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Modulation of Major Histocompatibility Complex (MHC) Class I Heavy Chain Gene Expression by Naringenin in Cells That Express Human Papillomavirus (HPV) E6 and E7 Genes

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Abstract. This study describes the effect of Naringenin on major histocompatibility complex (MHC) class I heavy chain gene expression in the presence of low risk or high risk Human Papillomavirus (HPV) E6 and E7 genes. Northern blot analysis showed that, in the presence of Naringenin, steady-state levels of MHC class I heavy chain mRNA transcripts were elevated in rat fibroblast (3Y1) cells. In transient expression assays of MHC class I heavy chain (H-2K^b) promoter activity, it was observed that there was a down-regulation of MHC class I promoter activity in 3Y1 cells co-transfected with either high risk HPV 16 E6 and E7 or low risk HPV 6 E6 and E7 expression plasmids. Treatment of control 3Y1 cells and cells co-transfected with high or low risk HPV E6 and E7 plasmids with 300μg/ml of Naringenin for 24 hours led to an increase in H-2K^b-driven CAT (chloramphenicol acetyl transferase) reporter gene activity. Thus, Naringenin retained the ability to increase H-2K^b promoter activity in the presence of either E6 or E7 expression constructs, indicating that Naringenin can over-ride the transcriptional repressor effects of HPV oncoproteins and may therefore have some potential immunostimulatory properties in HPV-associated malignancy.

Keywords. MHC Class I, papillomavirus, expression, naringenin

INTRODUCTION

Papillomaviruses are small DNA viruses associated with benign and malignant proliferative lesions of cutaneous epithelium. HPV 6 and 11 are found in benign lesions such as ano-genital condylomata acuminata and laryngeal papilloma while HPV 16 and 18 are commonly associated with cervical intraepithelial neoplasia (CIN), vaginal intraepithelial neoplasia (VIN), penile intraepithelial neoplasia (PIN), bowenoid papulosis and genital Bowen's disease, peri-anal and anal cancer (Howley and Lowy, 2001).

The E6 and E7 genes of genital HPVs interfere with cellular tumour suppressor proteins whose normal function is to regulate cell proliferation. Interactions of the E6 protein with p53 and the E7 protein with the retinoblastoma susceptibility gene product, p105 or pRb (Levine et al.,1991) are found to be more stable in the high risk HPV types 16 or 18 (associated with malignant cancers) than in the low risk HPV 6 or 11 derived from benign lesions (Werness et al., 1990).

Class I and class II molecules of the major histocompatibility complex (MHC) play a key role in the recognition of foreign antigens by T cell receptors carried by cytotoxic and helper T lymphocytes. The MHC class I gene products are a highly polymorphic family of cell surface proteins responsible for presentation of peptide antigens to cytotoxic T cells (Fourie and Yang, 1998). Several studies have examined the expression of MHC class I and II molecules in cervical lesions and any possible relationship between the presence of HPV and changes in MHC expression. A significant proportion of the tumours showed down-regulation of MHC class I molecules, which would prevent effective cytotoxic T cell activity against potential viral antigens (reviewed in Garrido and Algarra, 2001).

As well as playing a key role in subverting the cell cycle, the E7 gene products can repress multiple promoters in the MHC class I antigen processing and presentation pathway (Georgopoulus et al., 2000). This could lead to lowered levels of cell surface MHC class I molecules and thus the HPV-transformed cells could evade cellular immune surveillance mediated by CD8$^+$ cytotoxic T lymphocytes (Eiben et al., 2002).

Abbreviations: MHC, Major Histocompatibility Complex; HPV, Human Papillomavirus; CIN, cervical intraepithelial neoplasia.

