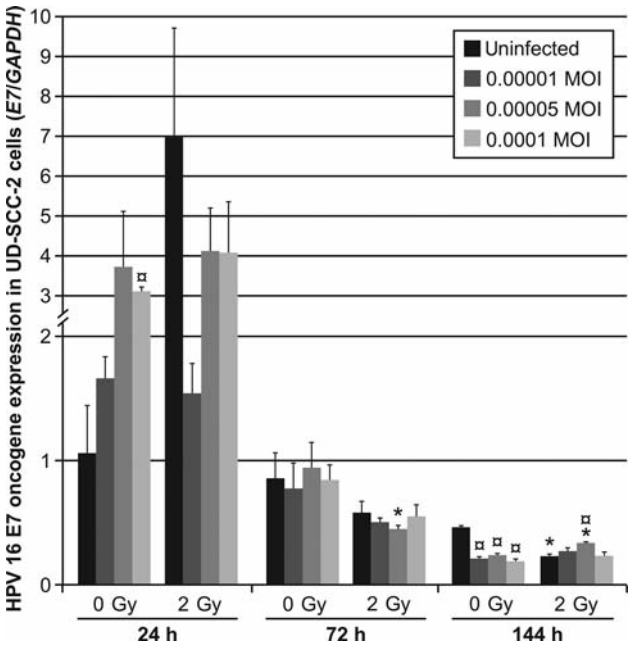

Figure 3. *HPV16 E7 expression. HPV16 E7 mRNA expression in UD-SCC-2 cells was measured using quantitative reverse-transcriptase PCR. The level is a normalized value calculated from E7/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression from triplicate cultures. The chart shows the different multiplicities of infection (MOI) of HSV-1 used to infect the cells irradiated (2 Gy) or not (0 Gy) at the time points indicated. Bars are the means±SEM. p-Values were calculated using the Mann–Whitney U-test. *p<0.05 comparing irradiated and non-irradiated cultures at the same time point and MOI; ¤p<0.05 comparing infected cultures to uninfected cultures at the same time point. Note the y-axis scale change.*

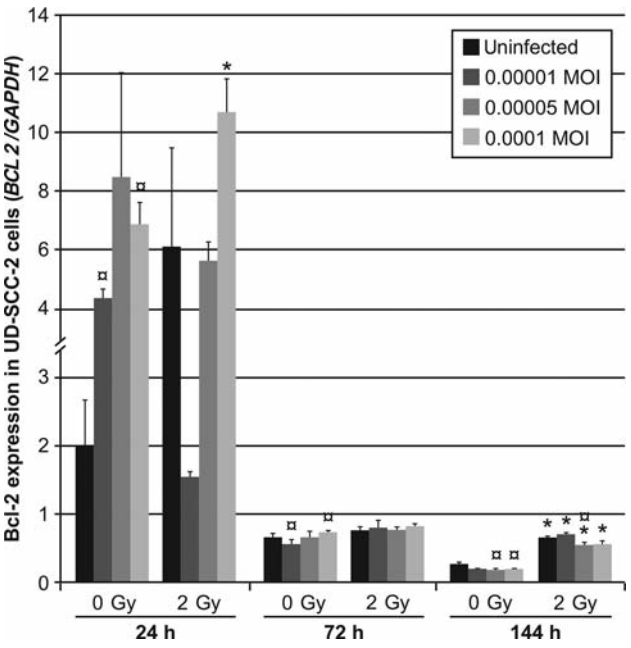

Figure 4. *Oncogene B-cell CLL/lymphoma 2 (BCL2) expression. BCL2 mRNA expression in UD-SCC-2 cells measured using quantitative reverse-transcriptase PCR. The level is a normalized value calculated from BCL2/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression from triplicate cultures. The chart shows the different multiplicities of infection (MOI) of HSV-1 used to infect the cells irradiated (2 Gy) or not (0 Gy) at the time points indicated. Bars are the means±SEM. p-Values were calculated using the Mann–Whitney U-test. *p<0.05 comparing irradiated and non-irradiated cultures at the same time point and MOI; ¤p<0.05 comparing infected cultures to uninfected cultures at the same time point. Note the y-axis scale change.*

