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HPV-16 E5 down-regulates expression of surface HLA class I and reduces recognition by CD8 T cells

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ABSTRACT

HPV-16 is the major causes of cervical cancer. Persistence of infection is a necessary event for progression of the infection to cancer. Among other factors, virus persistence is due the viral proteins fighting the immune response. HPV-16 E5 down-regulates MHC/HLA class I, which is much reduced on the cell surface and accumulates in the Golgi apparatus in cells expressing E5. This effect is observed also in W12 cells, which mimic early cervical intraepithelial progression to cervical cancer. The functional effect of MHC I down-regulation on human CD8 T cells is not known, because of the need for HLA-matched, HPV-specific T cells that recognise E5 expressing-cells. Here we employ a heterologous cell/MHC I system which uses mouse cells expressing both E5 and HLA-A2, and A2-restricted CTLs; we show that the E5-induced reduction of HLA-A2 has a functional impact by reducing recognition of E5 expressing cells by HPV specific CD8+ T cells.

Introduction

Human papillomavirus type 16 (HPV-16) is one of the major causes of cervical cancer (zur Hausen, 2009). Like most cancers, cervical carcinogenesis is a multi-step process which starts with viral infection and requires a number of events to occur for full cell transformation to take place. One of these events is persistence of viral infection. Papillomavirus persistence is due to several factors: the virus life cycle takes place fully within the thickness of the epithelium and therefore away from dermal immune cells (Stanley et al., 2007), the virus does not cause cell lysis and therefore no inflammatory response (Stanley et al., 2007), and the viral proteins actively fight the immune response (O'Brien and Campo, 2002). The E5 protein contributes to this fight by down-regulating MHC/HLA class I. MHC class I is much reduced on the cell surface and accumulates in the Golgi apparatus in cells expressing E5 (Ashrafi et al., 2005). The arrest of MHC class I in the Golgi apparatus is due to E5-induced alkalinisation of the endomembrane compartments (Schapiro et al., 2000), following E5-16k subunit c interaction, and to the direct interaction of E5 with the heavy chain of the MHC class I complex (Ashrafi et al., 2006; Cortese et al., 2010). These conclusions have been reached in cultured HaCaT cells and, in the case of bovine papillomas, also *in vivo* (Araibi et al., 2004). However, the functional effect of MHC I down-regulation on the activity of cytotoxic T lymphocytes (CTLs) has not been investigated, because HaCaT keratinocytes express primarily HLA-A3 (A31 B60 B51) and there are no readily available A3-restricted CTL clones that can be used which could recognise E5 expressing-HaCaT cells. Here we show that also in the cervical cell line W12 expression of E5 is accompanied by a decreased expression of HLA, and that E5 expression does reduce recognition by CD8 T cells.

Results and discussion

Expression of HPV-16 E5 is associated with down-regulation of HLA in the W12 cell system

Previous work on HPV-16 E5 (16-E5) and HLA class I has been done in HaCaT human keratinocytes. To study E5-HLA interaction in a more natural setting, we investigated W12 cells, a line of cervical keratinocytes derived from a cervical intraepithelial grade I (CIN I) lesion (Stanley et al., 2007). This cell line recapitulates the neoplastic progression of HPV-16-induced cervical lesions. Early passage W12

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cells (W12E) harbour episomal copies of HPV-16, and express all early viral genes, whereas late passage cells (W12G) harbour integrated HPV-16 genomes (Jeon et al., 1995) with the loss of the E2-E5 region. W12 cells are immortal but not transformed and can be grown and differentiated in three-dimensional organotypic raft cultures, in which W12E form CIN I-like epithelia and W12G form similarly structured epithelia but display reduced differentiation and some invasion (Aasen et al., 2003). In W12E we easily detected E5 mRNA by RT-PCR (Fig. 1A); on the contrary there was very little or no E5 mRNA in W12G despite the presence of very similar levels of GAPDH mRNA in both W12 cell lines (Fig. 1A) in agreement with the integration of HPV-16 genomes in the E2-E5 region of the viral genome (Jeon et al., 1995; Alazawi et al., 2002).

The availability of W12 cells with and without E5 expression prompted us to investigate if the presence of E5 had any effect on the surface expression of HLA class I.

