J. Comp. Path. 2008, Vol. 139, 231–237

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Expression of Platelet-derived Growth Factor-β Receptor and Bovine Papillomavirus E5 and E7 Oncoproteins in Equine Sarcoid

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Summary

Equine sarcoids are benign fibroblastic skin tumours that are recognized throughout the world. Infection with bovine papillomavirus (BPV) types 1 and 2 has been implicated as a major factor in disease development; however, the cellular mechanisms underlying fibroblast transformation remain poorly defined. The present study further characterizes aspects of the association with BPV in 15 equine sarcoids. BPV DNA was demonstrated in 12/15 tumours collected from different areas of Italy. Nine of these 12 tumours expressed the BPV oncoproteins E5 and E7, but these oncoproteins were not expressed by normal equine cells. The BPV E5 protein is known to bind to the platelet-derived growth factor-β receptor (PDGF-βR) and this molecule was expressed by 11 of the 12 sarcoids in which E5 was demonstrated. These findings add further weight to the theory that BPV and the PDGF-βR may have a role in the pathogenesis of this disease.

Keywords: bovine papillomavirus; horse; platelet-derived growth factor-β receptor; sarcoid

Introduction

Sarcoids are benign tumours of fibroblastic origin affecting the skin of horses, mules and donkeys and are considered to be the most common equine cutaneous neoplasm. The tumours most frequently arise from the skin of the head, ventral abdomen, legs and the paragenital region (Ragland *et al*., 1970). Sarcoids are locally invasive and often occur at sites of previous injury or scarring.

Bovine papillomavirus (BPV) is considered to be the aetiological agent of this tumour (Borzacchiello, 2008). Both BPV-1 and -2 have been detected in sarcoids with BPV-1 being predominant (Lancaster *et al*., 1979; Amtmann *et al*., 1980; Chambers *et al*., 2003). Equine sarcoid is an important tumour since it provides the only known example of natural cross-species infection by a papillomavirus. Moreover, while BPV infection in cattle produces benign lesions that may regress, the behaviour of the same virus in the horse is distinctly different as sarcoids are non-permissive for virus production, locally aggressive and rarely show regression (Borzacchiello, 2007).

Candidate molecules involved in BPV oncogenesis have been characterized. The BPV-1 *E5* oncogene encodes a 44 amino acid protein that is considered to be the major BPV oncoprotein (Schiller *et al*., 1986; Schlegel *et al*., 1986). E5 is a type II transmembrane protein that is expressed in the deep layers of infected epithelia (Burnett *et al*., 1992; Anderson *et al*., 1997; Venuti and Campo, 2002) and is largely localized to the membranes of the endoplasmic reticulum (ER) and Golgi apparatus (GA) of these cells (Burkhardt *et al*., 1989; Pennie *et al*., 1993). BPV E5 is expressed in the cytoplasm of both basal and suprabasal transformed epithelial cells (Bohl *et al*., 2001; Araibi *et al*., 2004) with a typical juxtanuclear pattern due to its localization in the GA (Borzacchiello *et al*., 2003).

The mechanism underlying BPV carcinogenesis is thought to relate to the binding (both *in vitro* and

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0021-9975/$ - see front matter
doi:10.1016/j.jcpa.2008.07.006

in vivo) of the E5 oncoprotein to the platelet-derived growth factor-β receptor (PDGF-βR) (DiMaio *et al.*, 2000; DiMaio and Mattoon, 2001; Borzacchiello *et al.*, 2006). This mechanism has been largely investigated *in vitro* and in naturally occurring bovine bladder cancer. However, in the latter situation, synergism between the virus and carcinogenic principles from bracken fern is required for full transformation (Campo *et al.*, 1992; Borzacchiello *et al.*, 2003).

