Presence of bovine papillomavirus type 2 DNA and expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows

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Samples of neoplastic and normal urothelium were obtained from cows originating from areas of southern Italy, a region in which chronic enzootic haematuria is endemic and bracken fern infestation is widespread. Specimens were analysed for bovine papillomavirus type 2 (BPV-2) DNA, BPV-2 E5 expression and telomerase activity. A total of 46 of 60 tumours and 17 of 34 normal bladder mucosa samples harboured BPV-2 DNA. Analysis of a subset of samples showed E5 protein expression and telomerase activity in tumour tissue only. No normal samples positive for BPV DNA showed E5 protein expression or telomerase activity, suggesting the presence of DNA in a latent state. Taken together, these data on naturally occurring bovine bladder tumours corroborate the hypothesis of their virus origin.

In cattle, tumours of the bladder are commonly associated with a syndrome known as chronic enzootic haematuria (CEH), which is caused as a result of prolonged ingestion of bracken fern (Confer & Panciera, 2001).

Bracken fern (genus Pteridium) is believed to be the only higher plant proven to cause cancer naturally in animals (Smith, 1997). The fern contains immunosuppressive, mutagenic, clastogenic and carcinogenic chemicals. Previous studies have pointed out a strong relationship between bovine papillomavirus (BPV) and bracken fern. BPV-2 DNA was found in 69 % of experimental bladder cancers and in 7 of 15 naturally occurring ones. This high degree of association between bladder cancers and BPV-2 suggested that this virus could play a role in bladder oncogenesis (Campo et al., 1992).

A possible aetiopathogenic mechanism is that BPV-1/2 infect the epithelium of the bladder and produce an abortive or latent infection, as already hypothesized for BPV-4 in gastrointestinal tumours (Borzacchiello et al., 2003; Campo et al., 1994). The immunosuppressants and chemical carcinogens in bracken act synergistically with the virus to induce the formation of pre-neoplastic lesions. During neoplastic progression, the ras gene is activated (Campos et al., 1990) and expression of the tumour suppressor fragile histidine tetrads locus is downregulated (Borzacchiello et al., 2001).

Most previous studies have been performed under experimental conditions and in few cases of naturally occurring bladder cancer; studies on large populations of cows are lacking.

In an attempt to validate the aetiopathogenic role of the association between BPV and bovine bladder cancers, our study was carried out on a large population of cattle from southern Italy, a region in which bracken fern is widespread and CEH is endemic. Samples were analysed for the presence of BPV DNA, E5 protein and telomerase activity. Telomerase is an enzyme that induces immortalization by preventing the progressive loss of telomere length during DNA replication (Bodnar et al., 1998).

Examination of bladders from 4- to 24-year-old cows was performed at public slaughterhouses in the south of Italy. Tumour samples from all animals affected and from a number of healthy cows were collected for both histological and molecular analysis.

PCR amplification of viral sequences using consensus primers was done according to methods described previously (Borzacchiello et al., 2001). These primers allow the amplification of a 307 bp DNA amplicon encompassing the BPV E5 sequence. A fragment corresponding to the expected size was amplified in 46 of 60 samples (77 %), from which DNA of PCR-quality was obtained. To determine the papillomavirus serotype, PCR amplicons were sequenced and proven to correspond to the expected region of BPV-2. An example of this analysis is shown in Fig. 1. Lower positivity (17 of 34) for BPV-2 sequences was detected in the
bladder mucosa of healthy cows as a control. The high incidence of BPV-2 DNA in all samples, both abnormal and normal mucosa, indicates clearly a high circulation of this virus in the areas examined. However, the difference between groups (pathologic versus normal samples) was statistically significant \( (P < 0.01, \text{Fisher's exact test and } \chi^2) \), strongly suggesting an association of BPV-2 with cellular abnormalities of the urinary bladder. There was no association between the presence of viral DNA and a particular histological type of tumour (data not shown), indicating that the different histological features may be related to other factors. Viral DNA was also detected in pre-neoplastic lesions, supporting its role in tumour development. The high incidence of BPV-2 DNA in normal bladder possibly reflects the presence of an abortive or latent infection that may be activated by the immunosuppressive and/or carcinogenic chemicals in bracken fern.