W12E and W12G cells were analysed by flow cytometry together with cervical carcinoma cell line CaSki (HPV-16 positive) and peripheral blood cells (PBMC) as control. As predicted from previous work, W12E cells showed much less surface HLA class I than W12G cells (Fig. 1B), which had similar levels of surface HLA as CaSki cells; all test cells expressed much less surface HLA class I than PBMC (Fig. 1B).

We also analysed surface HLA-A by immunohistochemistry in raft-cultured W12E and W12G cells, in conditions that mimic the natural architecture and the biological characteristics of early and late cervical lesions respectively. The E5-expressing W12E cells showed little or no HLA class I staining (Fig. 2C) and the staining was predominantly confined to the cytoplasm (inset) as it is the case in HaCaT cells expressing 16-E5 (Ashrafi et al., 2005). On the contrary, the W12G cells (no E5) showed abundant levels of HLA-A on their plasma membrane and in the cytoplasm (Fig. 2D and inset).

The sensitivity of immunohistochemistry is lower than that of flow cytometry and this is likely to be the reason why very little HLA class I is detected in W12E cells by the former technique compared to flow cytometry. Furthermore flow cytometry has been performed on undifferentiated cells while immunohistochemistry has been performed on raft-cultured differentiated cells.

In conclusion, also in the natural W12 system which recapitulates the neoplastic progression of cervical lesions E5 expression is associated with the down-regulation of surface HLA class I. The inverse relationship between expression of E5 and of surface MHC I has already been observed in bovine papillomas (Araibi et al., 2004) and in a small panel of naturally occurring CIN lesions (Araibi, 2006). Together with the present results, it appears that E5-associated down-regulation of surface MHC I in early lesions is a common occurrence. Introduction of exogenous E5 into W12E cells or its silencing in W12G cells should prove a cause-and-effect relationship between presence of E5 and absence of MHC I.

HPV-16 E5 down-regulates HLA-A2 but not HLA-E in a heterologous system

To study the recognition of infected cells by CD8 T cells, T cell lines are needed that are matched to the HLA type of the target cells. HaCaT keratinocytes express predominantly HLA-A3 (A31 B60 B51) and W12 cells express predominantly A11 (A11, 24 B8 Bw63) (M Stanley, personal communication). No CD8 T cell lines are available restricted to these HLA types and therefore neither HaCaT nor W12 cells can be

