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HPV-18 transformed cells fail to arrest in G1 in response to quercetin treatment

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Abstract

Previous work with primary human keratinocytes demonstrated that quercetin, a potent mutagen found in high levels in bracken fern (*Pteridium aquilinum*), arrested cells in G1 with concomitant elevation of the cyclin-dependent kinase inhibitor (cdki) p27^{Kip1}. Expression of the human papillomavirus type 16 (HPV-16) E6 and E7 oncoproteins, under transcriptional control of a heterologous promoter, in transformed keratinocytes failed to abrogate this arrest [Beniston, R., Campo, M.S., 2003. Quercetin elevates p27(Kip1) and arrests both primary and HPV-16 E6/E7 transformed human keratinocytes in G1. *Oncogene* 22, 5504–5514]. Given the link between papillomavirus infection, bracken fern in the diet and cancer of the oesophagus in humans, we wished to investigate further whether cells transformed by the whole genome of HPV-16 or HPV-18, with E6 and E7 under the transcriptional control of their respective homologous promoters, would be similarly arrested in G1 by quercetin. In agreement with earlier work, quercetin arrested HPV-16 transformed cells in G1 with an increase in the cyclin-dependent kinase inhibitor p27^{Kip1}. However, HPV-18 transformed cells did not arrest after quercetin treatment. The failure of HPV-18 transformed cells to arrest in G1 was linked to the up-regulation of the HPV-18 long control region (LCR) by quercetin, maintaining high expression of the viral transforming proteins. Transcriptional up-regulation of the HPV-18 LCR was mediated by a “quercetin responsive element” homologous to the one identified previously in the bovine papillomavirus type 4 (BPV-4) LCR. © 2005 Elsevier B.V. All rights reserved.

Keywords: Quercetin; HPV; p27^{Kip1}; LCR

Infection of the anogenital tract with high-risk human papillomaviruses (HPVs) can lead to the development of anogenital carcinomas and accounts for nearly all cervical cancers in women (zur Hausen, 1996). The high-risk HPVs most commonly linked with genital cancer are 16 and 18. These viruses have been linked also to oropharyngeal carcinoma (Gillison et al., 2000). High-risk HPV encode at least three different growth stimulating and transforming proteins: E5, E6 and E7. These proteins have an extensive number of cellular targets and functions (zur Hausen, 2000), however the expression of HPV-16 or HPV-18 E6 and E7 is sufficient to immortalise

primary human keratinocytes (Hawley-Nelson et al., 1989; Pecoraro et al., 1991).

Progression of HPV-induced lesions to carcinoma requires co-factors. Some co-factors have already been identified such as immunosuppression, smoking, oestrogen and progesterone (Dell and Gaston, 2001). Infection of the upper gastrointestinal (GI) tract of cattle with bovine papillomavirus type 4 (BPV-4) causes benign papillomas, which can progress to carcinoma in cattle feeding on bracken fern (Campo et al., 1994). Quercetin is a potent mutagen found widespread in nature and at its highest levels in bracken fern (Bjeldanes and Chang, 1977; Nakayasu et al., 1986). Quercetin can bind DNA causing single strand breaks (Fazal et al., 1990), DNA rearrangements (Suzuki et al., 1991) and chromosomal damage (Ishidate et al., 1988). Additionally, it can interfere with several kinases (Jackson and Campo, 1995; Davies et al., 2000). In our experimental model of BPV-4 associated oesophageal cancer, quercetin causes full oncogenic transformation of primary bovine cells partially transformed by BPV-4 (Pennie and Campo, 1992; Cairney and Campo, 1995) and this synergy is ascribed to the expression of BPV4 E7 abrogating a quercetin-induced G1 arrest mediated by p53 (Beniston et al., 2001).

