CLINICAL RESEARCH

Histological and cytological evidence of viral infection and human papillomavirus type 16 DNA sequences in cervical intraepithelial neoplasia and normal tissue in the west of Scotland: evaluation of treatment policy

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Abstract

Biopsy samples from 27 patients referred to a colposcopy clinic in Glasgow for cervical abnormalities were assessed for the relations among colposcopic appearances, cytological and histological diagnosis, expression of papillomavirus antigen, and the presence of human papillomavirus (HPV) types 6, 11, 16, and 18 deoxyribonucleic acid (DNA) sequences. Specimens were from colposcopically abnormal areas of the transformation zone and from colposcopically apparently normal areas of the zone in the same patients (paired matched internal control tissue). All 27 women referred for abnormal smears had colposcopic abnormalities.

HPV-16 or 18 DNA sequences were detected in 20 of the 27 colposcopically abnormal biopsy samples and 13 of the 27 paired normal samples. Twelve samples of colposcopically normal tissue contained histological evidence of viral infection but only four of these contained HPV DNA sequences. The other nine samples of colposcopically normal tissue which contained HPV DNA sequences were, however, histologically apparently normal. HPV-6 and 11 were not detected.

Integration of the HPV-16 genome into the host chromosome was indicated in both cervical intraepithelial neoplasia and control tissues. In two thirds of the HPV DNA positive samples

the histological grade was classed as normal, viral atypia, or cervical intraepithelial neoplasia grade 1. Papillomavirus antigen was detected in only six of the abnormal and three of the normal biopsy samples, and HPV DNA was detected in all of these.

The detection of HPV DNA correlates well with a combination of histological and cytological evidence of viral infection (20 of 22 cases in this series). A poor correlation between the site on the cervix of histologically confirmed colposcopic abnormality and the presence of HPV DNA sequences implies that a cofactor other than HPV is required for preneoplastic disease to develop.

A separate study in two further sets of biopsy samples examined the state of HPV DNA alone. The sets were (a) 43 samples from cervical intraepithelial neoplasia and nine external controls and (b) 155 samples from cervical intraepithelial neoplasia, cervical cancer, vulval intraepithelial neoplasia, and vulval cancer and external controls. HPV-11 was found in only two (4.7%) of the 43 specimens from cervical intraepithelial neoplasia, whereas HPV-16 was found in 90 (58%) of the other 155 specimens. These results also suggest that HPV subtype is subject to geographical location rather than being an indicator of severity of the lesion or of prognosis.

Introduction

The twin epidemics of cervical premalignancy and genital wart virus infection are increasingly being highlighted in the national press. There is little doubt that both diseases are sexually transmitted.^{1,2} Nevertheless, much of the evidence supporting the proposition that deoxyribonucleic acid (DNA) sequences associated with human papillomavirus (HPV) cause cervical intraepithelial neoplasia or genital cancers has lacked data on paired internal control tissues.^{3,5} Ferenczy *et al*⁶ and Macnab *et al*⁷ emphasised the importance of having two types of control tissues—that is, matched internal control tissue and external control tissue from women with no cytological or colposcopic evidence of disease—in assessing the relevance of HPV DNA in colposcopically abnormal areas of the cervix and adjacent colposcopically normal tissue. Some

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workers have used control tissue from age matched women with colposcopically and histologically normal cervixes.⁸ Such external samples cannot control for the state of histologically normal tissue in the same patient, though clearly they provide information about the prevalence of HPV in the general population.

Reid *et al* suggested that warty lesions may reliably be distinguished from cervical intraepithelial neoplasia by applying a colposcopic index to the appearance,⁹ but Kirkup *et al* found a poor correlation between the results of cervical cytology and colposcopy and histological evidence of viral infection.¹⁰ Crum *et al* thought that the presence of HPV-16 might be a good indicator of progression to grade 3 cervical intraepithelial neoplasia.⁴ HPV DNA, however, has been detected much more frequently than previously in normal human ectocervical tissue.¹¹ Thus the neoplastic potential of HPV on the cervical transformation zone may be mediated by expression of viral gene products coded from the early region of the viral genome.¹² Late gene products detected by structural viral antigen expression¹³ are usually found only in terminally differentiating cells. Histological evidence of viral infection of the cervix may be based on the observation of koilocytes.¹⁴

At present, therefore, the keystone of the association between HPV and cervical intraepithelial neoplasia is the detection of HPV DNA in abnormal tissue. This poses two questions: What is the significance of finding HPV DNA in apparently normal tissue? and What should the clinician do if confronted by a diagnosis of evidence of viral infection?