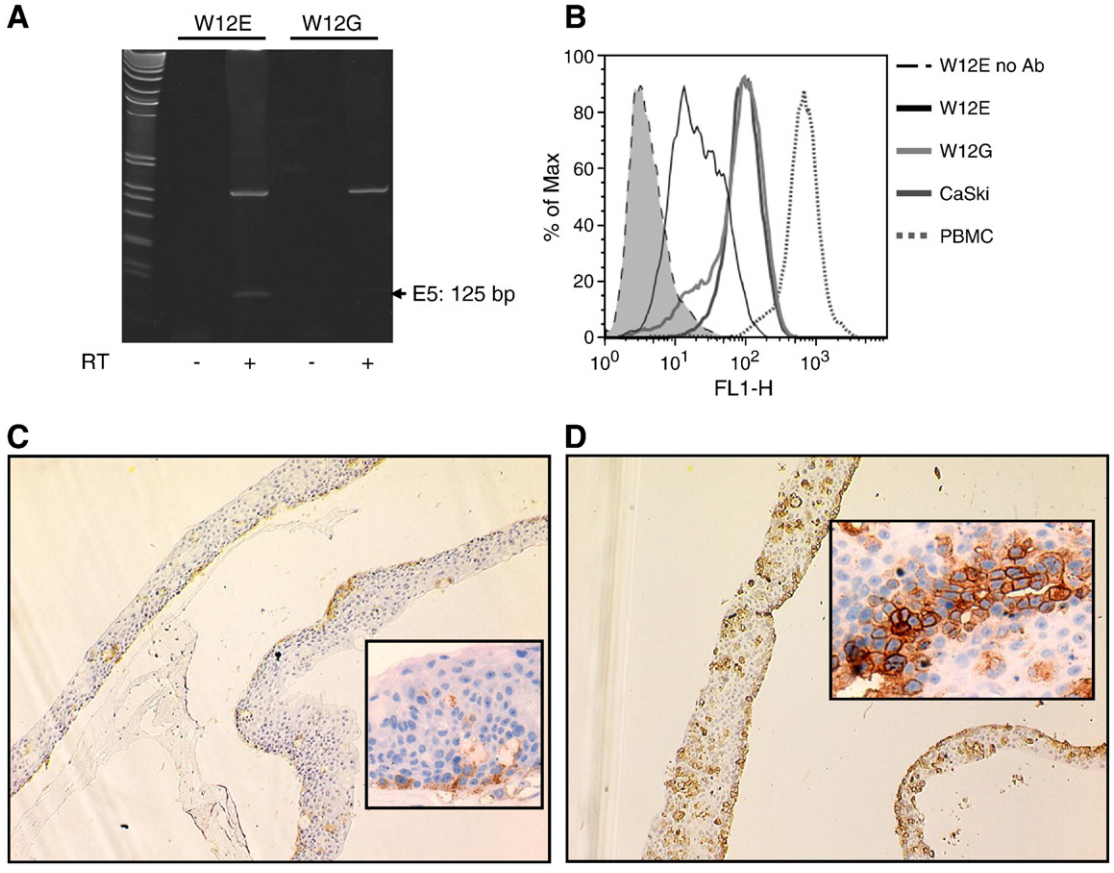

Fig. 1. HPV-16 E5 down-regulates HLA class I in W12 cells. (A) detection of E5 mRNA (125 bp band) by RT-PCR in W12E cells but not in W12G cells. The top band shows simultaneous amplification of GAPDH mRNA in the samples. (B) Flow cytometry analysis of surface HLA class I in W12E and G cells, with CaSki cells and PBMC as controls. (C and D) Immunohistochemistry of HLA-A in sections of organotypic cultures of W12E and W12G cells respectively. Main panels 5×; inset in C, 20×; inset in D, 40×. Note the reduction of HLA class I in W12E cells both by flow cytometry and immunohistochemistry.