Gene expression

HPV16 E6. HSV-1 infection at the highest MOI up-regulated HPV16 E6 expression at 24 h (p=0.05) in non-irradiated cells. At 144 h, down-regulation of E6 expression was seen in the presence of HSV-1 (p=0.05, 0.05 and 0.046 for 0.00001, 0.00005 or 0.0001 MOI, respectively). Irradiation of UD-SCC-2 cells down-regulated E6 expression at 144 h (Figure 2, p=0.046). However, when cells were infected with HSV-1 at 0.00001 MOI, 0.00005 MOI or 0.0001 MOI were irradiated, there was a 1.5-, 2.6- and 2.1-fold higher E6 expression at 144 h (p=0.05 for all) than in non-irradiated cultures infected with the respective MOIs.

HPV16 E7. HSV-1 infection up-regulated the HPV16 E7 expression at 24 h only at the highest MOI in non-irradiated cultures (p=0.05, Figure 3). At 72 h, E7 expression was lowest in irradiated cultures infected with 0.00005 MOI (p=0.05). At 144 h, HSV-1 down-regulated the E7 expression at all MOI (p=0.046 for all) in non-irradiated cultures but not in irradiated cultures. Infected cultures displayed a similar (0.0001 and 0.00001 MOI) or higher (0.00005 MOI, p=0.046) E7 expression 144 h postirradiation than uninfected irradiated cells. Irradiation also up-regulated E7 in cultures infected with 0.00005 MOI (p=0.05).

BCL2. HSV-1 infection at 0.00001 and 0.0001 MOI resulted in BCL2 up-regulation at 24 h. This was potentiated by irradiation in cells infected with 0.0001 MOI HSV-1 (Figure 4, p=0.05) but leveled at 72 h, where the lowest- and highest MOI led to down-regulation and up-regulation, respectively, in non-irradiated cultures (p=0.05 for both). At 144 h, irradiated cultures displayed elevated BCL2 compared to non-irradiated cultures irrespective whether HSV-1 was present (p=0.05, 0.05 and 0.046 for 0.00001, 0.00005 and 0.0001 MOI, respectively) or not (p=0.05). However, in non-irradiated cultures infected with HSV-1, a down-regulation in BCL2 was observed (p=0.046 for 0.00005 MOI and 0.0001 MOI). In the irradiated infected cells, a slight down-regulation due to HSV-1 was only found with 0.00005 MOI (p=0.05).

