Detection of E5 oncoprotein in human papillomavirus type 16-positive cervical scrapes using antibodies raised to synthetic peptides

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Polyclonal antibodies were raised to partial and full-length synthetic peptides of human papillomavirus type 16 (HPV-16) E5. Antiserum specificity for HPV-16 E5 was demonstrated by their ability to recognize not only their peptide immunogens but also full-length peptide and a glutathione S-transferase-E5 fusion protein. The most reactive antiserum, PE-6, raised to a full-length peptide, was used in Western blot analysis to identify HPV-16 E5 protein from exfoliated cervical cells. A strong, single band at approximately 20K was detected in two of six HPV-16-positive samples from women with a history of low-grade cervical intraepithelial neoplasia. The apparent Mr by SDS-PAGE suggests that HPV-16 E5 forms homodimers in vivo, but not through cysteine linkage.

Human papillomaviruses (HPVs) type 16 and 18 are believed to play a key role in the aetiology of cervical intraepithelial neoplasia (CIN) and cervical carcinoma (zur Hausen, 1988). The HPV DNA found in most cervical tumours is integrated into the host chromosomal DNA, often accompanied by deletions of part of the viral genome. Two early genes, E6 and E7, are usually retained in these tumours and their proteins are expressed (Schneider-Gadicke & Schwarz, 1986; Smotkin & Wettstein, 1986). They have been shown to play an essential role in the in vitro transformation of primary human keratinocytes (Hawley-Nelson et al., 1989; Munger et al., 1989) and rodent cell lines (Matlashewski et al., 1987) through their ability to interact with cellular growth regulatory proteins. E7 binds to, and inactivates, the product of the retinoblastoma gene, pRB (Dyson et al., 1989), a process required for rodent cell transformation but not for the immortalization of human keratinocytes (Jewers et al., 1992). E6 binds cellular p53, facilitating its breakdown through the ubiquitin-dependent pathway (Scheffner et al., 1990). Efficiency of transformation using E6 and E7 alone has been shown to be lower than that obtained using the whole genome (Bedell et al., 1989), suggesting a role for additional HPV genes.

Recent studies have identified HPV-16 E5 as a third oncogene (Leptak et al., 1991; Leechanachai et al., 1992), which acts by potentiating growth factor receptors, in particular that for epidermal growth factor (Straight et al., 1993). Although E5 has transforming activity in vitro, the gene is often deleted from the integrated viral genome in cervical cancers (Schwarz et al., 1985; Baker et al., 1987). This, and the finding that the most abundant mRNA transcripts in low grade CIN lesions are E5 and E4 (Stoler et al., 1992) suggest that the contribution of the E5 protein to tumour development occurs in the early stages of infection, when HPV DNA is episomal.

To study HPV-16 E5 protein expression, a panel of polyclonal antisera to both partial and full-length synthetic peptides was produced. Such antisera are not produced to extraneous cellular proteins and non-specific reactions are unlikely to be obtained (Johannsson, 1990). Seven peptides were synthesized, spanning the main hydrophobic areas of HPV-16 E5, as shown on a hydrophilicity plot (Fig. 1; Kyte & Doolittle, 1982). Fmoc amino acids (Milligen) were coupled on UltraSyn-C resin (Novabiochem), cleaved using trifluoroacetic acid with appropriate scavengers and freeze-dried, as previously described (Cason et al., 1993). Before their use as immunogens, peptides were conjugated to keyhole limpet haemocyanin (Sigma) using carboximide (Doolittle, 1986). A total of seven antisera were produced in New Zealand white female rabbits (Harlow & Lane, 1988) to either full-length or combinations of partial-length peptides (Table 1).

The reactivity of each antiserum with both partial and full-length HPV-16 E5 peptides was determined by enzyme immunoassays (EIA) as previously described (Cason et al., 1989). Briefly, polyvinyl microtitre plates
Fig. 1. Relationship of synthetic peptides to the Kyte-Doolittle plot of hydrophilicity for HPV-16 E5 protein. Shading below the line indicates hydrophobic regions. Numbers in parentheses are the peptide numbers. Numbering, from the first methionine residue, relates to the relative amino acid position on HPV-16 E5 to which the aligned peptide corresponds. The N terminus is on the left, and the C terminus on the right.