The object of this study was to assess the relevance of HPV DNA sequences in tissue diagnosed as colposcopically abnormal and in colposcopically normal (control) tissue from the same patient. Secondly, we aimed at studying the interrelations among the various criteria used for diagnosing HPV infection—that is, colposcopic appearances, histological appearances, expression of HPV structural antigen, and detection of HPV DNA by Southern blot hybridisation. Lastly, we wished to see whether relating HPV subtype to present strategy for assessing cervical smears was valid.

Patients and methods

The main study group comprised 27 patients referred to the colposcopy clinic at the Royal Infirmary, Glasgow, because of abnormal cervical cytology. Colposcopy was performed (LJC) and areas of aceto-white epithelium defined by applying 3% acetic acid. Paired adjacent colposcopically directed punch biopsy samples were taken from colposcopically abnormal and colposcopically normal areas of the cervical transformation zone (different biopsy forceps being used to avoid cross contamination). One specimen from each pair was processed for routine histological examination and then examined by immunohistochemistry for evidence of papillomavirus antigen expression (LJC). The other specimen from each pair was snap frozen in liquid nitrogen and stored at -70°C before DNA hybridisation studies (JBM).

A separate study to determine HPV subtypes only was carried out on 43 specimens from cervical intraepithelial neoplasia plus nine external controls and on 155 specimens from cervical intraepithelial neoplasia, vulval intraepithelial neoplasia, cervical cancer, and vulval cancer and internal controls and 30 external controls.

ANTIGEN STUDIES

Antigen studies were performed with an indirect peroxidase-antiperoxidase technique.¹⁵ Each specimen was fixed in Bouin's solution, embedded in paraffin, and examined histologically. Non-stained sections were dewaxed in xylene and dehydrated in serial alcohol solutions. Endogenous peroxidase was quenched with 0.5% hydrogen peroxide in methanol. The sections were incubated in normal swine serum to reduce non-specific background staining. Group specific bovine papillomavirus antiserum (Dako) was added in dilutions of 1/250 to 1/1000 and incubated overnight. Bridging antibody (swine antirabbit IgG) was applied for 30 minutes followed by the addition of peroxidase-antiperoxidase complex at 1/20 dilution for 10 minutes. The peroxidase reaction was developed with diaminobenzoic acid giving a dark brown intranuclear precipitate in cells in which the HPV antigen was expressed. Haematoxylin was used as counterstain.

MOLECULAR STUDIES

For the molecular studies we used HPV-6, HPV-11, and HPV-16 DNA cloned into the BamHI site of pBR322 and HPV-18 DNA cloned into the EcoRI site of pBR322. HPV DNA was released from the vectors by the appropriate restriction endonuclease digestion and purified twice by agarose gel electrophoresis and subsequent electroelution.