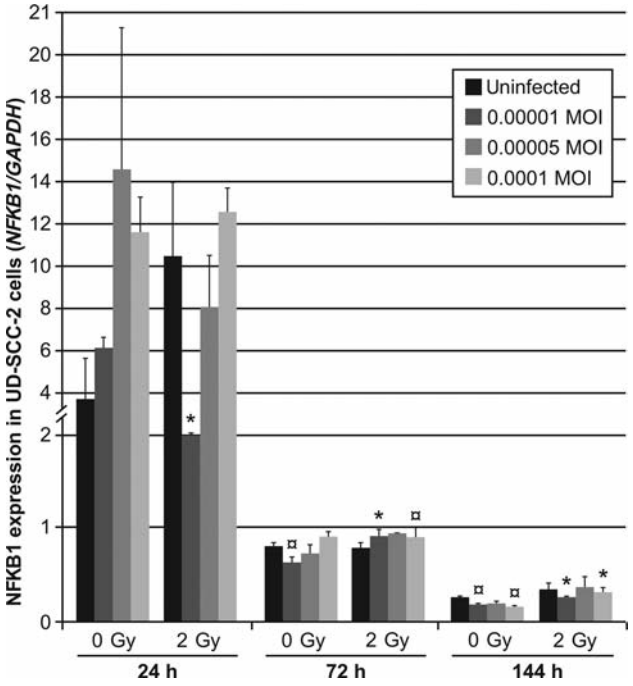

Figure 5. *Nuclear factor kappa-B, subunit 1 (NFKB1) expression. NFKB1 mRNA expression in UD-SCC-2 cells measured using quantitative reverse-transcriptase PCR. The level is a normalized value calculated from NFKB1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression from triplicate cultures. The chart shows the different multiplicities of infection (MOI) of HSV-1 used to infect the cells irradiated (2 Gy) or not (0 Gy) at the time points indicated. Bars are the means±SEM. p-Values were calculated using the Mann–Whitney U-test. *p<0.05 comparing irradiated and non-irradiated cultures at the same time point and MOI; ¤p<0.05 comparing infected cultures to uninfected cultures at the same time point. Note the y-axis scale change.*

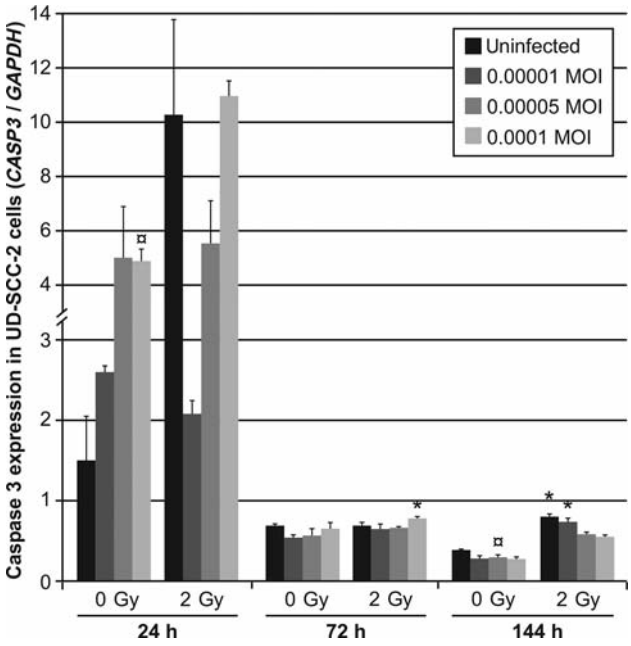

Figure 6. *Caspase 3 expression. Caspase 3 mRNA expression in UD-SCC-2 cells measured using quantitative reverse-transcriptase PCR. The level is a normalized value calculated from caspase 3/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression from triplicate cultures. The chart shows the different multiplicities of infection (MOI) of HSV-1 used to infect the cells irradiated (2 Gy) or not (0 Gy) at the time points indicated. Bars are the means±SEM. p-Values were calculated using the Mann–Whitney U-test. *p<0.05 comparing irradiated and non-irradiated cultures at the same time point and MOI; ¤p<0.05 comparing infected cultures to uninfected cultures at the same time point. Note the y-axis scale change.*

NFKB1. *NFKB1* levels were not significantly affected by the irradiation or infection at 24 h but combined irradiation and lowest MOI infection (0.00001) led to down-regulation (Figure 5; p=0.05). At 72 h, infection with the lowest MOI led to down-regulation of *NFKB1* (p=0.05) but not in irradiated cells. At 144 h, down-regulation was detected due to HSV-1 in the non-irradiated group (p=0.05 for MOI, except 0.0005).

This effect was not present when irradiated cultures were compared. However, *NFKB1* was up-regulated at this time point due to highest MOI HSV-1 and irradiation combined (p=0.05).

