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Down-regulation of MHC class I in bovine papillomas

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Summary

Bovine papillomavirus (BPV) induces papillomas, which in the great majority of cases regress due to the host immune response, but can persist and progress to malignancy. Even in the absence of malignant transformation, BPV infection persists for a significant period of time before activation of the host immune system, suggesting that the host immune system is unaware of, or disabled by, BPV. E5 is the major oncoprotein of BPV, which, in addition to its transforming properties, down-regulates the expression and the transport to the cell surface of major histocompatibility complex class I (MHC I). Here we show that co-expression of MHC I and E5 in papillomas caused by BPV-4 infection is mutually exclusive, in agreement with the inhibition of surface MHC I expression by E5 observed *in vitro*. The inhibition of MHC expression in E5-expressing papilloma cells could explain the long period required for the activation of the immune response and has implications for the progression of papillomas to the malignant stage: absence of peptide presentation by MHC I to cytotoxic T lymphocytes (CTL) would allow the infected cells to evade the host cellular immune response and the lesions to persist.

Text

Papillomaviruses are oncogenic viruses which induce benign proliferative lesions of epithelia, called papillomas or warts. Papillomavirus infections are usually eliminated by a cell-mediated immune response directed against viral antigens (O'Brien & Campo, 2002). However, in a minority of cases mucosal lesions do not regress and can progress to cancer. High risk human papillomavirus types 16 and 18 (HPV-16 and -18) are the main causative factor in the development of cancer of the cervix uteri (zur Hausen, 2002). Bovine papillomavirus types 1 and 2 (BPV-1 and -2) and BPV-4 are involved in carcinogenesis of the urinary bladder and the alimentary tract in cattle, respectively; BPV-1 is also involved

in cancer of the penis (Campo, 1997, Campo, 2002). In all cases, cancer is preceded by, and derives from, pre-existing papillomas.

Papillomas induced by BPV develop through four well defined stages, from stage 1 or plaque, the first clinical manifestation of infection, to stage 4 when the papilloma starts regressing (Jarrett, 1985). From infection to regression, papilloma development takes often more than twelve months. In the case of BPV-4-induced papillomas, regression is accompanied by infiltration of immune cells, primarily CD4⁺ T lymphocytes in the adjacent dermis, as well as CD8⁺ T lymphocytes infiltrating the keratinocytes (Knowles et al., 1996). Persistence, spread and progression of papillomas to cancer occurs mainly in animals grazing on bracken fern (*Pteridium* spp) (Campo et al., 1994). These animals are immunocompromised by immunosuppressants, such as sesquiterpenes, present in the plant. However, papillomas can also spread and persist in cattle not exposed to bracken fern (Tsirimonaki et al., 2003). Even in the absence of malignant transformation, BPV infection can persist for a significant period of time before activation of the host immune system. Lymphocytes from infected animals do not recognise early or late viral antigens until late in infection, despite the presence of numerous papillomas actively producing virus (Chandrachud et al., 1994, Chandrachud et al., 1995, Kirnbauer et al., 1996, McGarvie et al., 1995). This lack of recognition suggests that the host immune system is unaware of, or disabled by, BPV infection. It is now known that papillomaviruses can subvert the immune response indirectly, since viral replication is confined to the epithelial cells above the basal membrane and therefore in a site recognised poorly by immune cells (Frazer et al., 1999). In addition, papillomaviruses appear to interfere directly with host anti-viral immune mechanisms, including the interferon response and major histocompatibility complex class I (MHC I) antigen presentation to cytotoxic T lymphocytes (CTLs) (O'Brien & Campo, 2002, Tindle, 2002).

We have recently shown that expression of the E5 oncoprotein of BPV-1, BPV-4 and HPV-16 has a profound effect on the synthesis and transport of MHC I in cultured cells and therefore can potentially contribute to the ability of the virus to evade immune-recognition (Ashrafi et al., 2004, Ashrafi et al., 2002, Marchetti et al., 2002, O'Brien & Campo, 2002). To ascertain whether the down-regulation of MHC I observed in cultured cells takes place in tumours, we investigated the expression of BPV-4 E5 and MHC I in clinical samples of BPV-4 papillomas.

