Downregulation of major histocompatibility complex class I in bovine papillomas

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Bovine papillomavirus (BPV) induces papillomas in cattle; in the great majority of cases, these regress due to the host immune response, but they 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, downregulates the expression and transport to the cell surface of major histocompatibility complex class I (MHC I). Here, it is shown 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 that is observed in vitro. The inhibition of MHC expression in E5-expressing papilloma cells could explain the long period that is required for 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 would allow the infected cells to evade the host cellular immune response and allow the lesions to persist.

Papillomaviruses are oncogenic viruses that induce benign proliferative lesions of epithelia, called papillomas or warts. Papillomavirus infections are usually eliminated by a cell-mediated immune response, which is 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 factors in the development of cancer of the cervix uteri (zur Hausen, 1994). These have also been identified as factors in the development of cancer of the penis (Campo, 1997, 2002). Bovine papillomavirus type 1 and 2 (BPV-1 and -2) and BPV-4 are respectively involved in carcinogenesis of the urinary bladder and the alimentary tract in cattle; BPV-1 is also involved in cancer of the penis (Campo, 1997, 2002). In all cases, cancer is preceded by, and derives from, pre-existing papillomas.

Papillomas that are 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 to regress (Jarrett, 1985). From infection to regression, papilloma development often takes longer than 12 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 occur mainly in animals that graze on bracken fern (Pteridium spp.) (Campo et al., 1994). These animals are immunocompromised by immunosuppressants, such as sesquiterpenes, that are present in the plant. However, papillomas can also spread and persist in cattle that are 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 recognize early or late viral antigens until late in infection, despite the presence of numerous papillomas actively producing virus (Chandrachud et al., 1994, 1995; McGarvie et al., 1995; Kirnbauer et al., 1996). 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, as virus replication is confined to the epithelial cells above the basal membrane and therefore occurs in a site that is recognized poorly by immune cells (Frazer et al., 1999). In addition, papillomaviruses appear to interfere directly with host antiviral immune mechanisms, including the interferon response and major histocompatibility complex class I (MHC I) antigen presentation to cytotoxic T lymphocytes (O’Brien & Campo, 2002; Tindle, 2002).

We have shown recently 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., 2002, 2004; Marchetti et al., 2002; O’Brien & Campo, 2002). To ascertain whether the downregulation of MHC I that is 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 that were referred to the University of Glasgow Veterinary School. Tissue samples were fixed and stored in 10 % formaldehyde in PBS at pH 7·5 and embedded in paraffin wax for histological processing. Serial sections (1·5 μm) were cut and placed on microscopic slides that had been treated with VECTABOND (Vector). After deparaffinization in Histo-clear (National Diagnostics), sections were rehydrated in graded ethanol and incubated in 0·5 % H₂O₂/methanol for 20 min to quench endogenous peroxidase. Sections were subjected to antigen-retrieval treatment with 0·01 M sodium citrate buffer (pH 6) in a pressure cooker for 75 s at 103·4 kPa, blocked with 1 % normal unlabelled swine serum (Scottish Antibody Production Unit) in TBS containing 0·1 % Tween 20 for 30 min at room temperature and then incubated for 1·5 h 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) and streptavidin–biotin complex (Dako) for 45 min, following the manufacturer’s instructions. Immunoreactivity was visualized with diaminobenzene (Sigma). Sections were counterstained with Gill’s haematoxylin, dehydrated, cleared in Histo-clear and mounted permanently with DPX mountant under a coverslip prior to microscopic examination. A total of seven papillomas was 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 (Fig. 1a and b), fronds of transformed cells terminating in keratinized tips (Fig. 2d) and koilocytes, cells with highly enlarged cytoplasm that are typical of papillomavirus infection (Fig. 2d, black arrow). In contrast, the normal bovine alimentary mucosal epithelium had a typical architecture, composed of a regular basal cell layer and supra-basal, spinous and squamous layers (Fig. 1d).