HPV-16/HPV-18 has been associated with approximately 50% of oesophageal pre-cancers and cancers in humans (Chang et al., 1990; Togawa et al., 1994; Suzuk et al., 1996; Cooper et al., 1995; de Villiers et al., 1999; Syrjanen, 2002). Additionally, the presence of bracken fern in the human diet has been linked epidemiologically to cancer of the upper GI tract in several parts of the world (Galpin et al., 1990; Marliere et al., 2002; Alonso-Amelot et al., 1996; Hirayama, 1979; Villalobos-Salazar et al., 1995). These two observations suggest that oesophageal cancer in parts of the world may have the same aetiology as oesophageal cancer in cattle, namely infection by papillomavirus and dietary bracken fern. Here, we present data that support this suggestion.

Quercetin arrests HPV-16 but not HPV-18 transformed cells

Quercetin caused G1 cell cycle arrest of human keratinocytes transformed by HPV-16 E6 and E7 (HFKE6/E7) under the control of a retroviral LTR promoter (Beniston and Campo, 2003). As quercetin trans-activates the BPV4 LCR (Connolly et al., 1998), we decided to test human keratinocytes transformed by HPV-16 or HPV-18 whole

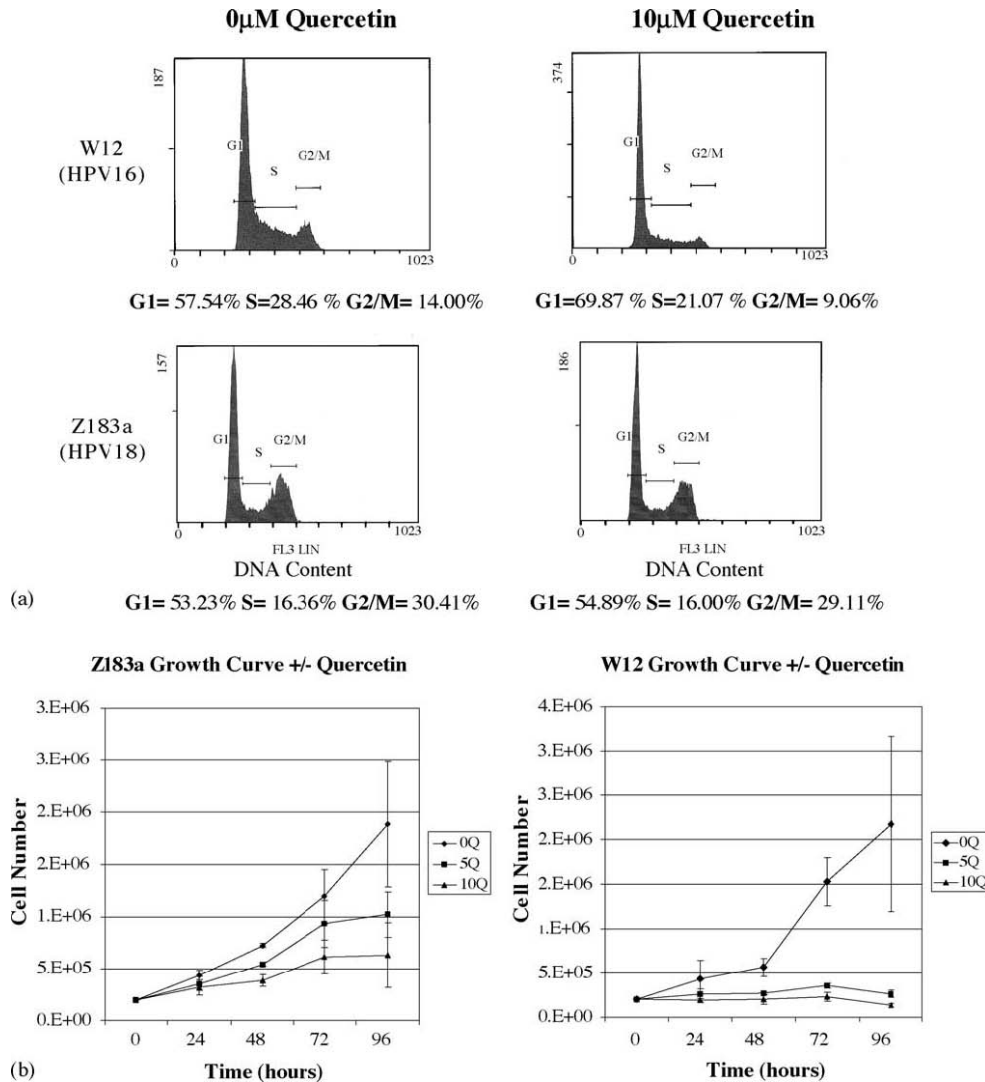


Fig. 1. (A) HPV-18 transformed cells do not cell cycle arrest in the presence of quercetin. Cells were treated with 0, 5, or 10 μM quercetin in ethanol over 36 h. Ethanol-fixed cells were stained with 20 $\mu\text{g/ml}$ propidium iodide (Sigma–Aldrich, Germany) and 200 mg/ml RNA-aseA (Kramel Biotech, Northumberland, UK), before being assayed on a Coulter Epics XL–MCL FACS machine. Results are shown only for 10 μM quercetin. (B) HPV-18 transformed cells continue to proliferate in the presence of quercetin. Cells were treated with 0, 5 or 10 μM quercetin in ethanol for up to 96 h. At each time point cells were trypsinised, stained with Trypan blue (Trypan blue exclusion indicates live cells) and manually counted in a haemocytometer. Each time point and quercetin treatment was carried out in duplicate and counted in duplicate for each experiment.