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Flavonoids form a large class of phenolic substances widely distributed in nature (Di Carlo et al., 1993). Naringenin is one of the major naturally occurring flavones. Naringin (4',5,7-trihydroxyflavanone 7-rhamno-glucoside), the so-called 'bitter principle' of grapefruit, is the most abundant of these compounds and is usually present in concentrations of up to 800 mg/l of juice (Rousseff et al.,1987). Following oral administration in humans, naringin is thought to be converted to the aglycone, Naringenin. Naringenin exhibits several biological effects such as anti-estrogenic activity, anti-ulcer actions, inhibition of human liver cytochrome P450 isoforms CYP 3A4, CYP 1A2 and other enzyme activities as well as inhibition of non-enzymatic lipid peroxidation (Saija et al.,1995; Huang et al.,1997).

Recognising the importance of the control of expression of MHC class I molecules in immune processes, in this study attention was directed towards an understanding of how expression of MHC class I heavy chain genes is regulated in both normal cells and cells expressing HPV oncogenes and, in particular, in any possible effects of Naringenin on MHC class I heavy chain gene expression. Since Naringenin is known to have multiple biological effects (such as anti-estrogenic and ulcer protecting effects), it was of interest to test its potential immunostimulatory and anti-viral effects, particularly on the expression of a key cellular MHC gene in the presence of the HPV 6 or 16 E6 and E7 genes. In this study we have shown that HPV E6 and E7 down-regulated MHC class I heavy chain gene expression and that Naringenin has the ability to overcome this repressive effect of HPV oncoproteins.

MATERIALS AND METHODS

Cell culture. The 3Y1 cell line which is a rat fibroblast (Kimura et al., 1975), was grown in RPMI supplemented with 10% foetal calf serum, penicillin and streptomycin (hereafter termed "complete medium") and maintained in a humidified atmosphere of 95% air, 5% CO_2. Cells were detached with trypsin-EDTA and seeded in 60mm dishes for experimental analysis. All cell culture media and materials were obtained from Gibco-BRL, U.K. Naringenin was obtained from the Sigma Chemical Company Ltd., USA. The required amount of Naringenin was dissolved in 100% ethanol and added to cell cultures at a concentration of 300μg/ml.

Transient expression assay. The plasmid H2K-CAT contains 2 kb of 5′ flanking region of the H-2K^b class I gene cloned in the multiple cloning site of pBLCAT 3 (Proffitt et al., 1996). The E6 or E7 open reading frames of HPV 6b or 16 were previously cloned into pUC 19 under the control of the Moloney murine leukaemia virus transcriptional regulatory elements (Barbosa et al., 1991). Prior to transfection, cells were plated at an appropriate density and grown for 24 hours to a confluency of 60% in 60 mm dishes. After 24 hours of incubation, the cells were transfected by the calcium phosphate method using a commercial kit (Pharmacia Biotech., U.S.A.) with either 15μg of H2K-CAT reporter gene plasmid and/or 15μg of the HPV 6b or 16 E6 or E7 expression plasmid. The cells were incubated with the calcium phosphate-DNA precipitate at 37°C in a humidified atmosphere containing 5% CO_2 for 6 hours. After 6 hours, the precipitate was removed, the cells were washed three times with RPMI medium and 3 ml of complete medium was added for the subsequent incubation at 37°C in a CO_2 incubator and treated with 100% ethanol as control and either 300μg/ml or 757.5μg/ml Naringenin . Twenty four hours later, the cells were processed for RNA analysis or assayed for chloramphenicol acetyltransferase (CAT), using a CAT ELISA Kit (Boehringer Mannheim, Germany).

Chloramphenicol Acetyl transferase (CAT) assay. Microtiter plate modules were used. Two hundred microliters of CAT standard working dilution or 200μl of cell extract was pipetted per well. The microtiter plate was incubated for one hour at 37°C. The solution was removed and the wells were rinsed with 250μl of phosphate buffer saline for 30 seconds. The washing buffer was removed and 200μl of anti-CAT-digoxigenin was added and incubated for one hour. The solution was then removed and rinsed with PBS. The washing buffer was removed and 200μl of anti-digoxigenin-POD was added and incubated for another hour. The solution was removed and 200μl of POD substrate was added until colour development was sufficient for photometric detection at 405 nm. The CAT activity was expressed as CAT concentration (ng/ml). The results were obtained from the average of three experiments. The fold-induction ratios of CAT activity were calculated by dividing the CAT activity from extracts of cells treated with Naringenin or 100% ethanol by that of the untreated, H2K-CAT-transfected cells.

