Identification of Human Papillomavirus Type 16 E7 Protein by Monoclonal Antibodies

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SUMMARY

A number of human papillomavirus (HPV) type 16 proteins have recently been identified in human cervical carcinoma cell lines using polyclonal antisera against papillomavirus gene products expressed in Escherichia coli. E7 protein has been found to be the most abundant papillomavirus protein in these cells. Here we describe a panel of monoclonal antibodies recognizing a 15K Mr, non-glycosylated cytoplasmic HPV-16 E7 protein. One of the antibodies cross-reacted with HPV-18 E7 protein.

Up to the present time more than 50 distinct types of human papillomaviruses (HPVs) have been identified. They have been found associated with a number of epithelial neoplasms: benign tumours like common warts and genital condylomas and malignant tumours like carcinomas of the skin and the uterine cervix (for review, see Gissmann, 1984; Broker & Botchan, 1986). HPV types 16 and 18 are those most commonly found in cervical carcinomas (Dürst et al., 1983; Boshart et al., 1984). They are also present in a number of cell lines derived from these tumours and are integrated into the chromosomal DNA. Viral sequences, notably the open reading frames E6 and E7, are actively transcribed in these cells (Schwarz et al., 1985). As tissue culture systems susceptible to efficient transformation by HPVs are not available, these cell lines serve as model systems for study of the expression of HPV genes in human tumour cells. As there is also no permissive tissue culture system available for the propagation of papillomaviruses in vitro, and because viral proteins are usually present only in very small quantities in naturally occurring lesions, interest has turned to the use of prokaryotic expression vectors as an alternative source of papillomavirus proteins. This approach has recently been used to raise polyclonal antibodies against a number of HPV-encoded gene products which led to the identification of some authentic viral proteins in HPV-positive cervical carcinoma cell lines. A phosphorylated cytoplasmic HPV-16 E7 protein has been found in HPV-16-containing CaSki (Patillo, 1977) and SiHa (Fried et al., 1979) cells (Smotkin & Wettstein, 1986, 1987; Seedorf et al., 1987; Firzlaff et al., 1987). In addition we recently detected the E6 and E4 proteins of HPV-16 as translation products of CaSki cell RNA in vitro as well as the E7 and E1 proteins of HPV-18 in HeLa cells using MS2 polymerase–HPV fusion proteins (Seedorf et al., 1987). As an extension of this work we now report a panel of monoclonal antibodies recognizing the 15K Mr, HPV-16 E7 protein.

About 500 hybridoma cell clones were obtained from one fusion of spleen cells of BALB/c mice immunized with MS2–HPV-16 E7 fusion protein with NS-1 myeloma cells (Kearney et al., 1979) using the standard protocol (Köhler & Milstein, 1975). In parallel ELISA tests (Engvall & Perlman, 1971) measuring reactivity with either MS2–HPV-16 E7 fusion protein or MS2 protein alone about 150 clones showed reactivity with the MS2 part of the fusion protein or with bacterial proteins present in the antigen preparation. Ten hybridomas producing antibody
Table 1. Isotypes of monoclonal antibodies and their reactivities with MS2–E7 fusion protein and E7 from CaSki and SiHa cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>ELISA</th>
<th>Western blot</th>
<th>Immunoprecipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7I</td>
<td>IgG1 (\kappa)</td>
<td>+</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>E7II</td>
<td>IgG2a (\kappa)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E7III</td>
<td>IgG1 (\kappa)</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>E7IV</td>
<td>IgG2a (\kappa)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E7V</td>
<td>IgG1 (\kappa)</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>E7VI</td>
<td>IgG1 (\kappa)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* NS, Non-specific.
† ND, Not determined.

against the E7 part of the MS2–HPV-16 E7 fusion protein were identified. Of the 10 clones six were stable after two rounds of cloning by limiting dilution (Table 1). In Western blots four of the six hybridomas identified in ELISA recognized the MS2–HPV-16 E7 fusion protein but not the MS2 protein alone (Fig. 1). In order to determine whether these antibodies recognized only type-specific epitopes, their reactivity with MS2–HPV-18 E7 fusion protein (Seedorf et al., 1987) was tested. One antibody (E7IV) recognized the HPV-18 E7 protein in ELISA. Absorbance values in this reaction were less than 50% of those for the reaction with HPV-16 E7 (data not shown) suggesting that the epitope recognized was not identical in the two different E7 proteins. As E7 proteins of further HPV types were not available it was impossible to demonstrate whether E7IV reacted with a group-specific epitope shared by all papillomaviruses. To obtain information on epitope discrimination of different monoclonal antibodies a series of competition binding assays was done in which we tested the ability of five unlabelled antibodies to inhibit the binding of three horseradish peroxidase-conjugated antibodies. In these assays all four monoclonal antibodies reacting with E7 protein in Western blots each significantly inhibited the others' binding (Fig. 2). The fifth antibody, E7III, which reacted with E7 protein in ELISA only, inhibited the binding of the others less efficiently but still significantly compared to a control antibody unrelated to E7. It was therefore most likely that the first four antibodies reacted with topologically closely related structures. The fact that E7IV cross-reacted with HPV-18 E7 in contrast to the other antibodies clearly showed that they did not recognize the same epitope.