Table 1. Nomenclature of polyclonal antisera produced, the peptides to which they were raised and their reactivity with GST–16 E5 fusion protein in Western blots

<table>
<thead>
<tr>
<th>Polyclonal antiserum</th>
<th>Peptide immunogen</th>
<th>Relative reactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE-6</td>
<td>6 (83–1)†</td>
<td>+ + + +</td>
</tr>
<tr>
<td>S-012</td>
<td>0, 1, 2 (83–59, 52–41, 72–59)</td>
<td>+ + +</td>
</tr>
<tr>
<td>L-12</td>
<td>1, 2 (52–41, 72–59)</td>
<td>+ +</td>
</tr>
<tr>
<td>C-4</td>
<td>4 (38–11)</td>
<td>+ +</td>
</tr>
<tr>
<td>LO-0</td>
<td>0 (83–59)</td>
<td>+</td>
</tr>
<tr>
<td>SW-1</td>
<td>1 (52–41)</td>
<td>–</td>
</tr>
<tr>
<td>B-2</td>
<td>2 (72–59)</td>
<td>–</td>
</tr>
<tr>
<td>PI-3</td>
<td>3 (83–41)</td>
<td>–</td>
</tr>
<tr>
<td>J-5</td>
<td>5 (38–1)</td>
<td>–</td>
</tr>
</tbody>
</table>

* Subjective assessment by eye of intensity of staining by 4-chloro-l-naphthol was as follows: + + + +, very strong through to –, no staining observed.
† Numbers in parentheses relate to the location of amino acids on the HPV-16 E5 protein.

(Falcon) were coated with either full-length peptide (peptide 6) or each of the partial-length peptide immunogens (peptides 0, 1, 2, 3, 4 and 5) at 10 mg/l in carbonate-bicarbonate buffer pH 9.6. Each antiserum reacted with its peptide immunogen(s) (data not shown) and recognized the full-length peptide, giving similar \( A_{\text{agg}} \) readings (Fig. 2a, b). Peak readings were up to threefold higher than those observed with the preimmune (normal) sera, which reacted only weakly with the full-length peptide (Fig. 2a, b).

To test the antisera against a source of HPV–16 E5 other than synthetic peptides, a glutathione S-transferase (GST)–HPV–16 E5 fusion protein, GST–16 E5, was prepared as follows. The HPV–16 E5 PCR product (Straight et al., 1993) was inserted into the BamHI site of pGEX-3X (Pharmacia; Smith & Johnson, 1988). When
grown in *Escherichia coli*, an IPTG-inducible fused product with an $M_r$ of approximately 30K was observed using SDS–PAGE. The fusion protein was purified under non-denaturing conditions by adsorption onto glutathione–agarose beads (Pharmacia) and elution in the presence of 5 mM reduced glutathione. The apparent $M_r$ of GST–16 E5 is lower (30K, Fig. 3a) than might be expected from the fusion of GST (26K) and E5 (9.5K; Halbert & Galloway, 1988). This discrepancy is most likely to be due to the strongly hydrophobic E5 moiety resulting in aberrant migration of the fusion protein on SDS–PAGE. GST control antigen was similarly prepared using pGEX-3X with no inserted DNA.

Antisera were tested against GST–16 E5 by Western blot analysis. Briefly, 1 µg each of GST–16 E5 and GST control antigen were loaded onto a 12% Tris–Tricine–SDS polyacrylamide gel (Schagger & von Jagow, 1987). Proteins were transferred to nitrocellulose membrane (Hybond-C super; Amersham) on a semi-dry blotter (Pharmacia). Membranes were then incubated in 5% (w/v) milk powder (Marvel; Premier Brands) in PBS with 0.01% (v/v) Tween 20 (PBST) for 2 h at room temperature, washed three times with PBST and exposed to rabbit antiserum at 1:50 dilution in PBST with 5% (w/v) milk powder, overnight at 4 °C. Membranes were washed as before and horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham) at 1:250 dilution in PBST was added for 1 h at room temperature. After washing three times as before, substrate was added to the membranes [0.5 g 4-chloro-1-naphthol (Promega), 20% v/v methanol and 0.05% v/v hydrogen peroxide per litre PBS] for about 10 min until a colour reaction was observed. Five antisera, PE-6, LO-0, L-12, S-012 and C-4, identified a single band at 30K, corresponding to GST–16 E5. PE-6 reacted most strongly, with the weakest reaction using antiserum LO-0. No reaction was observed with antisera SW-1, B-2, PI-3 or J-5 (Table 1). The identification of a single protein band on these blots suggests that the antisera are specific for HPV-16 E5 (Fig. 3b). Antisera were concluded to be specific for E5 by their inability to identify any protein in the GST control. Furthermore, as proof of specificity, attempts were made to absorb out antibodies specific to E5. By incubating antisera overnight with excess (1 mg/ml) full-length peptide (peptide 6) prior to their use in Western analysis, a reduction in reactivity rather than complete loss of activity was observed.