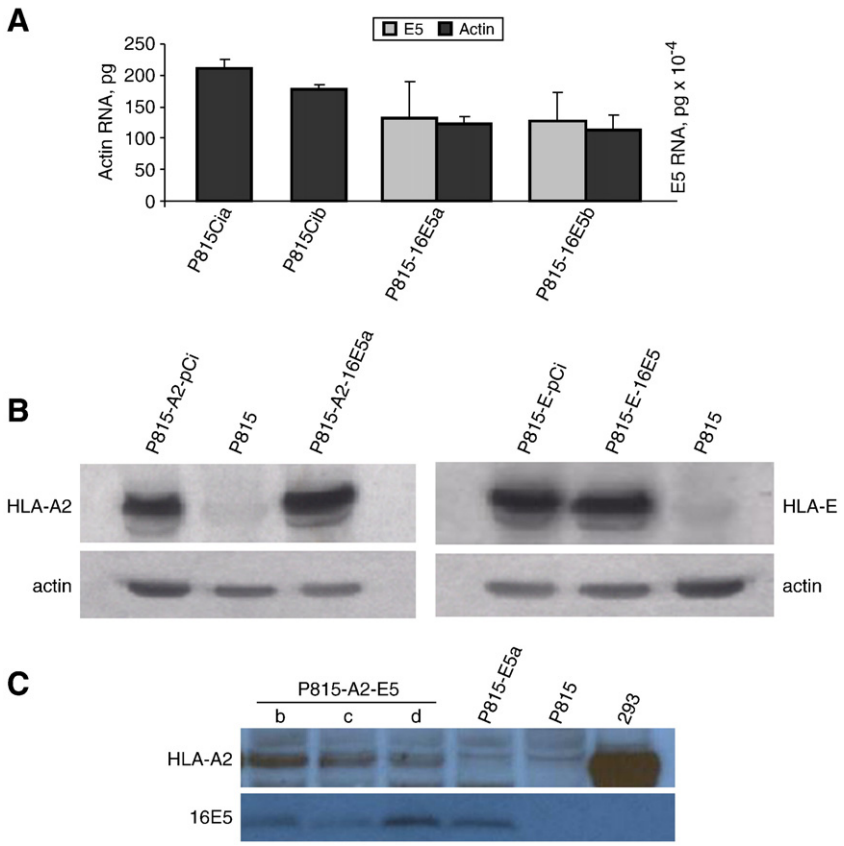

Fig. 2. HPV-16 E5 and HLA in P815 cells. (A) Quantitative RT-PCR of E5 RNA in two lines of P815 cells carrying pCineo empty vector (P815-pCia and P815-pCib) or pCi-16E5 (P815-16E5a and P815-16E5b). Note the absence of E5 mRNA in P815-pCi cells and the difference in scale between amount of actin (left y axis) and 16E5 (right y axis). (B) Western blotting for HLA-A2 and HLA-E in P815 cells with and without 16E5. Note the absence of HLA-A2 and HLA-E in P815 parental cells and the similar levels of HLA-A and HLA-E whether E5 is expressed or not. Mouse actin was the internal control. (C) Western blotting for 16E5 and HLA-A2 in three lines of P815-A2-16E5, one line of P815-16E5, P815 parental cells and human 293 cells for control. Note the absence of 16E5 in the parental cells and of HLA-A2 in P815-16E5 cells.

used in immunological assays to study the functional effect of E5-induced HLA class I down-regulation on the activity of CD8 T cells.

To overcome this problem, we used P815, a cell line derived from a mouse mastocytoma (Ellis et al., 1999), which has been successfully employed in the study of the interaction between BPV E5 and classical and non-classical bovine MHC class I (Araibi et al., 2006). To assess whether 16E5 behaves in P815 cells as it does in human cells, we transfected P815 cells with the heavy chain (HC) of HLA-A2 or the HC of HLA-E and 16E5, either alone or in combination. Heterologous MHC heavy chains can interact with mouse β2 microglobulin to form a functional complex (Ellis et al., 1999).

We derived several cell P815 lines by independent transfections. First we ascertained that P815 cells expressed 16E5; we detected E5 mRNA by qRT-PCR in two cell lines and E5 protein by western blotting in four additional cell lines, with or without HLA-A2. The levels of E5 mRNA were four orders of magnitude lower that those of actin (Fig. 2A), in agreement with E5 expression in HaCaT keratinocytes (Ashrafi et al., 2005). The amount of E5 protein was comparable in the other cell lines (Fig. 2C), although P815-A2-E5c cells appeared to have somewhat less E5.

Expression of A2 HC and E HC was assessed by western blotting. The two HC were expressed at approximately the same levels and, as in HaCaT keratinocytes, E5 did not affect their levels (Fig. 2B and C).

Next we assessed the effect of 16E5 on HLA-A2 or HLA-E surface expression by flow cytometry and immunofluorescence. It was clear that 16-E5 noticeably down-regulated the surface expression of HLA-A2, but not that of HLA-E (Fig. 3), confirming that 16E5 does not affect the transport of HLA-E to the cell surface (Ashrafi et al., 2005). Interestingly, in P815-16E5 cells the expression of surface mouse MHC I was also down-regulated compared to P815 parental cells (results not shown), corroborating previous results that both BPV and HPV E5 proteins are capable of interfering with the transport of MHC I to the cell surface independently of cell species (Ashrafi et al., 2005, 2002; Marchetti et al., 2002, 2009).

By immunofluorescence, both HLA-A2 and HLA-E were detected in the Golgi apparatus, as judged by the orange colour (merge of red Golgi and green HLA) and on the cell surface (green HLA) in the cells not expressing E5 (Fig. 3). On the contrary, HLA-A was detected only in the Golgi apparatus in cells expressing E5, while the location of HLA-E was not affected (Fig. 3). These results agree completely with those obtained in HaCaT keratinocytes.