Biopsy samples were chopped finely with a sterile blade and the DNA extracted in 0.4% sodium dodecyl sulphate, 0.1M sodium chloride, 0.01M edetic acid (disodium salt), 0.05M trometamol (TRIS) hydrochloric acid pH 8.0, proteinase K (Boehringer) 200 mg/l, and ribonuclease A 100 mg/l. The sample was sequentially extracted with phenol and chloroform and dialysed against $1\times\text{TE}$ buffer (0.01M trometamol pH 7.5 and 0.001M edetic acid (disodium salt)) before ethanol precipitation. Sample DNA was quantified against a known standard lambda DNA (Gibco, Paisley, Scotland) and 10 μg of each sample digested with a fourfold excess of the restriction endonuclease BamHI, PstI, or other multicut or no cut enzymes for HPV. The appropriate HPV reconstructions containing an equivalent amount of carrier mammalian DNA were similarly digested. Fragments were subjected to electrophoresis in full size 0.6% agarose gels. The DNA was denatured and transferred to the reproducible nylon membrane Genescreen Plus (New England Nuclear). Purified probes were radiolabelled with α -phosphorus-32-deoxyadenosine triphosphate and α -³²P-deoxythymidine triphosphate to a specific activity in excess of 1×10^8 counts/min/ μg DNA. Hybridisation of HPV clones was carried out in 50% formamide (Fluka), $5\times\text{SSC}$ ($1\times\text{SSC}=0.15\text{M}$ sodium chloride and 0.015M sodium citrate), HEPES buffered saline (300mM sodium chloride, 4mM potassium chloride, 0.8mM sodium hydrogen phosphate, 5mM dextrose, 20mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) pH 7.5), denatured salmon sperm DNA 100 mg/l (Sigma), and 0.5% sodium dodecyl sulphate at 42°C for three days. Blots were washed in $2\times\text{SSC}$ and 0.1% sodium dodecyl sulphate at 48°C (non-stringent conditions) and subjected to autoradiography with use of intensifying screens (Dupont) on X-Omat film (Kodak), then rewash in $2\times\text{SSC}$ and 0.1% sodium dodecyl sulphate at 68°C (stringent conditions) before repeat autoradiography.

Results

In the main study the routine histological diagnoses of colposcopically abnormal tissues from the 27 patients ranged from evidence of virus

TABLE 1—Results (main study)

	Patients with histological or cytological evidence of viral infection	Patients with evidence of viral antigen expression (abnormal/normal)	Hybridisation (stringent) to HPV-16 DNA (abnormal/normal)	Hybridisation (non-stringent) to HPV-18 DNA (abnormal/normal)
Histology of colposcopically abnormal tissue:				
Virus only (n=6)	6	1/1	3/3	0/0
Cervical intraepithelial neoplasia grade 1 (n=6)	4	1/0	4/3	1/0
Cervical intraepithelial neoplasia grade 2 (n=10)	10	3/1	6/4	1/0
Cervical intraepithelial neoplasia grade 3 (n=5)	2	1/1	4/3	2/0
Total (n=27)	22 (81%)	6/3 (22%/11%)	17/13 (63%/48%)	4/0 (15%/0%)
Histology of colposcopically normal tissue:				
Normal	15			
Virus only	9			
Cervical intraepithelial neoplasia grade 1 virus	3			
Total	27			

only in the absence of dysplasia (that is, koilocytotic atypia, individual cell keratinisation, multinucleation, parakeratosis, acanthosis, and papillomatosis¹⁴) to grade 3 cervical intraepithelial neoplasia (table I). Twelve samples of colposcopically apparently normal tissue were reported as showing histological features of viral infection, and three of these were classified as grade 1 cervical intraepithelial neoplasia. Altogether 22 of the 27 women with colposcopic abnormalities were reported as having histological or cytological evidence, or both, of viral infection (table I).

Six (22%) of the abnormal biopsy specimens were positive for papilloma-virus antigen (95% confidence interval 6.4% to 37.6%) compared with three (11%) of the normal specimens (95% confidence interval 0.8% to 22.0%) (table I).

Hybridisation to HPV-16 DNA under stringent conditions was found in 17 (63%) abnormal biopsy specimens (95% confidence interval 46.0% to

detected by staining for papillomavirus capsid antigen, indicating that the entire genome may not be transcribed in all cells with HPV DNA.

Samples which hybridised to the HPV-16 DNA probe under stringent conditions sometimes produced one to three bands on digestion, even with a fourfold excess of BamHI (fig 1). This suggests that the HPV DNA is integrated at multiple sites in the host cell chromosome.

Durst *et al* reported integration of HPV-16 into cell DNA in invasive cancer by cloning virus-cell junction fragments.¹⁶ Also by digesting cell DNA samples with both a restriction enzyme which cuts HPV and a combination of cut and non-cut enzymes they detected submolar bands representing virus-cell junction fragments. Insufficient DNA precluded detailed study of integration in all our samples, but in one case (case 1, sample A; fig 1) a sample cell DNA was digested with PstI alone (HPV contains recognition sites for PstI) and a second sample was digested with

FIG 1—Autoradiograph of BamHI digested DNA extracted from cervical intraepithelial neoplasia and normal tissues. Panels 1-4 show 5 µg DNA (digested with BamHI) extracted from histologically confirmed lesion of cervical intraepithelial neoplasia (A=abnormal) and from histologically normal adjacent control area (N=normal) from four different patients. HPV-16 radiolabelled probe hybridises to inserts in cervical intraepithelial neoplasia and normal samples. Specimens 1A, 1N, and 2N contain HPV DNA potentially integrated into different sites in cell genome. Track ct is control calf thymus DNA track and rc 10 is positive control for 10 copies of HPV genome present in control DNA per cell. Molecular weight markers in kb are to left of tracks.