mRNA analysis. Total RNA isolation was performed using TRIZOL (Gibco-BRL, U.K.) and analysed by Northern blotting using standard methods (Sambrook et al., 1989). DNA probes were either a 1.8 kb *Pst* I fragment of the murine H-2K^b gene (Ackrill and Blair, 1988) or a plasmid pAL41 containing a partial cDNA clone of mouse B-actin (Minty et al.,1983). Labelling was performed by nick translation (Sambrook et al., 1989) using [α-^{32}P]dCTP (Amersham International plc, U.K.). The specific activity of the labeled probes was approximately 10^8 dpm/μg.

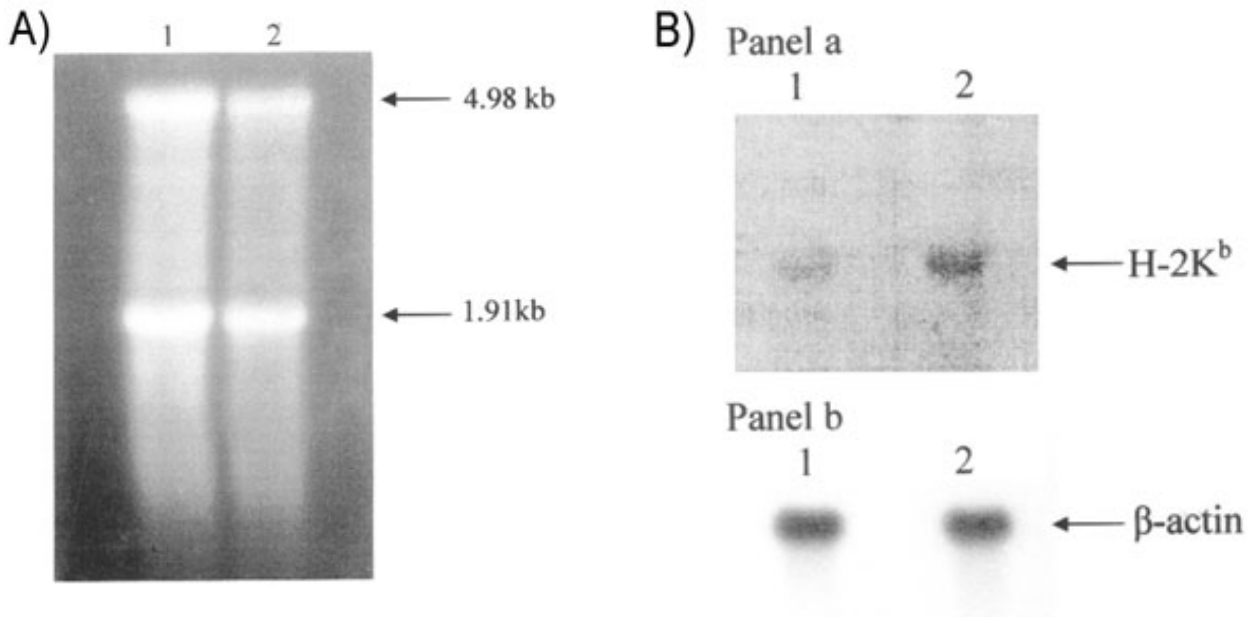

Figure 1. Steady-state levels of MHC class I heavy chain mRNAs in 3Y1 cells treated with Naringenin.
A. Ethidium bromide-stained formaldehyde 1%(w/v) agarose gel showing the integrity of total RNA isolated from Naringenin-treated and control 3Y1 cells. The cells were treated with 300 μg/ml of Naringenin for 24 hr. Total RNA was isolated. Lane 1: mRNA isolated from control 3Y1 cells. Lane 2: mRNA isolated from Naringenin-treated 3Y1 cells
B. Northern blot analysis of levels of mRNA expressed in Naringenin-treated and control 3Y1 cells. Total RNA was isolated from each culture and aliquots (10 μg) of RNA analysed by Northern blotting and hybridization with either a nick-translated H-2K^b insert (panel a) or β-actin insert (panel b). Lane 1: mRNA isolated from control 3Y1 cells. Lane 2: mRNA isolated from Naringenin-treated 3Y1 cells.