Papillomas from the palate, rumen and oesophagus, as well as samples of normal palate, tongue and buccal mucosa, were collected *post mortem* from animals referred to the University of Glasgow Veterinary School. Tissue samples were fixed and stored in 10% formaldehyde in phosphate-buffered saline (PBS) at pH 7.5 and then embedded in paraffin wax for histological processing. Serial sections (1.5 µm) were cut and placed on microscopic slides treated with VECTABOND (Vector, USA). After de-paraffinisation in Histo-clear (National Diagnostics, USA), the sections were re-hydrated in graded ethanol and incubated in 0.5% H₂O₂/methanol for 20 minutes to quench endogenous peroxidase. Sections were subjected to antigen retrieval treatment with 0.01M sodium citrate buffer (pH 6) in a pressure cooker for 75 seconds at 103.4 kPa, blocked with 1% normal unlabelled swine serum (Scottish Antibody Production Unit, UK) in TBS containing 0.1% Tween 20 for 30 minutes at room temperature and then incubated for 1.5 hours at room temperature with primary antibodies for detection of E5, E7, MHC I heavy chain and the proliferation marker Ki67 as detailed below. The sections were then incubated with biotin-labelled secondary antibody (Dako, UK) and streptavidin-biotin complex (Dako) for 45 minutes as per the manufacturer's instructions. Immunoreactivity was visualised with 3,3 diaminobenzidine (DAB, Sigma, UK). Sections were counterstained with Gills haematoxylin, dehydrated, cleared in Histo-clear and then permanently mounted with DPX

mountant and cover-slipped prior to microscopic examination. A total of seven papillomas were analysed, with at least three sections and three different section fields examined per papilloma; all papillomas were classified as stage 2/3, i.e. mature papillomas producing virus, and presented the typical features of BPV-4 infection (Jarrett, 1985), including an irregular basal layer (Figure 1A, B), fronds of transformed cells terminating in keratinised tips (Figure 2D), and koilocytes, cells with highly enlarged cytoplasm typical of papillomavirus infection (Figure 2D, black arrow). In contrast, the normal bovine alimentary mucosal epithelium had a typical architecture composed of a regular basal cell layer, suprabasal, spinous, and squamous layers (Figure 1D).

BPV-4 E5 was detected with each of two rabbit antisera, 274 and 275, raised against a synthetic peptide representing the 12 C-terminal amino acids of the protein, conjugated to keyhole limpet hemocyanin (KLH) (Anderson et al., 1997). Similar results were obtained with both anti-E5 antisera; only results obtained with antiserum 274 are shown. In agreement with previous results of ourselves and others (Burnett et al., 1992; Anderson et al., 1997), E5 was detected exclusively in the cytoplasm of epidermal cells, from the basal and parabasal layers to the spinous and squamous layers (Figures 1A,B, 2D). Expression was, however, discontinuous, as already reported (Anderson et al., 1997); no papilloma was stained in all the cell layers. To verify the specificity of the immunostaining of E5, several controls were carried out. There was no staining with pre-immune serum (data not shown) or when only the secondary antibody was used (Figure 1C) and the E5 antiserum did not react with normal mucosa (Figure 1D). Further, the E5 antiserum was pre-absorbed with the antigen peptide (3 µg peptide/ml antiserum). Pre-absorption eliminated reactivity both in immunoprecipitation of *in vitro* translated E5 labelled with S³⁵ methionine (Figure 1E), and immunostaining experiments (Figure 1F). E5 expression in the differentiated layers of the papillomas was often accompanied by expression of the proliferation antigen

Ki67 (Figure 3G), detected by monoclonal antibody MIB-1 (Dako). Expression of Ki67 confirmed the transformed nature of these cells: in normal epithelia cells cease to proliferate once they leave the basal layer.

To analyse expression of MHC I in papillomas we incubated sample sections with mAb IL-A88, which detects the heavy chain of bovine MHC I (Toye et al., 1990). In normal epithelium, there was strong staining of the capillaries and of stromal cells; the epithelium was stained throughout most of its thickness (Figure 2A) and staining on the surface of the cells was particularly clear in cells of the basal and suprabasal layers (Figure 2B). There was no staining without primary antibody (data not shown). In sections of papillomas, MHC I could be detected in stroma and capillaries (Figure 3E), and on the surface of some epithelial cells, as in normal tissue, but not in cells expressing E5 (Figures 2D,E; 3A,B), independently of whether the cells were in the deeper layers (Figure 3A,B) the suprabasal transit and lower spinous layers (Figure 2D,E, boxed area 1), or the more superficial layers (Figure 2D,E; 3D,E). These results suggest that expression of E5 is incompatible with the expression of MHC I.