BPV-4 E5 was detected with each of two rabbit antisera, 274 and 275, that were raised against a synthetic peptide representing the 12 C-terminal amino acids of the protein, conjugated to keyhole limpet haemocyanin (Anderson et al., 1997). Similar results were obtained with both anti-E5

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**Fig. 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 [³⁵S]E5. Lane 1, input E5 protein; lane 2, E5 immunoprecipitated with antiserum 274; lane 3, E5 immunoprecipitated with antiserum 274 pre-absorbed with an E5 C-terminal peptide. (f) Serial section of papilloma 386 incubated with pre-absorbed antiserum, showing loss of reactivity. Magnification, × 40 (a); × 20 (b, c and f); × 10 (d).
antisera; only results that were obtained with antiserum 274 are shown. In agreement with previous results by us 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 (Figs 1a and b, and 2d). Expression was, however, discontinuous, as reported previously (Anderson et al., 1997); no papilloma was stained in all 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 secondary antibody was used (Fig. 1c), and the E5 antiserum did not react with normal mucosa (Fig. 1d). Furthermore, when the E5 antiserum was pre-absorbed with the antigen peptide by using 3 μg peptide (ml antiserum)$^{-1}$, pre-absorption eliminated reactivity, both in immunoprecipitation of in vitro-translated E5 labelled with $[S^{35}]$methionine (Fig. 1e) and in immunostaining experiments (Fig. 1f). E5 expression in the differentiated layers of the papillomas was often accompanied by expression of the proliferation antigen Ki67 (Fig. 3g), detected by mAb 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 stromal cells; the epithelium was stained throughout most of its thickness (Fig. 2a) and staining on the surface of the cells was particularly clear in cells of the basal and suprabasal layers (Fig. 2b). There was no staining without primary antibody (data not shown). In sections of papillomas, MHC I could be detected in stroma and capillaries (Fig. 3e) and on the surface of some epithelial cells, as in normal tissue, but not in cells expressing E5 (Figs 2d and e, and 3a and b), independent of whether the cells were in the deeper layers (Fig. 3a and b), the suprabasal, transit and lower spinous layers (Fig. 2d and e, boxed area 1) or the more superficial layers (Figs 2d and e, and 3d and e). These results suggested that expression of E5 is incompatible with expression of MHC I.

The E7 proteins of HPV-16 and -11 have been implicated, respectively, in the downregulation 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, which were raised against a β-galactosidase–E7 fusion protein (Anderson et al., 1997). Similar results were obtained with both antisera; only results that were obtained with antiserum 11547 are shown. E5 and E7 are co-expressed in the same cells (Anderson et al., 1997) and, accordingly, in
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 (Fig. 3a–c). Conversely, cells that expressed E7, but not E5, still had detectable MHC I (Fig. 3d–f and h–j). Thus, it appears that expression of E7 is not responsible for downregulation of MHC I.

Although the lack of MHC I in the uppermost layers of the papillomas (Figs 2e and 3e) was consistent with the differentiated state of the cells, its absence in the basal (Fig. 3b) and the immediate suprabasal (transit and lower spinous) (Fig. 2e, boxed area 1) layers could not be attributed to cell differentiation, as MHC I was present in these areas of normal mucosa (Fig. 2a). Furthermore, MHC...
Fig. 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; a, d and h), anti-bovine MHC I heavy chain mAb IL-A88 (1 : 200; b, e and i) anti-E7 antiserum 11547 (1 : 250; c, f and j) or with anti-Ki67 mAb MIB-1 (1 : 200; 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 differentiated keratinocytes, but not in 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 (d) and the corresponding areas in (e) and (f) are shown at higher magnification in (h–j), respectively. (g) Serial section of papilloma 1810, showing expression of Ki67 in basal cells and in E5-expressing differentiated keratinocytes. Magnification, × 40 (a–c, h–j); × 20 (d–g).

I was absent in similar areas of papillomas where E5 was not expressed (Fig. 2d and e, boxed area 2).

We conclude that E5 inhibits the expression of MHC I in BPV-induced papillomas, corroborating and validating our observations on downregulation 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 downregulation has been observed in CIN, but E5 expression was not investigated (Bontkes et al., 1998). Given that HPV-16 E5 downregulates 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 also responsible for MHC I downregulation in CIN.

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

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