genome in which expression of the viral oncogenes is under the control of the native LCR. Thus, we treated either W12 cells, which maintain HPV-16 episomally (Stanley et al., 1989), or Z183a cells, which have transcriptionally active integrated HPV-18 (Pecoraro et al., 1989), with 5 or 10 μM quercetin for 36 h. Quercetin treatment of W12 cells lead to an increase in the cell population in G1 and a corresponding decrease in cell numbers in S and G2/M indicative of a G1 arrest in the cell cycle (Fig. 1a; shown only for 10 μM quercetin). On the contrary, quercetin treatment of Z183a cells did not noticeably change their cell cycle profile (Fig. 1a), showing that the cells failed to stop cycling. These observations were confirmed by direct count of proliferating cells. Cells were treated with 5 or 10 μM quercetin and counted at 24 h intervals, for up to 96 h. Again, quercetin treatment caused a complete

cessation of cell proliferation in W12 cells, whereas Z183a cells continued to proliferate albeit at a diminished rate compared to untreated cells (Fig. 1b).

The arrest of W12 cells in G1 agrees with what already observed with HFKE6/E7 (Beniston and Campo, 2003). These results suggest that HPV-16 oncoproteins cannot abrogate quercetin-induced G1 arrest, as neither the transcriptional promoter (HPV LCR in W12 versus retrovirus LTR in HFKE6/E7) nor the status of the viral DNA (episomal in W12 versus integrated in HFKE6/E7) affected the outcome of quercetin treatment.

Quercetin inhibits expression of Skp2 in W12 but not in Z183a cells

High concentrations of quercetin (such as 50 μ M) induce cell cycle arrest through p53 (Beniston et al., 2001; Plaumann et al., 1996) while lower concentration of quercetin (5–20 μ M) arrest cell cycle through p27^{Kip1} (Beniston and Campo, 2003). To determine which component(s) of the cell cycle regulatory machinery was affected by quercetin, we analysed by immunoblotting the levels of a number of negative regulators of the cell cycle in quercetin-treated cells. Cells were treated with 0, 5 or 10 μ M quercetin for 36 h. Cell lysates were run in SDS-PAGE gels, blotted and probed for p53, p16^{INK4a}, p21^{Waf1}, p27^{Kip1}, Skp2(p45), E7, and β Actin, using appropriate antibodies (Fig. 2).