HPV-16 E5 reduces recognition by CD8 T cells

Having established that 16E5 behaves in P815 cells as it does in human keratinocytes, we conducted $CD8^+$ T cell activation assays, in which the read-out for functional activity was the production of γ-interferon (γIFN) (Thomas et al., 2008). This is more stringent than measuring cytotoxic function because more peptide:HLA complexes are required to induce cytokine release when compared to cytotoxicity (Valitutti et al., 1996). We used the well-established $CD8^+$ T cell clone 7E7 isolated from a cervical cancer patient which recognises a peptide epitope from HPV16 $E6_{29–38}$ (TIHDIILECV) presented by HLA-A2 (Evans et al., 2001). This T cell clone was co-cultured with P815 parental cells, P815-A2 and P815-A2-E5 cells after they had been loaded with increasing amounts of the $E6_{29–38}$ peptide. Consistent results were obtained in several repeat experiments; namely that the parental cells P815 (HLA-A2 negative) did not induce any γIFN production by 7E7 T cells, whereas P815-A2 cells did elicit γIFN production in a peptide dose-dependent manner (Fig. 4). The magnitude of the latter response was comparable

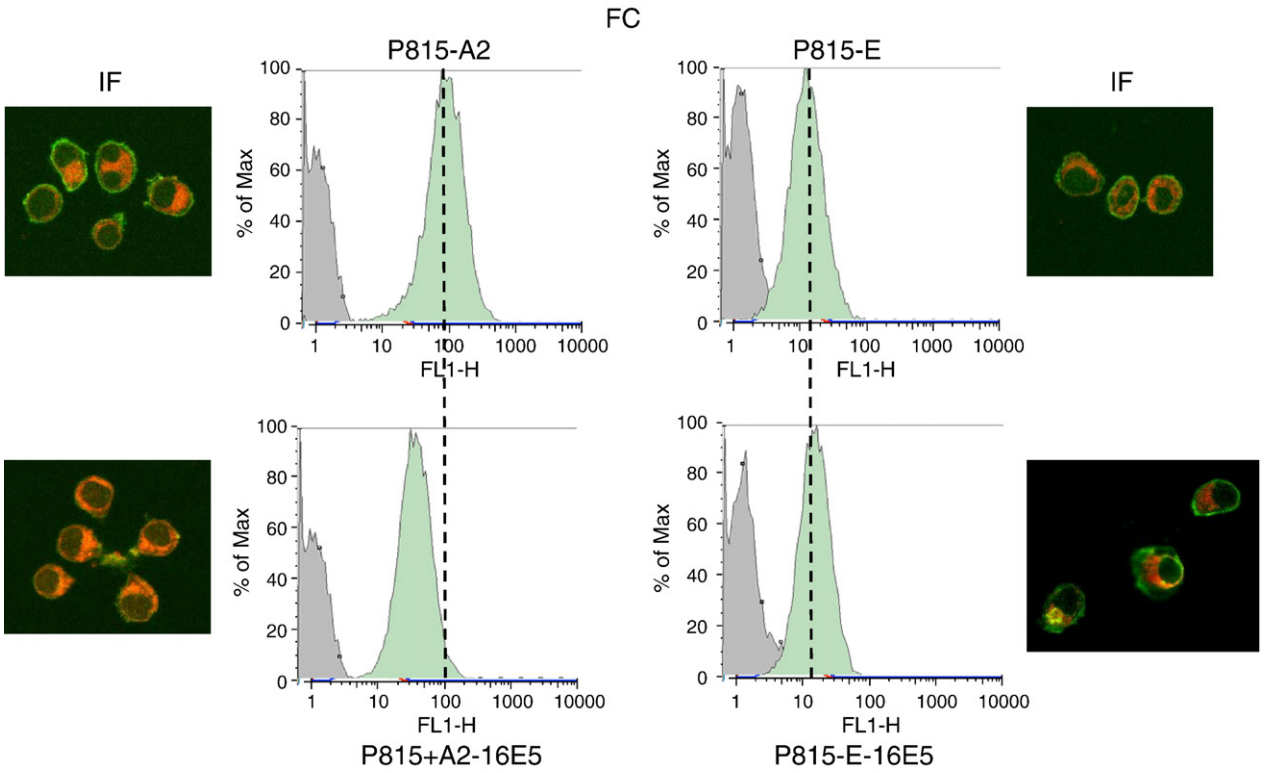

Fig. 3. HPV-16 E5 down-regulates HLA-A2 but not HLA-E in P815 cells. FC: flow cytometry for surface HLA-A and HLA-E with and without 16E5. 16E5 decreases the amount of surface HLA-A2 but not surface HLA-E. The dashed vertical line indicates the mean forward fluorescence. IF: immunofluorescence; the Golgi apparatus in P815 cells was stained red with BODIPY-TR-ceramide and HLA-A2 and HLA-E were stained green by FITC-conjugated secondary antibodies. HLA is detected on the cell surface in P815-A2, P815-E and P815-E-16E5 cells but not in P815-A2-16E5 cells. Note that the IF panel showing P815-E-16E5 cells is a composite of two fields, put together for ease of comparison with the other panels.

to those seen using peptide-loaded human HLA-A2 positive cells (data not shown). However a clear reduction in γIFN production by 7E7 was observed for the P815-A2-E5 cells at every dose of peptide tested (Fig. 4). This ranged from 50% to 70% of the maximum response (highest dose of peptide with P815-A2) to no response at the lowest dose of peptide in repeat experiments. The reduction in γIFN production correlated with the decrease in surface HLA-A2 expression in the P815-A2-E5 cells (Fig. 3).