To identify the authentic HPV-16 E7 protein in HPV-16-containing cell lines Western blotting and immunoprecipitation experiments were performed (Table 1) using previously described protocols (Towbin et al., 1979; Koch & Hämmerling, 1982). In Western blots the E7 protein of the described size of 15K (Seedorf et al., 1987) was detected in CaSki and SiHa cells by all four monoclonal antibodies which had previously recognized the fusion protein (Fig. 3). In immunoprecipitation tests using CaSki cell extracts two of the three tested antibodies precipitated E7 (Fig. 3). The amount of E7 protein in CaSki cells was estimated to be less than 0.01% of total cellular protein. In control cell lines [simian virus 40-transformed human fibroblasts (SV80) and HPV-18-containing HeLa cells] no signals were detected. The binding of monoclonal antibodies could be blocked either by the addition of an excess amount of MS2–E7 fusion protein or addition of polyclonal rabbit antibody against HPV-16 E7 (Seedorf et al., 1987) to the reaction (Fig. 3, lanes 7 and 8). The E7IV monoclonal antibody which had shown some cross-reactivity with HPV-18 E7 in ELISA did not detect the authentic protein in HeLa cells in Western blots. This may be explained by a low affinity of the antibody for the HPV-18 E7 protein. In order to prove further the specificity of the monoclonal antibodies for HPV-16 E7 protein the E7 coding sequence was inserted into the RNA transcription vector pSPT18 (Promega, Biotec, Geneva, Switzerland). E7-specific RNA was obtained and translated in vitro
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Fig. 1. Reactivity of hybridoma culture supernatants containing monoclonal antibodies E7II to E7VI (lanes 1 to 5, respectively) with MS2-E7 fusion protein (a) and MS2 (b) in Western blots. Approximately 50 μg of protein per cm of gel was applied for 15% SDS–PAGE (Laemmli, 1970).

Fig. 2. Inhibition of binding of peroxidase-conjugated monoclonal antibodies (a) E7IV-PO, (b) E7V-PO and (c) E7VI-PO, 0.5 μg/ml each, in ELISA by addition of unconjugated antibodies E7II to E7VI (columns II to VI) at 50 μg/ml each. Binding without addition of unlabelled antibody was considered to be 100% (column 0). A monoclonal antibody with specificity for HPV-16 L1 protein was used as a negative control (column C). A$_{492}$ was measured.

Fig. 3. Identification of HPV-16 E7 protein by monoclonal antibodies: lanes 1 to 8, CaSki cell extracts were separated by 10% SDS–PAGE, transferred to nitrocellulose and incubated with monoclonal antibodies. A horseradish peroxidase conjugate was used as a second antibody. Lanes 1 to 3, E7II (3.2, 1.6 and 0.8 μg/ml, respectively); lane 4, E7IV; lane 5, E7V; lane 6, E7VI; lane 7, blocking of reaction from lane 3 by addition of polyclonal rabbit anti HPV-16 E7 antibody (20 μg/ml); lane 8, blocking of reaction from lane 3 by addition of MS2–E7 fusion protein (50 μg/ml). Lanes 9 to 21, identification of HPV-16 E7 protein in immunoprecipitates of [35S]methionine-labelled cellular extracts. Lane 9, E7II applied to extracts of SV80 cells and lane 10, HeLa cells; lanes 11 to 19, CaSki cell extracts: lanes 11 to 13, incubated with E7II, E7IV and E7VI; lane 14, fraction not bound by lentil lectin Sepharose 4B plus E7II; lane 15, fraction bound plus E7II; lane 16, with W6/32 antibody (anti-human MHC class 1); lane 17, without antibodies added; lane 18, with polyclonal rabbit anti-HPV-16 E7 antibody; lane 19, E7II and E7IV. Lane 20, SiHa cell extract plus E7II and E7IV (exposure time, 5 days). Lane 21, HeLa cell extract plus polyclonal rabbit anti-HPV-16 E7 antibody (exposure time, 2 days). Lanes 22 to 26, identification of [35S]methionine-labelled HPV-16 E7 protein as a translation product in vitro from RNA synthesized in vitro, and precipitated by a mixture of E7II and E7IV; lane 22, no RNA added; lanes 23 to 25, 0.5, 2.0 and 10 μg of E7-specific RNA added to the translation reaction; lane 26, tobacco mosaic virus RNA (exposure time, 10 days). M, are ×10$^{-3}$.