In agreement with our previous observations (Beniston and Campo, 2003), both cell lines showed a decrease of p16^{INK4a} and no p53/p21^{Waf1} response (Fig. 2). On the contrary, p27^{Kip1} was elevated in W12 cells in response to quercetin, but the increase was much less noticeable in Z183a cells (Fig. 2). The difference in the p27^{Kip1} response in the two cell lines is likely due to the difference in the levels of Skp2(p45), the F box protein which negatively controls p27^{Kip1}: after quercetin treatment, Skp2(p45) was absent in W12 cells, as reported for HFKE6/E7 cells (Beniston and Campo, 2003), whereas it was not affected in Z183a cells (Fig. 2). In the absence of a functional

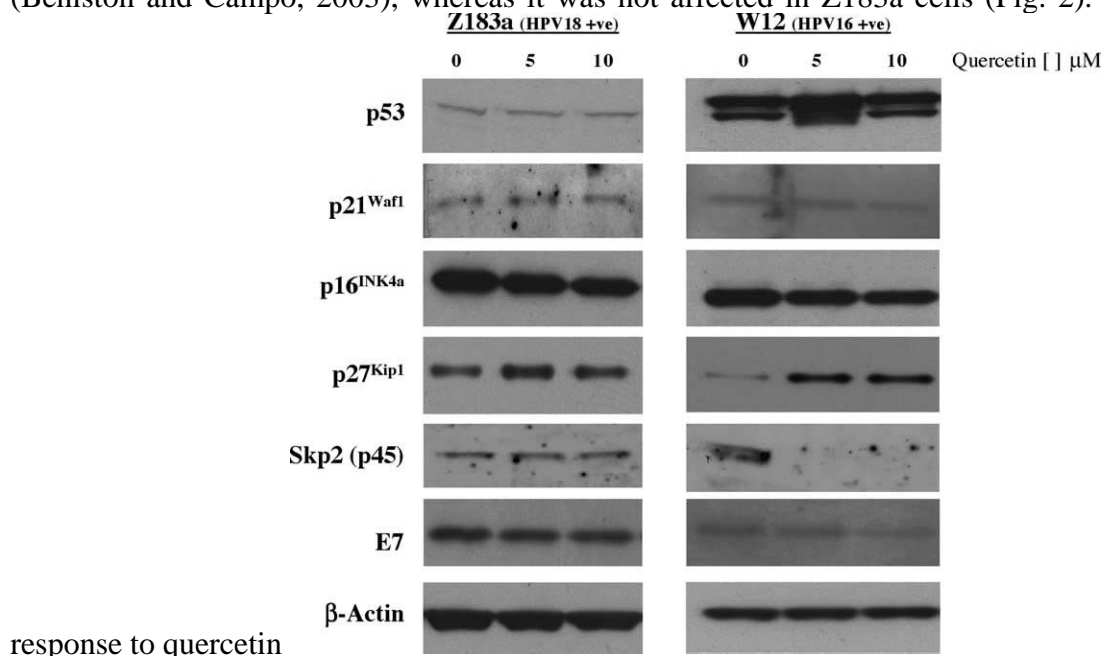


Fig. 2. Differential effect of quercetin on the G1 cdk inhibitors. Cells were treated with 0, 5 or 10 μ M quercetin for 36 h before being harvested in NP40 lysis buffer (0.1% NP40, 150 mM NaCl and 50 mM Tris-HCl pH 7.6) with protease inhibitor cocktail (Roche, Mannheim, Germany). 25–100 μ g of cell lysate was run on Tris-glycine buffered

SDS-PAGE gels in MOPS pH buffer (Invitrogen, Groningen, Holland), transferred to nitrocellulose and blocked in 3% BSA or 5% marvel milk in PBS for 1 h at room temperature. Membranes were probed for p53 (antibody D0-1; Serotec, Kidlington, Oxford), p21^{Waf1} (Ab3; Oncogene Research Products, La Jolla, CA), p27^{Kip1} (Clone 57; Transduction Laboratories, Heidelberg, Germany), p16^{INK4a} (DCS50; Sigma–Aldrich), Skp2(p45) (N19; Santa Cruz Biotechnology, Santa Cruz, CA), β -Actin, (AB1; Oncogene Research Products, Cambridge, MA), HPV-16 E7 (8C9; Zymed Laboratories, San Francisco, CA), HPV-18 E7 (N19; Santa Cruz). Secondary antibodies were anti-mouse IgG-HRP (Amersham-Pharmacia, Bucks, UK) and anti-mouse IgM-HRP (Santa Cruz). Membranes were developed using chemiluminescent peroxidase substrate (Sigma–Aldrich).