We conclude that E5 expression has an impact on functional recognition by an HPV specific T cell clone, by reducing the number of HLA molecules available to present HPV peptide epitopes. In this respect E5 is similar to many other viral proteins which down-regulate HLA/MHC I, reduce antigen presentation and prevent recognition and killing by CTL (reviewed in Lilley and Ploegh, 2005). In humans, E5 could play an important role in the establishment and persistence of HPV infection by allowing the infected cells to escape host immunosurveillance. The persistent lesions arising from this immune evasion would be at greater risk of undergoing additional oncogenic events which would trigger malignant transformation. Attempts to treat malignant disease caused by oncogenic papillomaviruses through use of therapeutic vaccines have met with limited success. This may be explained in part by the powerful suppressive mechanisms exerted both locally and systemically in advanced cancer. However it is clear from animal models of papillomavirus infection that infiltrating T cells are involved in regression of papillomavirus lesions (Knowles et al., 1996; Nicholls et al., 2001; Selvakumar et al., 1997). Furthermore, a recent trial using a synthetic peptide vaccine in pre-malignant disease (VIN3) demonstrated clinical efficacy in 47% of vaccinated patients, with these clinical responses correlating with boosted T cell immunity (Kenter et al., 2009). This suggests that strong T cell responses against oncogenic HPVs would be beneficial in early disease, when the immune system has not been suppressed by cancer cells. Further research on E5 is warranted, particularly in developing methods to specifically block E5 expression. It may be possible to combine these with vaccination approaches to maximise the efficacy of any induced HPV specific T cell responses and thereby prevent the development of malignant disease.

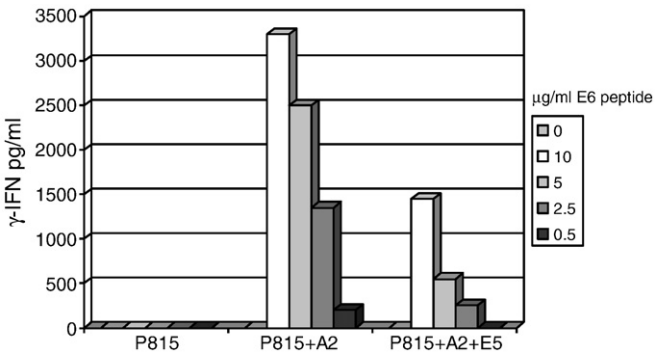

Fig. 4. HPV16 E5 reduces recognition by CD8+ T cells. Parental P815, P815-A2 or P815-A2-E5 cells were loaded with varying doses of HPV16 $E6_{29-38}$ peptide before co-culture with the HPV16 $E6_{29-38}$ specific T cell clone 7E7 for 20 hours. Supernatants from duplicate culture wells were assayed for γIFN by ELISA. Note the poor response of 7E7 T cells to P815-A2-E5 cells. Note too the lack of γIFN production by 7E7 in the absence of peptide, showing the specificity of the assay.