using commercially available reagents (Amersham Buchler). Monoclonal antibodies were used for immunoprecipitation of E7 protein from translation reactions in vitro. An approximately 12.5K M, protein was precipitated (Fig. 3). It migrated slightly faster than the HPV-18 E7 protein detected in HeLa cells but was considerably smaller than the authentic HPV-16 E7 protein derived from CaSki and SiHa cells (Fig. 3). In agreement with previous reports (Smotkin
& Wettstein, 1986, 1987; Bernard et al., 1987) the E7 protein was shown to be located in the cytoplasm (Fig. 4). To exclude the possibility that E7 was a glycoprotein, labelled extracts of CaSki cells were chromatographed over lentil lectin Sepharose 4B prior to immunoprecipitation (Hayman & Crumpton, 1972). No binding was observed (Fig. 3).

The $M_r$ of the E7 protein found in our experiments (15K) was in agreement with that determined with polyclonal antisera against MS2 fusion proteins (Seedorf et al., 1987) or against trpE fusion proteins (Firzlaff et al., 1987). The $M_r$ of 12.5K estimated for the HPV-16 E7 protein as a translation product in vitro was considerably lower but closer to the value of 11K expected from the DNA sequence. In HPV-18 the calculated $M_r$ of E7 was 11.9K. The estimate of 12.5K for the authentic protein found in HeLa cells was consistent with the predicted value. The higher than expected $M_r$ of 15K of the authentic HPV-16 E7 protein in CaSki and SiHa cells may be due to post-translational modifications. The amino acid sequence contains one possible glycosylation site at the asparagine residue at position 28. The results of Lens culinaris chromatography indicate that this site is not used, which is in line with the evidence that E7 is a cytoplasmic protein. Whether the known phosphorylation of E7 (Smotkin & Wettstein, 1986, 1987) is the only reason for the higher than calculated $M_r$ in SDS gels or if other modifications are involved remains to be elucidated. Only one of the antibodies obtained cross-reacted with HPV-18 E7 protein. From sequence comparisons between HPV-16 E7 and HPV-18 E7 (Seedorf et al., 1987) a majority of type-specific epitopes but also some conserved ones would be expected. Polyclonal antisera to HPV-16 E7 do cross-react with HPV-18 E7 (K. Seedorf, unpublished results). The competition binding assays suggested that all antibodies obtained recognized the same epitope or closely neighbouring structures. This may indicate that monoclonal antibodies against HPV-16 E7 are preferentially directed to one site. Such observations have been made with other antigens such as the CD 19 antigen of T-lymphocytes (G. Moldenhauer, personal communication).

The E7 protein is the most abundant protein in HPV-16- or HPV-18-containing cell lines (Smotkin & Wettstein, 1986, 1987; Seedorf et al., 1987). Genetic studies of bovine papillomavirus suggest a function in the maintenance of high copy numbers of episomes in transformed mouse cells (Lusky & Botchan, 1986). An additional function must be assumed in human cervical cancer cell lines in which no episomal viral DNA is present. Its permanent high level of expression suggests a role in the maintenance of the transformed phenotype of these cells. The availability of monoclonal antibodies may well resolve this question. Monoclonal antibodies may also be applied for diagnosis of HPV-16 infection which is so far based exclusively on DNA–DNA hybridization (Wagner et al., 1984). Unfortunately the amount of E7 protein in the tested cells is too low to be detected by indirect immunofluorescence or other immunocytochemical methods (T. Oltersdorf et al., unpublished data). Studies to detect HPV-16
E7 protein in tumour extracts by immunoprecipitation or Western blotting are under way. Furthermore if patients show a humoral immune response to viral proteins, monoclonal antibodies may be useful for detection of antibodies in sera by competition binding assays.

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