from p53 with no increase in p21^{Waf1} or p16^{INK4a}, we conclude that p27^{Kip1} is the most likely mediator of quercetin-induced G1 arrest in W12 cells.

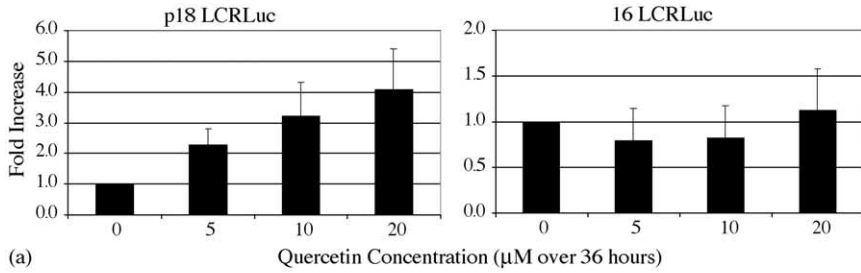
Given the function of HPV E7 in overcoming G1-S control (Jones and Munger, 1996), we analysed the levels of this protein in W12 and Z183a cells after quercetin treatment. E7 levels decreased markedly in W12 cells with quercetin treatment, whereas they remained constant in Z183a cells (Fig. 2). The reason why E7 levels should decrease in W12 cells but not in Z183a cells are unknown, but it is reasonable to assume that the E7 reduction observed in W12 cells may in part explain the inability of these cells to overcome quercetin-induced G1 arrest. Cell type specific variations in oncoprotein levels between HPV-16 and HPV-18 transformed cells cannot be discounted and, due to the lack of antibodies that cross-react with both HPV-16 and HPV-18 E6 and E7, cannot be thoroughly investigated. However, in our hands, and in contrast to the HPV-18 oncoproteins, HPV-16 oncoproteins have consistently failed to abrogate quercetin-induced cell cycle arrest.

Quercetin up-regulates the HPV-18 long control region

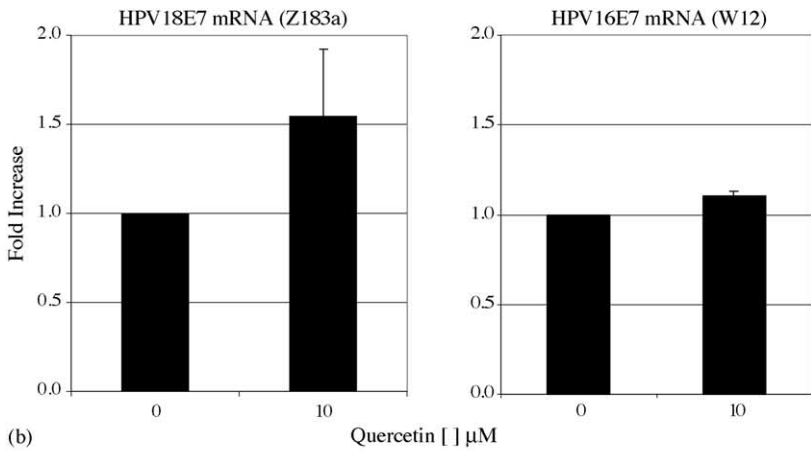
Given that quercetin affects the activity of the BPV-4 LCR (Connolly et al., 1998), and to see if the different levels of E7 in W12 and Z183a cells after quercetin treatment were due to differences in transcription, we investigated the transcriptional activity of HPV-16 and HPV-18 LCRs in luciferase reporter assays. p18LCRLuc or p16LCRLuc contain the HPV-18 or HPV-16 LCR, respectively, controlling the expression of the luciferase gene. The constructs were transfected into C33a cells (HPV negative cervical cancer cells) and cells were treated with 0–20 μ M quercetin for 36 h. C33a cells were used rather than human keratinocytes as they do not arrest in G1 in response to quercetin treatment (data not shown), whereas human keratinocytes do (Beniston and Campo, 2003) and are therefore unsuitable for transient transfection reporter assays.