The E7 protein of HPV-16 and HPV-11 has been implicated, respectively, in the down-regulation of MHC I, either through inhibition of the transcriptional promoter of the MHC I heavy chain (Georgopoulos et al., 2000), or indirectly through inhibition of TAP, the transporter associated with peptide (Vambutas et al., 2001). To ensure that the absence of MHC I in bovine papillomas was due to E5 and not to E7, we stained papilloma sections with rabbit antisera 11547 and 11823 raised against a β -gal-E7 fusion protein (Anderson et al., 1997). Similar results were obtained with both antisera; only results with antiserum 11547 are shown. E5 and E7 are co-expressed in the same cells (Anderson et al., 1997), and, accordingly, in this study cells that expressed either E5 or E7 alone were seldom detected. Nevertheless, in cells that expressed E5 and did not express E7, or expressed it at

levels below detection, there was little or no MHC I (Figure 3A-C). Conversely, cells that expressed E7, but not E5, still had detectable MHC I (Figure 3D-F, H-J). Thus, it appears that expression of E7 is not responsible for down-regulation of MHC I.

Although the lack of MHC I in the uppermost layers of the papillomas (Figures 2E, 3E) is consistent with the differentiated state of the cells, its absence in the basal (Figure 3B) and in the immediate suprabasal (transit and lower spinous) layers (Figure 2E, boxed area 1) cannot be attributed to cell differentiation, as MHC I is present in these areas of normal mucosa (Figure 2A). Furthermore, MHC I is absent in similar areas of papillomas where E5 is not expressed (Figure 2D,E, boxed area 2).

We conclude that E5 inhibits the expression of MHC I in BPV-induced papillomas, corroborating and validating our observations on down-regulation of MHC I by E5 *in vitro*. HPV-16 and other high risk HPV types induce cervical intraepithelial neoplasia (CIN), the precursor lesion of cervical cancer (zur Hausen, 2002). MHC I down-regulation has been observed in CIN, but E5 expression was not investigated (Bontkes et al., 1998). Given that HPV-16 E5 down-regulates MHC I (Ashrafi et al., 2004), and that HPV-16 E5 can be found in CIN samples (Chang et al., 2001), it can be speculated that E5 is responsible for MHC I down-regulation also in CIN.

It remains to be seen whether the E5-induced down-regulation of MHC I leads to evasion of the host immune response, thus allowing the virus to establish the infection, and infection to persist.

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Figure Legends

Figure 1. Expression of E5 in BPV-4-induced papillomas. Representative papilloma sections stained with anti-E5 antiserum 274 (1:2000). A, papilloma 1803, showing E5 expression in basal and suprabasal layers. B, papilloma 386, showing E5 expression in suprabasal layers. C, serial section of papilloma 386 incubated with secondary antibody only; D, normal buccal mucosa, showing no expression of E5. E, Autoradiograph of in vitro labelled S³⁵-E5: lane 1, input E5 protein; lane 2, E5 immunoprecipitated with antiserum 274; lane 3, E5 immunoprecipitated with antiserum 274 pre-absorbed with E5 C-terminus peptide. F, serial section of papilloma 386 incubated with pre-absorbed antiserum, showing loss of reactivity. Magnification: x40 (A), x20 (B,C,F) and x10 (D).

Figure 2. Expression of MHC I in BPV-4-induced papillomas. Representative papilloma sections stained with anti-MHC I heavy chain mAb IL-A19 (1:200). A, section of normal buccal mucosa showing MHC I expression through most of the thickness of the epithelium. B, same as in A, at higher magnification, showing staining of MHC I on the cell surface. C, serial section of papilloma 386, stained with mAb IL-A19, showing lack of reactivity. D, section of papilloma 1804 stained with antiserum 274, showing E5 expression in suprabasal transit and lower spinous layers (boxed area 1) and in differentiated keratinocytes (black arrow). Boxed area 2 shows transit and lower spinous layers without E5 expression. E, serial section of papilloma 1804, stained with mAb IL-A19, showing MHC I expression in cells lacking E5 (boxed area 2), and lack of MHC I expression in cells expressing E5 (boxed area 1). Magnification: x10 (A,D,E) and x20 (B,C).