Caspase 3. At 24 h, infection with the highest MOI caused an up-regulation of caspase 3 (Figure 6, p=0.05). This effect was not detected in other groups. Caspase 3 was unaffected at 72 h. However, at 144 h, 2 Gy irradiation led to a 2.1-fold up-regulation of caspase 3 (p=0.05) which was reduced to one half, losing significance when HSV-1 was present. HSV-1 infection *per se* led to a slight down-regulation of caspase 3 at highest MOI compared to uninfected non-irradiated cells (p=0.05), but not when the cultures had been irradiated.

Caspase 8. At 24 h, HSV-1 infection up-regulated caspase 8 (Figure 7, p=0.05) and more effectively in the combined effects group (2.1 *vs*. 7.3-fold up-regulation, p=0.05 for both). At 72 h, HSV-1 at lowest MOI led to caspase 8 down-regulation in non-irradiated cultures (p=0.05). Irradiation led to down-regulation of caspase 8 in uninfected (p=0.05) and infected (p=0.05) cultures except at 0.00005 MOI. At 144 h, down-regulation was observed only due to highest MOI infection in non-irradiated cultures (p=0.05) and lowest MOI infection in the irradiated group (p=0.05).

Caspase 9. HSV-1 infection led to a significant up-regulation of caspase 9 at 24 h (Figure 8, p=0.05). Simultaneously, lowest MOI infection down-regulated caspase 9 when

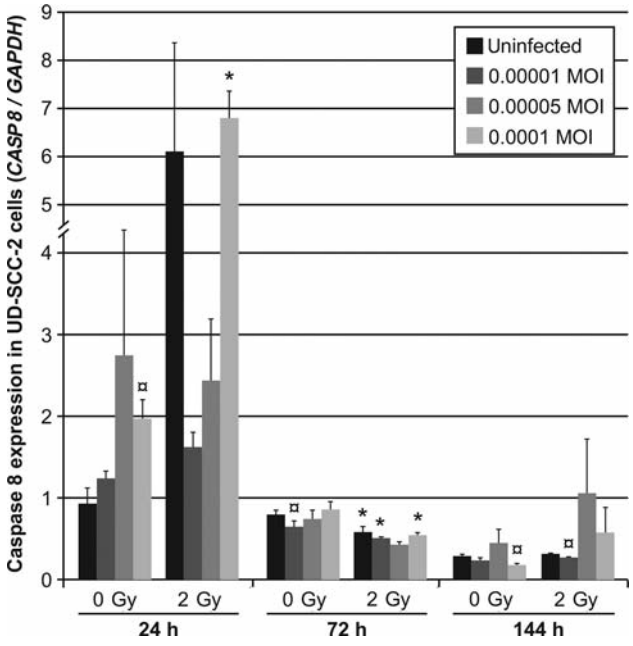

Figure 7. *Caspase 8 expression. Caspase 8 mRNA expression in UD-SCC-2 cells measured using quantitative reverse-transcriptase PCR The level is a normalized value calculated from caspase 8/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression from triplicate cultures. The chart shows the different multiplicities of infection (MOI) of HSV-1 used to infect the cells irradiated (2 Gy) or not (0 Gy) at the time points indicated. Bars are the means±SEM. p-Values were calculated using the Mann–Whitney U-test. *p<0.05 comparing irradiated and non-irradiated cultures at the same time point and MOI; ¤p<0.05 comparing infected cultures to uninfected cultures at the same time point. Note the y-axis scale change.*