Although, as previously reported (O'Connor et al., 1998), the transcriptional activity of the HPV-16 LCR was low (Fig. 3a), there was no significant change in its activity in response to quercetin. On the contrary, the HPV-18 LCR showed a dose-dependent increase in transcriptional activity with quercetin (Fig. 3a). Although quercetin affected transcription from the HPV-18 LCR, it had no effect on the replication of an HPV ori containing plasmid (data not shown).

To confirm that the quercetin-induced increase in the transcriptional activity of HPV-18 LCR seen in C33a cells reflected a true increase in oncogene transcription, real-time RT-PCR was carried out on E7 RNA in Z183a and W12 cells. After quercetin treatment, the level of E7 mRNA consistently increased on average 50% in Z183a cells but not in W12 cells (Fig. 3b), confirming that quercetin activates transcription from the LCR of HPV-18 but not of HPV-16. As quercetin treatment appears to reduce the level of HPV-16 E7 in W12 cells (through an as yet unknown mechanism), it is likely that this contributes to the resultant G1 arrest induced by quercetin. As the transcriptional activity of the HPV-16 LCR appears to be unchanged with quercetin exposure (Fig. 3a), alterations in protein translation or degradation may underpin the decrease in E7. However, further investigation is required in order to determine the mechanism by which quercetin causes loss of HPV-16 E7 expression. In Z183a cells, increased E7 mRNA transcription may help maintain E7 protein levels (see Fig. 2) and, thus, together with the poor response of p27^{Kip1}, bypass quercetin-induced G1 arrest.



(a) Quercetin Concentration (μM over 36 hours)



(b) Quercetin [] μM

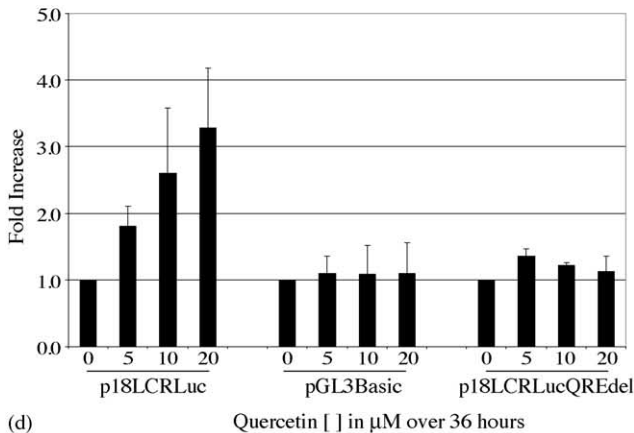
BPV 4 LCR: 241 atatacgttg catcccatc ggctgtaccg aatcgggtgc atataaagg agagcagtgc
 301 ggatttggtg catgaggcag tagctctcat catgctttg tggcttatct atgtttgtt
 361 gctttctgg tgtccttta atttcttgc actgtattt gcaattattg tgcacttct atg=E5 at 332
 421 gtaatttct actattactc ggtagatgg atgggattga attttattt attgtgtta

HPV18 LCR: 1 attaatactt ttaacaattg tagtatataa aaaaggagf aaccgaaaac ggtcgggacc
 61 gaaaacggtg tatataaag atgtgagaa cacaccaca tactatgpcg cgcttgagg atg=E6 at 105
 121 atccaacacg gcgacctac aagctacctg atctgtgac ggaactgaac acttactgc