Materials and methods

Plasmids

pCi-16E5 has the HPV-16 E5 (16E5) ORF tagged with the HA epitope at its 5' end cloned in pCineo (Promega) in which E5 is expressed under the control of the immediate early (IE) promoter of

human cytomegalovirus (hCMV) (Ashrafi et al., 2006). The plasmid confers G418 resistance.

The HLA-A2 and HLA-E coding sequences were excised from pAL356HLA-A2 and pAL269HLA-E (Tomasec et al., 2000; Youde et al., 2005) and cloned into pcDNA6 under the transcriptional control of hCMV IE promoter to generate pcDNA6/HLA-A2 and pcDNA6/HLA-E. Both plasmids confer blasticidin resistance.

Antibodies

Monoclonal antibody (mAb) W6/32 (Serotec) is raised against HLA-A, B and C; mAb DT9 (kind gift of Dr V. Braud, Sophia Antipolis, France) is raised against HLA-E. mAb HC10 (Cancer Research UK) is raised against HLA-class I heavy chain (HC) and mAb MEM-E/02 (Abcam) against HLA-E HC. The mAb Ab-1 (Calbiochem) is raised against mouse actin.

Cell lines

W12

W12 is a line of human cervical keratinocytes explanted from a CIN I lesion (Stanley et al., 2007). W12E is an early passage clone (p17) that harbours episomal copies of HPV-16, W12G is a late passage clone (p25) with integrated HPV-16 genomes (Jeon et al., 1995). W12E and G cells were co-cultured with 4 μg/ml mitomycin C-treated J2 3T3 fibroblasts at a ratio of 1:5 where 2×10^5 W12 cells were added per 10 cm culture dish. Cells were grown for 10 days in DMEM, 10% foetal calf serum, 2 mM glutamine, cholera toxin (8.4 ng/ml), hydrocortisone (0.4 ng/ml), and epidermal growth factor (5 ng/ml) (McPhillips et al., 2004).

P815

P815 is a line of mouse mastocytoma cells (ATCC). P815 cells were grown in suspension in RPMI medium supplemented with 10% FCS (Invitrogen) and penicillin/streptomycin. Cells were maintained at 37 °C 5% CO_2. Stably transfected cells were maintained as parental cells with the addition of selective antibiotics where appropriate: G418 (Invitrogen) at 500 μg/ml in case of E5 expression plasmids, and/or blasticidin (InvivoGen) at 10 μg/ml in case of HLA-A2 and HLA-E.

CTL clone 7E7

The CTL clone 7E7 was isolated from a cervical cancer patient; it recognises a peptide epitope from HPV16 $E6_{29-38}$ (TIHDIILECV) presented by HLA-A2 (Evans et al., 2001). The cells were grown as previously described (Evans et al., 2001). Briefly, 1×10^6 7E7 CTL were cultured in tissue culture flasks containing 20×10^6 irradiated allogeneic peripheral blood mononuclear cells, rIL2 (20 IU/ml), phytohaemaggultin (Murex, 1 μg/ml) in RPMI1640 media with 10% human AB serum. After 7 days, the CTL were transferred to 24 well plates, and culture continued in fresh media supplemented with 100 IU/ml rIL2 for up to 14 days.

Organotypic raft cultures

W12E and W12G cells were grown in organotypic raft cultures basically as described before (Aasen et al., 2003). Briefly, 2.5×10^5 cells were seeded on a contracted lattice of collagen type I containing primary human epidermal fibroblasts (3×10^5 cells per ml collagen) in 3:1 DMEM: Ham's F12 medium containing 10% FCS, 0.1 mg/ml L-glutamine, 0.4 ng/ml hydrocortisone, 10 ng/ml cholera toxin, 5 μg/ml insulin, 180 nM adenine, 5 μg/ml transferrin and 5 ng/ml EGF. The lattice was lifted to the air–liquid interface after 3 days and fixed in 4% (v/v) formaldehyde after 12–14 days of stratification. Sections were paraffin embedded and processed for routine H&E.

Transfections

One $x10^6$ P815 cells were transfected using an Amaxa nucleofector (Amaxa GmbH) according to the manufacturer's instructions. Cells were allowed to grow for 48 hrs before the addition of selective media. Selection was carried out for approximately three weeks. Several independent transfections were carried out and polyclonal lines derived from them.