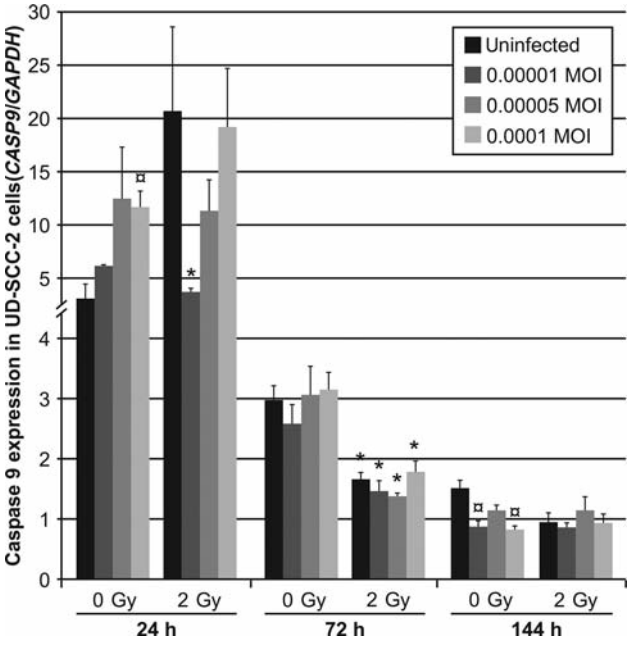

Figure 8. *Caspase 9 expression. Caspase 9 mRNA expression in UD-SCC-2 cells measured using quantitative reverse-transcriptase PCR. The level is a normalized value calculated from caspase 9/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression from triplicate cultures. The chart shows the different multiplicities of infection (MOI) of HSV-1 used to infect the cells irradiated (2 Gy) or not (0 Gy) at the time points indicated. Bars are the means±SEM. p-Values were calculated using the Mann–Whitney U-test. *p<0.05 comparing irradiated and non-irradiated cultures at the same time point and MOI; ¤p<0.05 comparing infected cultures to uninfected cultures at the same time point. Note the y-axis scale change.*

combined with irradiation (p=0.05). At 72 h, irradiated groups displayed lower caspase 9 expression regardless of HSV-1 presence (p=0.05 for all) but this effect was not present at 144 h, when progressive HSV-1 infection led to down-regulation of caspase 9 in all non-irradiated groups except at 0.00005 MOI (p=0.05).

Immunocytochemistry. At 144 h, cultures infected with 0.0001 MOI HSV-1 or irradiated with 2 Gy or infected and irradiated, were examined with immunocytochemistry (Table I). Ki-67 protein was expressed in the nuclei of 76% of UD-SCC-2 cells. HSV-1 infection reduced this to 48%. Irradiation reduced expression to 64% and the combined effect with HSV-1 infection did not change this expression level (65%). Expression of BCL2 protein was up-regulated as a result of HSV-1 infection (68% *vs*. 55% cytoplasmic positivity). Irradiation reduced the BCL2 level only slightly in both HSV-1-infected and uninfected cells. Activated caspase 3 was expressed in 6% of UD-SCC-2 cells and surprisingly, also in 6% of HSV-1-infected cells (Figure 9). Irradiation increased expression of caspase 3 protein to 35% of cells but combining irradiation with HSV-1 infection reduced this to only 15%. $p16^{INK4A}$ expression was up-regulated by both HSV-1 and irradiation alone from a baseline of 43% to 60% of cells, whereas combined treatment resulted in protein expression in 56%. Cyclin E was expressed in 2% of UD-SCC-2 cells. HSV-1 infection raised expression of nuclear cyclin E to 4%, while irradiation raised this to 9% and the combined effect of irradiation and HSV-1 infection resulted in expression in 12%. MDM2 was detected in 2% of the nuclei of untreated cells. HSV-1 infection reduced this to 1%. Irradiation led to 6% nuclear expression but combined treatment resulted in expression in 2%. The levels were similar when both nuclear and cytoplasmic staining were taken into consideration: 11% in UD-SCC-2 cells, 5% in HSV-1-infected cells, 20% in irradiated cells and 18% in HSV-1 infected and irradiated cells. At 144 h, HSV-1 staining was detected in all cells infected with HSV-1 at 0.0001 MOI regardless of their irradiation status.