HPV16 LCR: 1 actacaataa tcatgtata aaactaagg cgtaaccgaa atcggttgaa ccgaaaccgg
 61 ttagtataaa agcagacatt tatgacca aaagagaact gcaatgttc aggaccaca atg=E6 at 83
 121 ggagcgacc agaaagtac cacagttatg cacagagctg caaacaacta tacatgat

TRE Consensus: TGAGTCA
BPV-4 QRE Sequence: TGAGGCA
HPV-18 'QRE' Sequence: TGTGAGA

(c)



(d) Quercetin [] in μM over 36 hours

Fig. 3. (A) Quercetin increases the transcriptional activity of the LCR of HPV-18. p18LCRLuc and p16LCRLuc contain the HPV-18 or HPV-16 LCR, respectively, cloned into pGL3Basic (Promega, Madison, WI) upstream of the firefly luciferase reporter gene. p18LCRLuc

was derived from a CAT reporter plasmid (Thierry and Howley, 1991), and p16LCRLuc was obtained from Dr. I.M. Morgan, Glasgow, UK. 2×10^5 C33a cells were transfected with 0.1 μ g of p18LCRLuc, p16LCRLuc or pGL3Basic using Lipofectamine (Invitrogen) for 5 h at 37 °C in serum free medium. Cells were treated with 0–20 μ M quercetin for 36 h, harvested and assayed for luciferase activity with luciferase assay buffer (Promega, Madison, WI) in a Dynex-MLX luminometer. The activity of p16LCRLuc was consistently approximately 10-fold higher than that of pGL3Basic, while the activity of p18LCRLuc was approximately 1000-fold higher (data not shown). The activity of the LCR constructs in the absence of quercetin was arbitrarily defined as 1 and the increase in activity is shown as “fold increase”. (B) Quercetin increases HPV-18 E7 mRNA transcription. Total RNA was isolated using the RNeasy Minikit (Qiagen, Sussex, UK) from Z183a or W12 cells. The RNA was treated with DNA-aseI (Invitrogen, Glasgow, UK) and analysed by real time RT-PCR for HPV-18 E7, or HPV-16 E7 RNA, respectively, and for β -Actin as control using Taqman $\text{\textcircled{A}}$ EZ RT-PCR Kit (Applied Biosystems, Foster City, CA). HPV-16 E7 primers were E7FOR 5-AAGTGTGACTCTACGCTTCGGTT-3 and E7REV 5-TTTGGAAGACCTGTTAATGGGC-3 with probe 5-TGCGTACAAAGCACACACGTAGACATTGTA-3. HPV-18 E7 primers were 18E7FOR 5-TGTATTGCATTTAGAGCCCCAA-3 and 18E7REV 5CTTCCTCTGAGTCGCTTAATTGC-3 with probe 5-ATGAAATTCGGTTGACCTTCTATGTCACG-3. Commercially available β -Actin primers and probe were used (Applied Biosystems, Cheshire, UK). DNA contamination in samples was tested by using β -Actin primers and Taq polymerase in reactions with no RT. RT-PCR was performed using an ABI Prism 7700 Sequence detector. Each experiment used 8–10 10-fold serial dilutions of the appropriate plasmid DNA for each set of primers and probes to generate standard curves, and sample amplification was carried out in triplicate. The relative amounts of HPV-16 or HPV-18 E7 RNA or β -Actin RNA were determined by comparison to the standard curves. Levels of HPV-16 or HPV-18 E7 RNA were normalised to β -Actin RNA levels. The level of E7 RNA in the absence of quercetin was arbitrarily taken as 1 and its increase in the presence of quercetin is shown as “fold increase”. (C) The HPV-18 LCR contains a “quercetin responsive element”. Sequence comparison of the BPV-4, HPV-16 and HPV-18 basal promoter regions. Numbers indicate position as defined by NCBI Genome sequence database (NC 004711, NC 001526 and NC 001357, respectively). The QRE sequences are in bold and underlined; the TATA box is underlined and the starting codon of the first early gene is boxed. Comparison of the TRE/QRE sequences is shown below where identical nucleotides are underlined. (D) The QRE of the HPV-18 LCR mediates the quercetin-induced increase of transcriptional activity. A deletion mutant of the HPV-18 LCR (QREdel) was generated by PCR, using first primer 1 (HPV-18mutfor2 5-CGCTCGAGCGCTGGCACTATTGCA-3) and primer 2 (GL2 Primer 2, Promega), and then primer 3 (HPV-18QREdelYA 5-CGCCATAGTATTGTGGTGTGTTTCTTTTATATACACCGTTTTTCGGTCCC-3) and primer 4 (HPV-18QREdelB 5-AACACACCACAATACTATGGCG-3). The deletion of the putative QRE site was confirmed by DNA sequencing. p18LCRLuc or p18LCRLucQREdel were transfected into C33a cells and quercetin treatment was started 24 h later with 0–20 μ M quercetin for 36 h. Cells were harvested and assayed for luciferase activity as described in the legend to panel A. The activity of p18LCRLucQREdel in the absence of quercetin was approximately 1000-fold higher than that of pGL3Basic and similar to that of p18LCRLuc (data not shown). The activity of the LCR constructs in the absence of quercetin was arbitrarily defined as 1 and the increase in activity is shown as “fold increase”.