To verify this hypothesis, the expression of E5, the major oncogenic protein of BPV-1/2, was analysed in different urinary bladder samples. E5 is a small hydrophobic protein (44 aa) that is capable of inducing in vitro cell transformation through the activation of several kinases, from growth factor receptors to cdk cyclins (Venuti & Campo, 2002). E5 interacts physically with the cellular protein 16k ductin/subunit c, a component of the gap junction and of the V0 sector of vacuolar H\(^+\)-ATPase (Conrad et al., 1993; Faccini et al., 1996; Finbow et al., 1991; Goldstein et al., 1991), causing the downregulation of gap junction communication (Ashrafi et al., 2002; Faccini et al., 1996; Oelze et al., 1995) and the lack of acidification of endosomes and Golgi apparatus (GA) (Schapiro et al., 2000; Straight et al., 1995). However, its function in vivo is still unknown. Recent reports show E5 expression in both equine and bovine BPV-infected cancers (Bohl et al., 2001; Carr et al., 2001), suggesting a possible role of this viral protein in malignant transformation in vivo. A total of 36 BPV-positive (including pre-neoplastic lesions) and 10 BPV-negative tumours were analysed together with 15 BPV-positive and 10 BPV-negative normal mucosa samples. Briefly, paraffin sections were deparaffinized, rehydrated and heated in a microwave oven twice for 5 min each at 750 W to allow

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\caption{PCR amplification of urinary bladder samples. Lanes: M, molecular mass marker type VI (Roche Diagnostics); 1–7, tumour samples; 8, negative control with no DNA added; 9 and 10, normal mucosa samples; 11, positive control of BPV-2 plasmid. The arrow indicates the position of the 307 bp BPV-2 PCR product. The lower part of the figure shows the sequence similarity between the sequence of the amplicon in lane 2 (top line) and the sequence of prototype BPV-2 (GenBank NC_001521) (bottom line). Primer sequences are in bold.}
\end{figure}
antigen unmasking. Slides were then incubated with a 1:50 dilution of rabbit anti-E5 antiserum (kindly provided by R. Schlegel, Georgetown University, USA) and thereafter with FITC-conjugated secondary antibody (Chemicon). Immunofluorescence was analysed with a confocal laser scanning microscope (LSM 510, Zeiss). The pattern of immunostaining demonstrates clearly the presence of the E5 protein in BPV-positive tumours. The typical immunostaining pattern for the E5 protein is shown in Fig. 2. Almost all neoplastic cells from BPV DNA-positive tumour samples displayed cytoplasmic E5 immunoreactivity. In both pre-neoplastic and neoplastic lesions, a clear immunopositive signal for E5 was observed (Fig. 2a, b). E5 was located mostly within the cytoplasm of basal and suprabasal cells. In particular, a typical juxtanuclear immunoreactivity was also observed. No E5 immunostaining was detected in BPV-negative tumours, nor was it detected in normal urinary bladder mucosa positive for BPV DNA (Fig. 2c, d).

This is the first demonstration of E5 expression in naturally occurring BPV-associated urinary bladder tumours. The localization of E5 in the basal layer of the transformed urothelium is similar to that reported for papillomas during early stages of infection in the deep layers of the infected epithelium (Anderson et al., 1997; Burnett et al., 1992; Chang et al., 2001). It has been shown in vitro that, in agreement with its hydrophobic nature, E5 is localized in the endomembrane compartments of the endoplasmic reticulum and GA of the host cell (Burkhardt et al., 1989; Pennie et al., 1993). The cytoplasmic reactivity that is evident in pre-neoplastic and neoplastic samples is consistent with those findings, suggesting that in vivo membrane association is also likely. Moreover, some of the cells show accentuated staining in a perinuclear region, which could be the GA. It has been suggested that cancer cells may escape host immune control by E5-mediated downregulation of MHC class I molecules. This downregulation seems to be related to the interaction of E5 with 16k ductin/subunit c, which may lead to improper glycosylation and processing of MHC class I molecules (Marchetti et al., 2002). Since the cows from

**Fig. 2.** E5 expression in urinary bladder mucosa. (a) BPV-2-positive severe dysplasia. Most dysplastic cells express a strong cytoplasmatic E5 immunoreactivity (white arrows). (b) BPV-2-positive urothelial carcinoma grade I. Scattered neoplastic cells showing a juxtanuclear E5 staining are shown (white arrows). (c) BPV-2-negative urothelial infiltrating carcinoma grade II. No E5 immunoreactivity is seen. (d) BPV-2-positive normal urothelium. No E5 immunoreactivity is seen. Bar, 10 μm.
which we collected the samples had been grazing on bracken fern-infested pasture, the immunosuppression caused by the plant could enhance the escape of the E5-expressing tumour cells from the immune system.