RESULTS

Steady-state levels of MHC class I heavy chain mRNAs in 3Y1 cells treated with Naringenin. Since the main aim of this investigation was to screen for any immunostimulatory or anti-viral effects produced by Naringenin, it was therefore of interest to investigate whether elevated levels of MHC class I transcripts could be detected in Naringenin-treated 3Y1 fibroblasts. 3Y1 cells were chosen for this analysis since previous studies had shown that both endogenous MHC class I gene expression and transfected class I promoters could be modulated by adenovirus E1A oncoproteins (13). 3Y1 cells were treated with 300 μg/ml of Naringenin for 24 hours. Analysis of total cellular RNA samples by formaldehyde-agarose gel electrophoresis confirmed that intact total RNA had been isolated from cells and two predominant species (of 4.98 kb and 1.91 kb, corresponding to 28S and 18S ribosomal RNAs) could be detected at equal level in both control and Narigenin-treated cells (Figure 1A). Northern blot hybridisation using the nick-translated class I DNA probe showed a modest but reproducible increase in MHC class I transcript levels after 24 hours of Naringenin treatment (Figure 1B). Control hybridisation using nick-translated β-actin cDNA showed that the β-actin mRNA level in Naringenin treated and control 3Y1 cells was only slightly affected by Naringenin treatment. Densitometric analysis of autoradiographs showed that the class I/β-actin ratio increased by approximately 1.4 fold upon Naringenin treatment of 3Y1 cells. This suggested that there could be an increase in transcription of class I genes in Naringenin-treated 3Y1 cells. This directed our attention towards analysing the activity of an MHC class I promoter linked to a CAT reporter gene in control and Naringenin-treated cells.

The effect of naringenin on the MHC class I promoter activity. Experiments were therefore performed to investigate the effects of different concentrations of Naringenin on the MHC class I promoter activity using transient expression assays in 3Y1 cells. When 3Y1 cells were transfected with a plasmid in which 2 kb of 5' flanking region of the mouse H-2K^b class I heavy chain gene was linked to a CAT reporter gene (H2K-CAT) and treated with 100% ethanol as control and either 300μg/ml or 757.5μg/ml Naringenin (Fig. 2), it could be seen that there was a stimulation of H-2K^b promoter activity at the lower concentration of Naringenin (300μg/ml), giving a two-fold increase in CAT activity after 24 hrs of treatment. However

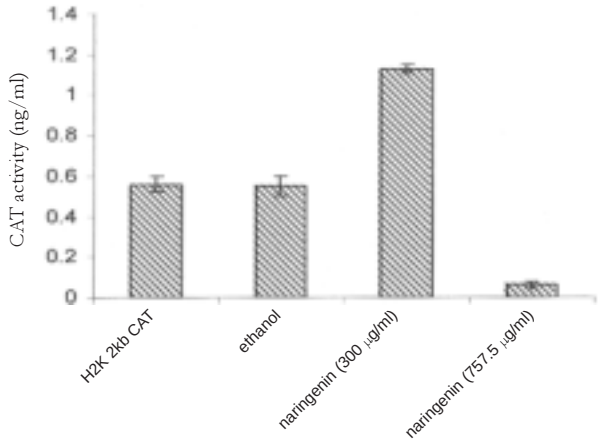

Figure 2. The effect of different concentrations of Naringenin on MHC class I heavy chain promoter activity in 3Y1 cells. The cells were treated for 24 hr with 100% ethanol as a control and either 300μg/ml or 757.5 μg/ml Naringenin and the CAT assay was performed. The CAT activity was expressed as CAT concentration (ng/ml). The results were obtained from the average of three experiments. The fold-induction ratios of CAT activity were calculated by dividing the CAT activity from extracts of cells treated with Naringenin or 100% ethanol by that of the untreated, H2K-CAT-transfected cells.