 antisera raised to combinations of peptides were often

![Fig. 3. Western blot analysis of GST–16 E5 fusion protein using antiserum PE-6. (a) Coomassie blue-stained SDS-polyacrylamide gel with: lane 1, $M_r$ standards (Rainbow markers; Amersham); lane 2, GST; lane 3, GST–16 E5. (b) Western blot of the gel from (a) incubated with antiserum PE-6. The GST lane (2) is apparently clear and the GST–16 E5 lane (3) has a single band corresponding to the expected $M_r$ of the fusion protein.](image)

Table 2. Analysis of cervical scrapes by Western blot assay using antiserum PE-6

<table>
<thead>
<tr>
<th>Patient</th>
<th>Previous diagnosis*</th>
<th>Previous treatment</th>
<th>Current diagnosis*</th>
<th>Current HPV type present</th>
<th>Western blot assay using PE-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Normal</td>
<td>None</td>
<td>CIN II</td>
<td>16</td>
<td>$\dagger$</td>
</tr>
<tr>
<td>B</td>
<td>Normal</td>
<td>None</td>
<td>Mild dyskaryosis</td>
<td>16</td>
<td>$\times$</td>
</tr>
<tr>
<td>C</td>
<td>CIN</td>
<td>Cone biopsy</td>
<td>Normal</td>
<td>16</td>
<td>$\times$</td>
</tr>
<tr>
<td>D</td>
<td>CIN</td>
<td>Cone biopsy</td>
<td>Normal</td>
<td>16/18</td>
<td>$\checkmark$</td>
</tr>
<tr>
<td>E</td>
<td>CIN</td>
<td>Laser</td>
<td>Normal</td>
<td>16</td>
<td>$\times$</td>
</tr>
<tr>
<td>F</td>
<td>CIN</td>
<td>Laser</td>
<td>Normal</td>
<td>16</td>
<td>$\times$</td>
</tr>
<tr>
<td>G</td>
<td>CIN</td>
<td>Laser</td>
<td>Normal</td>
<td>18</td>
<td>$\times$</td>
</tr>
<tr>
<td>H</td>
<td>CIN</td>
<td>Lietz loop†</td>
<td>Normal</td>
<td>ND§</td>
<td>$\times$</td>
</tr>
</tbody>
</table>

* Cytological diagnosis.
† Indicates the presence of a single band at approximately 20K, suggesting dimerization of E5.
‡ Large loop excision of the transformation zone.
§ ND, No HPV type detected.
more reactive with GST–16 E5 fusion protein than those raised to a single peptide spanning the same region. For example, no reactivity was observed using antiserum raised to either peptides 1 or 2 alone (SW-1, B-2) yet antiserum raised to peptides 1 and 2 together showed a moderately strong reaction (L-12). Furthermore, antiserum to peptide 0 (LP-0) showed some reactivity with GST–16 E5, but reactivity was greater when peptides 1 and 2 were co-inoculated with peptide 0 (S-012) (Table 1). It was surprising that antiserum to peptide 3 (PI-3), a peptide that spans the same region as peptides 0, 1 and 2, failed to recognize GST–16 E5. This may be due to the tertiary structure of the hydrophobic, 42 amino acid peptide 3, which may compromise its ability to act as an immunogen. The use of a combination of peptides spanning this region may result in aggregates that no longer share the tertiary structure of the single peptide and hence are capable of functioning as immunogens.

From the Kyte–Doolittle plot of HPV–16 E5, the only hydrophilic, and therefore probably the strongly immunogenic areas, are located in the N-terminal region. Indeed, immunogenicity of this region has been confirmed by pepscan analysis (Geysen et al., 1987) which has identified a major reactive epitope at amino acids 10 to 14 (data not shown), included in peptide 5 and in part in peptide 4. It may be deduced from our observations that this region contributes significantly to the immunogenicity of the full-length synthetic peptide and explains the greater reactivity of antiserum PE-6. Antiserum raised to peptide 4 (C-4) showed moderately strong reactivity with GST–16 E5 on Western analysis, whereas no reactivity was observed using antiserum raised to peptide 5 (J-5). This result is probably because of hindrance caused by the relatively large GST molecule fused to the N-terminus of HPV–16 E5.