Quercetin up-regulation of the HPV-18 LCR is mediated by a “QRE” element

Quercetin activates the LCR of BPV-4 through an initiator-like element downstream of the TATA box, defined as quercetin response element or QRE, and mutation or deletion of this element abrogate the ability of quercetin to up-regulate the BPV-4 LCR (Connolly et al., 1998). Analysis of the HPV-16 and HPV-18 LCR showed that a QRE-like element is present in the HPV-18 LCR in a similar position relative to other controlling elements, but not in the HPV-16 LCR (Fig. 3c). To determine whether this QRE-like element was the mediator of quercetin trans-activation of the HPV-18 LCR, we constructed a deletion mutant of the LCR lacking the element. The “QRE Del” mutant LCR completely lost its response to quercetin (Fig. 3d) showing that, as in BPV-4, quercetin activates the HPV-18 LCR via this site.

Further investigation is required to determine, not only the mechanism by which this modulation of the HPV-18 LCR activity occurs, but also whether it is necessary and/or sufficient for abrogation of the G1 arrest in HPV-18 positive cells.

In conclusion, HPV-16 transformed cells fail to proliferate in the presence of quercetin, independently from whether the oncoproteins are expressed from the HPV-16 LCR or a retrovirus LTR, and from the status of the viral DNA (episomal in W12 cells and integrated in HFKE6/E7 cells).

On the contrary, the HPV-18 containing Z183a cells are not affected by quercetin and continue proliferating,

likely leading to the accumulation of quercetin-induced mutations and thus increased cell transformation. We ascribe the ability of the Z183a cells to continue proliferating in the presence of quercetin to the persistence of Skp2, which negatively regulates p27^{Kip1}, and to the increased activity of the HPV-18 LCR, which keeps the levels of E7 constant to counteract the relatively low levels of p27^{Kip1}. The differences in levels of Skp2 after quercetin treatment is likely to be due to the different oncoproteins of the two HPVs, but this remains to be established. While some of the observed differences between W12 and Z183a cells may be due to the status of the viral DNA (episomal HPV-16 in the former and integrated HPV18 in the latter, and therefore potentially subject to different cellular controls), the different response to quercetin of the isolated LCRs is inherent to the LCRs themselves and cannot be ascribed to the status of the viral DNA.

It would be interesting to analyse HPV-18 transformed cells at different progression stages to delineate the relationship between the status of the viral genome (integrated/episomal) and ability to abrogate quercetin-induced cell cycle arrest also in the case of HPV-18. Such an analysis would allow a greater understanding of the interactions between these two oncogenic viruses and environmental cocarcinogens.

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