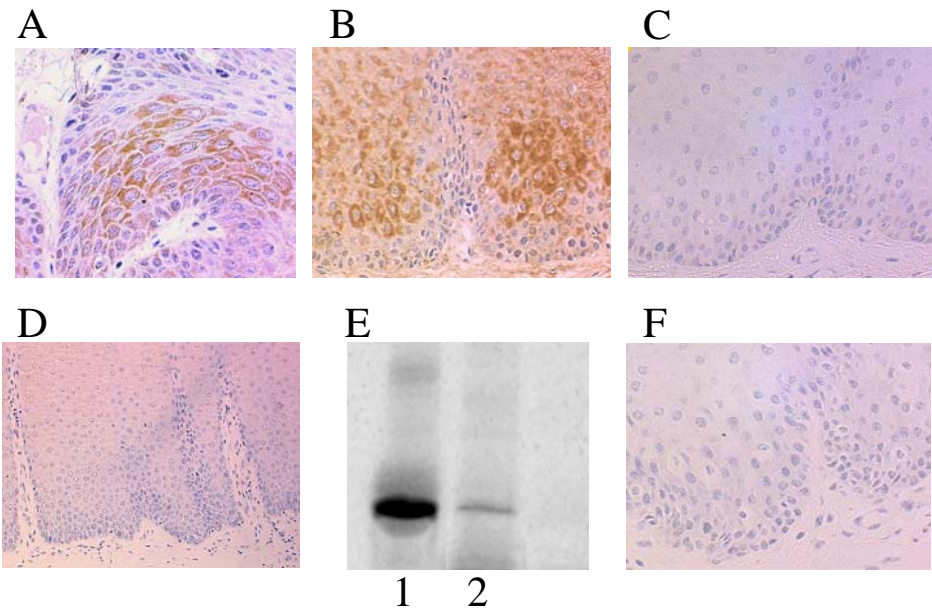
Figure 3. Expression of E5, E7, MHC I and Ki67 in BPV-4-induced papillomas. Representative papilloma sections stained with anti-E5 antiserum 274 (1:2000; panels A,D and H); with anti-bovine MHC I heavy chain monoclonal antibody IL-A88 (1:200; panels B,E and I); anti-E7 antiserum 11547 (1:250; panels C,F and J), and with anti-Ki67 monoclonal antibody MIB-1 (1:200; panel G). A, papilloma 1803, showing expression of E5 in the basal layers. B, serial section of papilloma 1803, showing lack of expression of MHC I in cells expressing E5. C, serial section of papilloma 1803, showing lack of expression of E7 in the basal layers. D, papilloma 1810, showing E5 expression in the differentiated keratinocytes but not in the basal cells. E, serial section of papilloma 1810, showing expression of MHC I in basal cells. F, serial section of papilloma 1810, showing expression of E7 in basal cells and co-expression with E5 in differentiated keratinocytes. The boxed area in panel D and the corresponding areas in panels E and F are shown at higher magnification in panels H-J respectively. G, serial section of papilloma 1810, showing expression of Ki67 in basal cells and in E5-expressing differentiated keratinocytes. Magnification: x40 (A-C, H-J) and x20 (D-G).

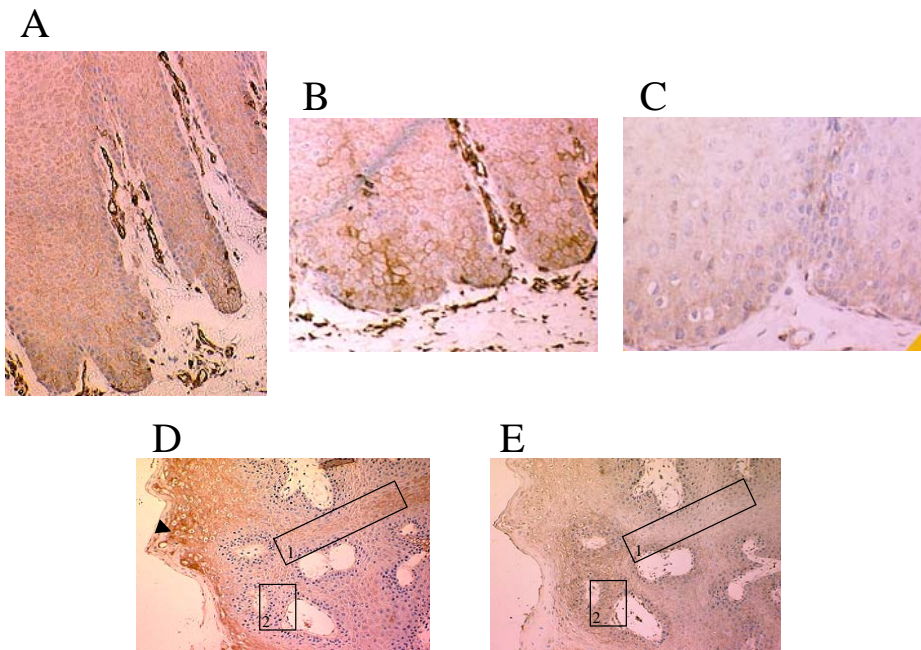
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Araibi et al, Figure 1





Araibi et al, Figure 3