Antisera PE-6, L-12, S-012, LO-0 and C-4 reacted with both full-length E5 peptide and GST–16 E5. To investigate whether these antisera could identify HPV–16 E5 protein produced in vivo, cervical cell samples from eight women with current or previous history of low grade CIN (grades I or II) were tested by Western blot analysis (Table 2). Cells were scraped from the endocervical canal and posterior vaginal vault with sterile wooden spatulas and suspended in 5 ml of sterile distilled water. The HPV DNA status of the samples was initially determined using the consensus primers MY09 and MY11 in a PCR (Manos et al., 1989). Positive samples were then typed using the method of van den Brule et al. (1990). Of the eight samples, five were HPV–16-positive, one HPV–18-positive, one positive for both HPV–16 and HPV–18, and one was HPV-negative (Table 2). All samples were tested with β-globin primers to ensure that there was sufficient DNA for analysis (Saiki et al., 1986). A 15 µl volume of each sample was subjected to SDS–PAGE and Western blots were performed, as described above. To ensure that sufficient protein was present, identical gels were run in parallel and stained with Coomassie Blue. All samples were tested with PE-6, L-12, S-012 and LO-0 (C-4 was not tested in these studies). Of these only PE-6, the antiserum that was most reactive with GST–16 E5, detected E5 protein. A single, strong band corresponding to 20K was detected in cervical cells from two of six patients (Table 2; Fig. 4). Of these, one patient was HPV–16-positive and the other was both HPV–16- and HPV–18-positive. Preimmune rabbit antisera did not identify this or any other protein band. Although E5 protein was detected in a sample that was both HPV–16- and HPV–18-positive, PE-6 antiserum was considered to be specific for HPV–16 E5 because of the low amino acid sequence homology (31.3%) between HPV–16 E5 and HPV–18 E5. It was considered most unlikely that the antiserum was reacting with a cellular E5 homologue, as identified in mice by Kahn et al. (1992), as no protein was identified in the HPV-negative sample or indeed in four of the HPV–16-positive samples.

The detected protein was twice the expected Mr of HPV–16 E5. Similarly, histidine-tagged HPV–16 E5 fusion protein from the expression of pET (Rosenberg et al., 1987) with a similar E5 insert also ran at approximately 20K (data not shown). These results suggest that HPV–16 E5 forms homodimers. Cysteine-linked dimerization has been demonstrated for bovine papillomavirus type 1 E5 (Burkhardt et al., 1987) and has been postulated for HPV–16 E5 (Bubb et al., 1988). However, the putative dimer was not denatured under the reducing conditions of SDS–PAGE (2-mercaptoethanol and dith-
iothreitol). It is therefore considered unlikely that HPV-16 E5 dimerization occurs through the linkage of cysteine residues. Preliminary secondary-structure analysis confirmed that cysteine residues in HPV-16 E5 are not positioned to support dimerization (C. Ullman and R. Jewers, unpublished results). Indeed, Conrad et al. (1993) have shown that HPV-6 and HPV-16 E5 fusion proteins, produced in COS cells, fail to form disulphide-linked dimers and oligomers. It is possible that dimerization occurs through protein aggregation because of the severely hydrophobic regions found in HPV-16 E5.

Evidence of HPV-16 E5 gene activity in cervical cell samples from patients A and D (Table 2) supports the findings of Stoler et al. (1992) that mRNAs encoding E5 and E4 are most abundant in low grade CIN lesions. However, HPV-16 E5 protein, indicating viral early gene activity, was observed in cells from patient D although the cytology was reported to be normal (Table 2). HPV-16-positive samples in which no E5 protein was detected (patients B, C, E and F) were considered either to lack expression of this early gene or, more likely, to have levels of E5 protein not detectable by the Western blot analysis used here. Lack of HPV-16 E5 protein may be due to integration of the viral genome, an event associated with disease progression but unlikely to occur at the early stages of CIN sampled in this work, or may be indicative of an inactive or latent viral infection. Further studies are required to determine to what extent HPV-16 E5 protein expression is associated with disease progression and whether detection of this oncoprotein might be used as an early diagnostic marker of active HPV-16.

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References


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