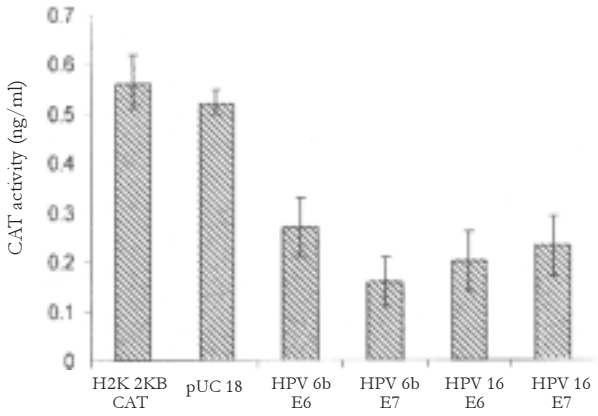

Figure 3. The effect of co-transfection of HPV E6 or E7-expression plasmids on MHC class I heavy chain promoter activity in 3Y1 cells. The H2K-CAT plasmid was co-transfected with either the control plasmid (pUC19) or HPV 6 E6, HPV 6 E7, HPV 16 E6, HPV 16 E7 expression plasmid.

at the higher concentration of Naringenin (757.5 μg/ml), H-2K^b promoter activity appeared to be down-regulated. Therefore standard conditions for Naringenin treatment were adopted, typically 24 hrs of treatment with 300 μg/ml of Naringenin. Interestingly it was also observed that high concentrations of Naringenin (700 μg/ml) seemed to be toxic to the cells.

The effect of HPV 6b and 16 E6 and E7 oncoproteins on MHC class I promoter activity. To ascertain the effect of the E6 and E7 oncoproteins on MHC class I promoter activity, co-transfection of 3Y1 cells was performed with equivalent (10 μg) masses of either control pUC19, HPV 6b E6, HPV 16 E6, HPV 6b E7 or HPV 16 E7 expression plasmid and 15 μg of H2K-CAT plasmid DNA. When the pUC19 and H2K-CAT plasmids were co-transfected into 3Y1 cells, it was observed that there was no significant reduction of CAT expression, which shows that the cells are unaffected by the pUC19 plasmid. On the other hand, in cells co-transfected with pUC19, H2K-CAT and either the HPV6b or 16 E6 or E7 plasmids, CAT expression was significantly reduced. The CAT expression level was suppressed to about 0.48-fold for HPV 6 E6 co-transfected cells, 0.38-fold for HPV 16 E6 co-transfected cells, 0.29-fold for HPV 6 E7 co-transfected cells and 0.42-fold for HPV 16 E7 co-transfected cells (Fig. 3). This therefore implied that the down-regulation in MHC class I heavy chain gene expression could be directly related to the presence of the E6 and E7 gene products, with the resulting CAT activity being repressed the most in the presence of the HPV 6 E7 gene product.

The effect of Naringenin on MHC class I promoter activity in cells expressing the HPV E6 and E7 oncoproteins. Experiments were performed to determine the effect of Naringenin in cells expressing the HPV E6 or E7 gene products (Fig. 2). Co-transfections of 10 μg masses of either pUC19, HPV 6 E6, HPV 16 E6, HPV 6 E7 or HPV 16 E7 plasmid and 15 μg of H2K-CAT plasmid into the 3Y1 cells were performed and then treated with Naringenin for 24 hrs. When 3Y1 cells were co-transfected with pUC19 and H2K-CAT followed by treatment with Naringenin for 24 hrs, an increase in CAT activity was observed and the CAT induction level was about 2-fold, again showing that CAT expression driven by H2K-CAT was again increased in the presence of Naringenin. In addition, in all co-transfected cells, an increase in CAT activity over the basal levels was observed in the presence of Naringenin. The CAT induction level was about 25-fold for the HPV 6 E6 co-transfection, 6.7-fold for the HPV 16 E6 co-transfection, 35-fold for the HPV 6 E7 co-transfection and 3.6-fold for HPV 16 E7 co-transfection (Fig. 4). Thus, 3Y1 cells co-transfected with HPV 6 E7 or HPV 6 E6 exhibited much higher CAT activities compared to the same cells with HPV 16 E6 or HPV 16 E7. In summary, it was therefore established that Naringenin could overcome the effect of HPV 6 or 16 E6 and E7 gene products on MHC class I expression.