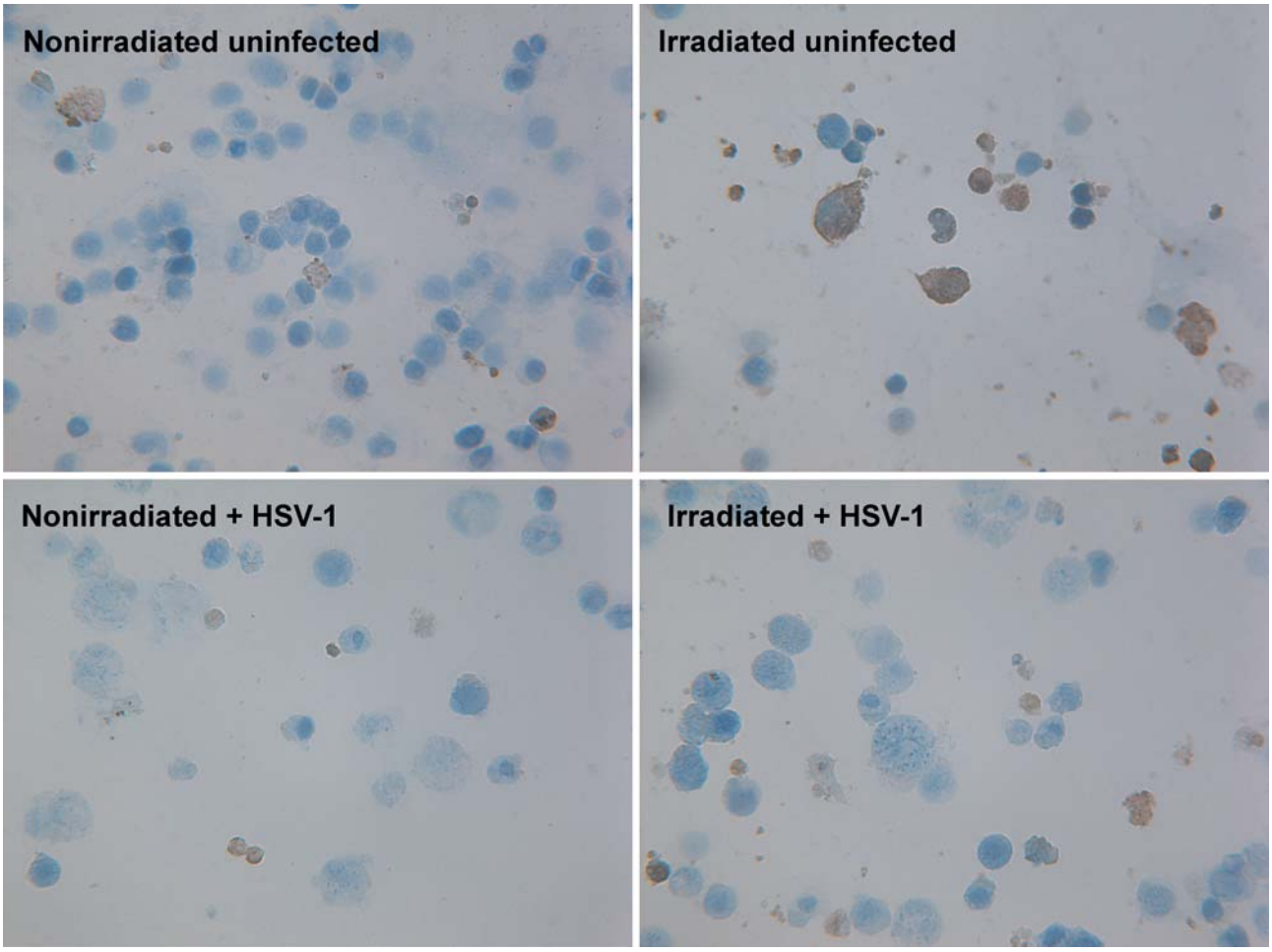

Figure 9. *The micrographs show the immunocytochemical analysis of UD-SCC-2 cultures expressing activated caspase 3 at 144 h postirradiation. Taken at ×400 magnification with a Leica DC500 camera with Leica application suite v4.2 without editing.*