RT-PCR

RNA was prepared from W12 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) and PCR-amplified using E5 forward primer 5'-ATGACAAATCTTGATACTGCA-3' and E5 reverse primer 5'-AATGATGTGTATGTAGACACAG-3' and GAPDH forward primer 5'-TCCACCACCCTGTTGCTGTA-3' and GAPDH reverse primer 5'-ACCACAGTCCATGCCATCAC-3' with Taq DNA polymerase (Invitrogen). RT-PCR reactions were fractionated on acrylamide gels.

Quantitative RT-PCR

The expression of E5 in P815 cells was analysed by quantitative RT-PCR (qRT-PCR) using primers/probes for 16E5 and for mouse actin as described previously (Araibi et al., 2006; Ashrafi et al., 2005).

Western blotting

E5 expression in P815 cells was also analysed by western blotting using mAb HA11 (Sigma) against the HA epitope. The expression of HLA-A2 and HLA-E was analysed using mAb HC10 and MEM-E/02 respectively.

Flow cytometry

Flow cytometry analysis of P815 cells was basically carried out as described in (Araibi et al., 2006), with the difference that mAb W6/32 (1:100) was used for the detection of HLA-A2 and mAb DT9 (1:10) for the detection of HLA-E. After the addition of anti-mouse FITC-conjugated IgG (1:100; Sigma) at 4 °C for 30 min in the dark, the cells were washed and examined in a Beckman Coulter EPICS Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

Immunofluorescence

Immunofluorescence analysis of P815 cells was carried out as described in (Araibi et al., 2006). Briefly, the Golgi apparatus was visualised with 1:1000 dilution of BODIPY Ceramide Texas Red (TR) (in 2.5% HEPES in serum-free medium) for 30 min at 4 °C. After removal of BODIPY Ceramide, cells were incubated in 2.5% HEPES in serum-free RPMI 1640 medium for 30 min at 37 °C and then incubated with W6/32 for HLA-A2 or DT9 for HLA-E. After the addition of anti-mouse FITC-conjugated IgG secondary antibody, cells were washed three times and the coverslips were mounted onto slides using Citifluor. Images were captured using a Leica TCS SP2 true confocal scanner microscope (Leica Microsystems, Heidelberg, Germany) at a wavelength of 488 nm (MHC class I: FITC) or 543 nm (Golgi apparatus: Texas Red). The merge between the fluorescent signals was achieved using the accompanying software.

Immunohistochemistry

This procedure was performed by the Pathological Services of the Institute of Comparative Medicine. Briefly, sections were de-paraffinised, heated in a pressure cooker for antigen retrieval for HLA detection. Endogenous peroxidase was blocked using peroxidase block (Envision Kit DakoCytomation) and then the sections were washed first with Tris/Tween then twice with water. mAb HC10 (1:2000) was added and after washing with Tris/Tween the secondary antibody conjugated with horseradish peroxidase was applied; dual anti-mouse/rabbit (Dako) was used for all antibodies. Sections were washed as above and DAB was added (Envision Kit, DakoCytomation). Then sections were counter-stained with haematoxylin, de-hydrated and mounted. The stained sections were analysed using a Zeiss Axioskop 2 microscope and images were digitally captured with the accompanying camera and

software KS300 version 3. Absence of primary antibodies resulted in no staining in all cases (not shown).

γIFN release assays with P815 cells

P815 parental cells, P815-A2 and P815-A2-E5 cells were resuspended in 1 m aliquots (10^6/ml) of serum free RPMI1640 media in the presence of varying doses of $E6_{29-38}$ peptide (Severn Biotech). After 2 hours incubation at 37°C, the cells were washed three times in serum-free RMPI1640 before resuspending in tissue culture media (RPMI1640 + 10% human AB serum). The peptide loaded P815 parental, P815-A2 and P815-A2-E5 transfectant cells (5×10^4 cells/well) were co-incubated with 7E7 T cells (10^5 cells/well) in 48 well plates for 18–20 h. γIFN release by 7E7 T cells was measured using an γIFN ELISA kit (Mabtech, Nacka Strand, Sweden) as previously described (Thomas et al., 2008).

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