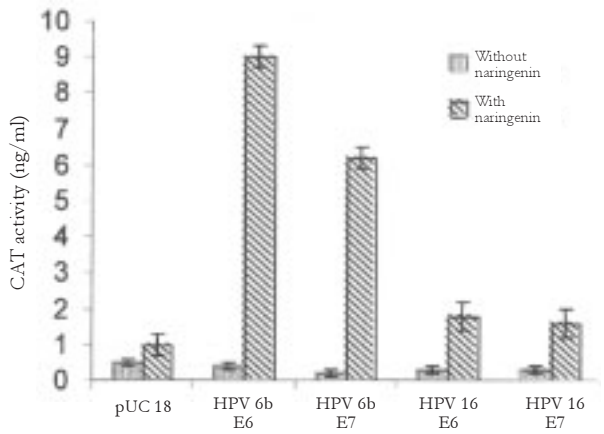

Figure 4. The effect of Naringenin on MHC class I promoter activity in 3Y1 cells expressing HPV E6 or E7 gene products. The H2K-CAT plasmid was co-transfected with either the control plasmid (pUC19) or HPV 6 E6, HPV 6 E7, HPV 16 E6, HPV 16 E7 plasmids and was treated for 24hrs with 300µg/ml naringenin

DISCUSSION

Northern blot results obtained with the Naringenin-treated and control 3Y1 cells showed that there is an elevated level of MHC class I heavy chain transcripts in Naringenin-treated 3Y1 cells. This suggested that the class I mRNA transcription might be increased in Naringenin-treated 3Y1 cells, leading to the observed elevated levels of class I heavy chain mRNA. This directed our attention towards analyzing transcription of class I genes in transfected cells by studying an MHC class I promoter (derived from the mouse H-2K^b gene) linked to a CAT reporter gene.

The expression of MHC class I genes was known to be reduced in HPV 16-associated tumours and it has been proposed that this allows HPV 16-transformed cells to escape immune surveillance and to form tumours in the human host (Cromme et al., 1993). The results presented here demonstrate that HPV 16 E6 and E7 and HPV 6 E6 and E7 gene products have the ability to down-regulate the MHC class I promoter in co-transfection experiments in 3Y1 cells. This suggests that the E7 gene and E6 gene of both the low- and high-risk HPV genotypes may play a role in the repression of MHC class I antigen processing and presentation pathway. The ability of Naringenin to restore the activity of the class I heavy chain promoter indicates that the cells are not deficient in MHC class I expression (for example, by mutations or rearrangements in class I genes) but rather that E6 and E7 are actively repressing class I transcription and that Naringenin can overcome these repressory effects.

The mechanism(s) responsible for the repression of class I heavy chain promoters and their up-regulation by Naringenin remain to be elucidated. One possibility is that the level or activity of transcription factors that are common to multiple class I antigen processing and presentation genes might be modulated by HPV oncoproteins and Naringenin. Such a well-characterised candidate transcription factor is NF-κB, whose activity is regulated by phosphorylation and binding of inhibitory proteins (Ghosh and Baltimore, 1990; Liou et al., 1992). Future work will focus on studies of DNA binding and activity of this and other transcription factors involved in regulating expression of MHC genes in HPV-transformed and Naringenin-treated cells.

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