Table I. *Immunocytochemical analysis. Immunocytochemical staining of UD-SCC-2 cells infected at 0.0001 multiplicities of infection (MOI) with HSV-1, irradiated or non-irradiated and analyzed at 144 h postirradiation. The percentage represents the fraction of positive cells in each field and is an average of two independent measurements from four cell cultures. The specific staining is shown as nuclear (nucl) and cytoplasmic (cyt) staining.*

	Ki67 (nucl.)	Caspase 3 (cyt.)	BCL2 (cyt.)	$P16^{INK4A}$ (Nucl.+cyt.)	Cyclin E (Nucl.)	MDM2 (Nucl.)	MDM2 (Nucl.+cyt.)	HSV-1 (cyt.)
Uninfected, non-irradiated	76%	6%	55%	43%	2%	2%	11%	N/A
HSV-1-infected, non-irradiated	48%	6%	68%	60%	4%	1%	5%	100%
Uninfected, 2 Gy irradiation	64%	35%	53%	60%	9%	6%	20%	N/A
HSV-1-infected, 2 Gy irradiation	65%	15%	66%	56%	12%	2%	18%	100%

N/A: No specific staining.

Viral load. At 144 h postirradiation, before the cells were harvested for immunocytochemistry, samples of the medium were analyzed using standard plaque titration assays on Vero cells. No differences in HSV-1 titers were detected between irradiated and non-irradiated cultures (Figure 10).

Discussion

Radiotherapy for HNSCC is effective, particularly against HPV-positive cases. Yet a sub-group of these patients develops rapidly-advancing disease despite treatment. Therefore, any factors that could potentially hinder the efficacy of such therapies are of great interest. In the present study, the effects of HSV-1 infection and irradiation on an HPV16-positive hypopharyngeal carcinoma cell line were assessed in cultures infected at low MOI. The effects can be summarized as follows.

HSV-1. HSV-1 infection lowered the viability of UD-SCC-2 cells. Eventually, HPV16 E6 and E7 expression were down-regulated at 144 h at all MOIs, implying that progressive infection increases apoptosis by down-regulating the key HPV oncogenes as reported previously (27), down-regulation of which is also known to lead to apoptosis of cervical carcinoma cells (28, 29). The mRNA expression of *BCL2*, *NFKB1* and caspases 3 and 9 simulated the pattern of E6 and E7 expression, while that of Ki67 was reduced. This might signify the role of HSV-1 inhibiting the intrinsic apoptotic pathway while simultaneously lowering HPV-specific anti-apoptotic gene expression in these cells. Moreover, the lower Ki-67 and MDM2 protein positivity along with the decrease in *NFKB1* mRNA and increased $p16^{INK4A}$ protein indicates increased senescence (30, 31). The net effect would increase apoptosis and also be detected by the viability assays (Figure 1).

Irradiation. Irradiation resulted first in up-regulation of E6, E7, BCL2 and caspase 9 at 24 h. This has been reported previously in cervical carcinoma cells after irradiation (31) and may reflect the residual p53 response that is critical for HPV-mediated radiosensitivity (32,33). At 144 h, both E6 and E7 and *NFKB1* were down-regulated. BCL2 mRNA, but not protein, and caspase 3 mRNA and activated protein along with MDM2, cyclin E and $p16^{INK4A}$ proteins were up-regulated. This overall expression pattern favors apoptosis demonstrated by down-regulation of Ki67 and cell viability, and up-regulation of $p16^{INK4A}$ (30).