The absence of E5 staining in tumours that lacked BPV-2 DNA ruled out artefactual results, such as cross-reactivity with cellular proteins.

Interestingly, no normal urinary bladder with BPV DNA sequences showed positivity for E5 protein. These data demonstrate that the viral DNA is transcriptionally active only in abnormal tissue and reinforce the hypothesis that BPV is capable of infecting the urinary mucosa and remaining latent in normal tissue until some factors trigger viral gene expression. It is not known how latent viral DNA is activated but the chemicals of bracken fern are strong candidates. The flavonoid quercetin is present in high concentrations in the fronds of the fern. Quercetin is mutagenic and clastogenic, leading to single-strand DNA breaks (Campo, 1997; Plaumann et al., 1996) and chromosomal rearrangements (Leal et al., 2003). It promotes the full in vitro transformation of primary bovine cells infected with BPV-4 DNA (Cairney & Campo, 1995; Pennie & Campo, 1992) and upregulates viral DNA transcription via a cis-acting element in the viral transcriptional promoter (Connolly et al., 1998). The consequent increased expression of the viral oncoproteins forces the cells to proliferate, despite quercetin-induced DNA damage (Beniston et al., 2001). These findings have led to the suggestion that quercetin is a co-carcinogen of BPV-4 in vivo. However, the actual role of quercetin in bracken-related carcinogenicity is still an open question (Shahin et al., 1999).

The hypothesis of a role of BPV in the pathogenesis of urinary bladder tumours seems also to be supported by the analysis of telomerase activity in the urothelial tumour samples. The increased activity of cellular telomerase, a ribonucleoprotein enzyme that synthesizes telomeric DNA, is well documented in various immortalized cell lines. Telomerase activity is also detected in various animal and human cancers, and telomerase activation is thought to be a crucial factor in immortalization of cells and tumourigenesis (Argyle & Nasir, 2003; Kim et al., 1994).

Recent reports indicate that papillomaviruses may also affect telomerase activity: the E6–E7 genes of human papillomavirus type 16 (HPV-16) increase telomerase activity during epithelial cell immortalization (Baege et al., 2002), whereas the E2 gene downregulates the human telomerase reverse transcriptase promoter (Lee et al., 2002).

Telomerase activity was analysed in bladder samples using a commercial telomerase detection kit (Trapeze, Intergen) that utilizes the telomeric repeat amplification, whose sequence is highly conserved through mammalian species (Moyzis et al., 1988). Briefly, specimens from a subset of tumours and controls, from which protein could be extracted, were lysed in CHAPS buffer, and after protein content normalization, were subjected to PCR amplification according to the manufacturer’s instructions. Analysis of each sample consisted of two assays; one with a protein extract and one with an extract that had been heat-treated at 80 °C. Heat treatment inactivates the telomerase and serves as a negative control. Aliquots of the reactions were subjected to electrophoresis on a non-denaturing 10 % polyacrylamide gel. After electrophoresis, the gel was stained with ethidium bromide. A representative assay of positive tumour samples is shown in Fig. 3.

Telomerase activity was detected in 4 of 12 BPV-2-associated urinary bladder cancer. This is the first report of the detection of telomerase activity in bovine cells. Moreover, this activity was detected only in BPV-positive samples and not in normal specimens (0 of 10) or BPV-negative tumours (0 of 7). Although the number of samples examined is rather limited, this result seems to indicate that telomerase activity is upregulated at least in some BPV-associated cancers. However, further investigations are needed.

The presence of viral DNA, of endocyttoplasmic E5 protein and the alteration of telomerase activity only in BPV-associated bladder tumours strongly supports that, in areas of CEH and of bracken fern infestation, more than 70 % of tumours are caused by BPV infection. The virus and chemicals in bracken fern appear to act synergistically in inducing cell transformation and immune escape.

These results open the perspective to start a vaccination programme to eradicate infection. Since E5 seems likely to play a role in urinary bladder carcinogenesis, it would...
represent a target for immunomodulated therapies. Recently, it has been reported that mouse vaccination with HPV-16 E5 reduced the growth of tumours induced by E5-expressing cells (Liu et al., 2000). The potential for E5 vaccination should be validated in naturally occurring animal models and, moreover, the development of therapeutic/preventive immunophylaxis for these mucosal tumours would represent a model for human pathologies and could add precious indications for their treatment.

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