The BPV-1 *E7* gene encodes a 127 amino acid zinc-binding protein that co-operates with E5 and E6 in inducing cell transformation. In natural infection, BPV-1 E7 expression occurs in the cytoplasm and nucleoli of cells of the basal and lower spinous layers of squamous epithelium. Once E7 is co-expressed with E5 and E6, the transformation capacity of the virus increases many fold (Bohl *et al.*, 2001). Such co-expression may also occur in bovine tumours of mesenchymal origin (Borzacchiello *et al.*, 2007). Mutants lacking the *E7* open reading frame are still able to induce transformation but at a lower efficiency, and produce transformants with altered characteristics (Sarver *et al.*, 1984).

The mechanisms described above have been largely investigated in bovine tumours and there are no studies published to date concerning the molecular mechanisms underlying BPV transformation in equine sarcoids. The aim of the present study was therefore to determine whether the mechanisms described in bovine carcinogenesis might also occur in equine sarcoid by examining the expression of key oncogenic molecules in the equine tumour.

Materials and Methods

Tumour Samples

Examples of equine sarcoid ($n = 15$) were derived from the archives of the Department of Pathology and Animal Health, Naples University, and the Department of Comparative Biomedical Sciences, University of Teramo. Formalin-fixed tissue embedded in paraffin wax was available from each case. Sections taken from these blocks and stained by haematoxylin and eosin (HE) were re-evaluated by two observers (GB and VR) and the original diagnosis of sarcoid was confirmed. The 15 samples were derived from 13 different animals. The breeds of these animals included: Arabian thoroughbred ($n = 3$), Maremmano ($n = 1$), draft horse ($n = 6$), thoroughbred ($n = 3$), standard bred ($n = 2$), and one donkey and one mule. Seven were stallions, five were geldings and three were mares. The median age of the animals was 6 years old.

Detection of Viral DNA

DNA was extracted from the tissue embedded in paraffin wax with the DNeasy Tissue Kit™ (Quiagen, Milan, Italy) according to the manufacturer's protocol. Samples were lysed using proteinase K. Lysates were loaded onto DNeasy spin columns and, after two washes, pure DNA was eluted in low salt buffer.

Amplification of the *E5* open reading frame (ORF) was carried out by polymerase chain reaction (PCR) using a BPV-1 and -2 consensus primer pair, BPVE5F (TTGCTGCAATGCAACTGCTG corresponding to BPV nucleotides 3915–3934) and BPVE5R (TCATAGGCACTGGCACGTT corresponding to BPV nucleotides 4208–4225) amplifying a fragment of 311 bp from nucleotide 3915 to 4225. To evaluate the adequacy of the DNA, a control PCR for equine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was performed using the primers published by Yuan *et al.* (2008).

Aliquots (200–300 ng) of purified DNA were amplified in a 50 µl reaction system containing 1× *Pfx* amplification buffer, 0.3 mM dNTP mixture, 1 mM $MgSO_4$, 0.3 µM primer mixes and 1 unit platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Cycling conditions were as follows: denaturation for 2 min at 94°C, followed by 28 cycles of PCR amplification comprising denaturation at 94°C for 15 s, annealing at 50°C for 30 s and extension at 68°C for 50 s, followed by a final extension at 68°C for 7 min. Detection of the amplified products was carried out by electrophoresis in an ethidium bromide-stained agarose gel. Each experiment included a blank sample consisting of reaction mixture without DNA. PCR products were purified by SwitchCharge™ beads (Invitrogen) and sequenced using the Applied Biosystems (Foster City, CA, USA) Big Dye™ terminator cycle sequencing reagents and sequences were obtained by the use of an ABI 3100 automated sequencer. Sequence analysis was performed with Basic Local Alignment Search Tool (BLAST).

Immunohistochemistry

Sections were prepared from the 12 sarcoids in which BPV DNA had been detected. Briefly, the sections were de-waxed and then endogenous peroxidase activity was blocked by incubation in H_2O_2 0.3% in methanol for 20 min. Antigen retrieval was performed by pretreating with microwave heating (twice for 5 min each at 750 W) in citrate buffer pH 6.0. The primary antibodies were applied overnight at 4°C in a humid chamber. The polyclonal rabbit anti-E7 (a gift from Professor P. Howley, Harvard Medical School, Boston, USA) was applied at a dilution of 1 in 2000; the polyclonal rabbit anti-PDGF-β receptor