HSV-1 infection and irradiation combined. The combined effects differed from those described above. Firstly, despite the up-regulation in *BCL2*, caspases were up-regulated and viability lowered at 24 h. The cells seemingly recovered at 72 h, as caspases 8 and 9 decreased and NF-κB1 increased, although viability was lowered. Finally, at 144 h, the viability of UDSCC-2 cells after combined exposures did not significantly differ from that of cells exposed to HSV-1 or irradiation only, as Ki-67 proliferative marker displayed less down-regulation than the single exposures would cause. This attenuation of toxicity seems to be mediated by a higher expression of E6 and *NFKB1*. This is likely indicated by the higher cyclin E level and, more importantly, protection from activated caspase 3 protein and mRNA up-regulation caused by irradiation. Irradiation is known to activate the ATM pathway *via* the MRN complex, leading to NFκB1 activation (34), which has been implicated in UV-radioresistance of keratinocytes (35). AKT-MDM2 pathway can also be activated by the entry of HSV-1 or irradiation and could theoretically synergize E6 in increasing postirradiation survival (36, 37), therefore requiring future studies. Both irradiation and HSV-1 cause apoptosis by activating the intrinsic apoptotic pathway by removal of up-stream BCL2 inhibition, leading to activation of caspase 9- and down-stream caspase 3, and cell death (38, 39). These changes reflect the down-stream intrinsic apoptotic pathway, suggesting that this pathway could mediate the cellular response to these combined effects.

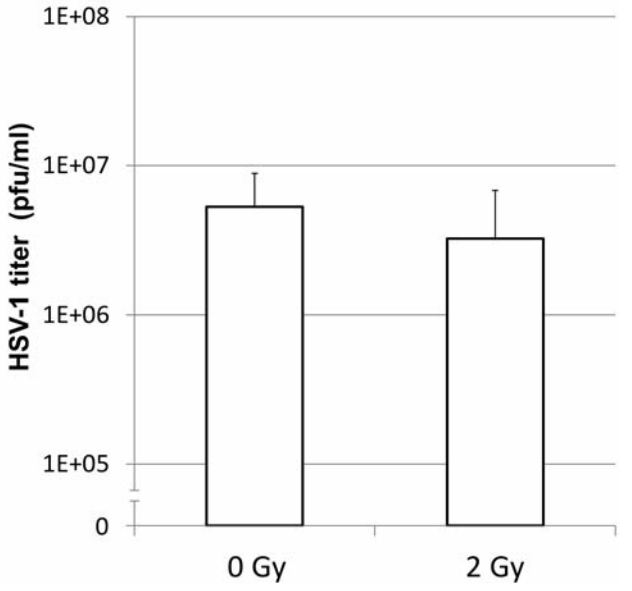

Figure 10. *Effect of irradiation on HSV-1 titer. The HSV-1 titer measured from quadruplicate medium samples taken from 144 h cultures of UD-SCC-2 immediately before fixation for immunocytochemistry (Table I). The titration was performed on b-Vero cells using a standard protocol. No statistically significant differences in HSV-1 titer were found between irradiated and non-irradiated cultures (Mann–Whitney U-test, p=0.564).*

The most important time point in this study was at 144 h when most cells are infected (Table I) as at this point the effects are more likely to be mediated by HSV-1. Because of similar HSV-1 presence in the 144-h cultures, irrespective of whether irradiated or not, HSV-1 infection kinetics are likely not responsible for these findings. A general shutdown in gene expression due to HSV leading to the observed changes is unlikely because this would also be seen in irradiated and infected cultures. Although symptomatic outbreaks of HSV can be partly inhibited by antiviral medications during cancer treatment, it appears that HSV replication in oral tissues

cannot be fully prevented (40, 41). Our results are not entirely compatible with studies testing HSV-1 mutants in the treatment of HNSCC, as the viruses in question are genetically modified for enhanced cancer cell killing usually by deletion of neurovirulence and immunosuppressive genes (42-44).

To conclude, apoptosis might be impaired following radiation in HPV-positive HSV-1-infected cells *in vitro*. Therefore, these results may have important clinical implications, advocating further research on the subject.

Conflicts of Interest

None declared.

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