80.0%) and 13 (48%) colposcopically normal internal control specimens (95% confidence interval 29.2% to 66.8%) (table I). In three cases in which hybridisation to HPV-16 was not detected under stringent conditions hybridisation to HPV-18 was detected under non-stringent conditions only (table I). In one further case in which hybridisation to HPV-16 was detected under stringent conditions HPV-18 was also detected under non-stringent conditions only. This may represent a mixed HPV-16/HPV-18 infection or cross hybridisation between the HPV-18 probe and HPV-16 in the sample or to another HPV subtype. No colposcopically normal specimen hybridised to HPV-18. HPV-11 DNA failed to hybridise to any samples in this group. As HPV-6 and HPV-11 share a base pair homology of 80% and both stringent and non-stringent hybridisation conditions were used with HPV-11 it may be concluded that HPV-6 was also not present in this group. In total 20 (74%) of the colposcopically abnormal samples from the 27 patients hybridised to HPV-16 or HPV-18 or to both probes.

Thus of the 22 patients who had cytological or histological evidence, or both, of viral infection in abnormal biopsy specimens, 20 (91%) had HPV DNA sequences. By contrast, nine of the 13 (69%) colposcopically normal internal control samples containing HPV DNA sequences were reported as histologically normal. Hence a combination of cytological and histological analysis accurately detected evidence of HPV DNA sequences but histological assessment of individual biopsy specimens was comparatively inefficient. Six of the 20 HPV DNA positive cervixes were

PstI plus XhoI, SstI, and HindIII (HPV DNA does not contain recognition sites for XhoI, SstI, and HindIII). Six submolar bands were identified suggesting that the HPV genome was inserted into three sites in the host chromosome in this sample of cervical intraepithelial neoplasia (fig 2).

In some cases—for example, case 1 (fig 1)—the genome copy number in the abnormal biopsy specimen exceeded that in the normal specimen. By contrast, however, other patients showed a higher HPV copy number in control tissues than in colposcopically abnormal tissue—for example, case 2 (fig 1). Thus the site on the cervix of maximum colposcopic abnormality is not necessarily the site at which the maximum viral genome copy number is found. Maximum copy number may reflect replicating DNA.

In the separate study of hybridisation (stringent) only two of the 43 specimens from cervical intraepithelial neoplasia contained HPV-11 DNA sequences (4.7%) and 90 (58%) of the 155 other abnormal genital samples had HPV-16 DNA sequences.

Discussion

Our main study was designed to assess criteria used in the diagnosis and treatment of cervical intraepithelial neoplasia and also of patients with “evidence of viral infection” and to assess the

relations among the colposcopic appearance of the cervix, the detection of HPV DNA sequences, and the importance of HPV in the host cell. The results show that the combination of cervical cytology and histological assessment of colposcopically abnormal tissue is efficient in detecting HPV DNA sequences in a cervix. We did, however, find that HPV DNA is frequently detectable in apparently normal tissue (tables I and II) and that the HPV copy

number may be higher in normal than abnormal tissue. Adjacent pairs of punch biopsy samples were taken for histological analysis and for DNA studies. Clearly, the histological grading may vary in adjacent samples, and histological grades from normal to grade 3 cervical intraepithelial neoplasia may be found in a single tissue section. In situ DNA-DNA hybridisation studies on histologically graded sections may correlate detection of viral DNA with cellular histological abnormalities. Such studies, however, cannot disclose viral copy number, restriction pattern, and state of the HPV genome and relate these to colposcopic appearances. Ferenczy *et al* showed that HPV is found in normal skin adjacent to wart lesions and that it is associated with recurrent warts.⁶ These findings are consistent with the belief that HPV causes viral atypia but do not prove that the virus causes coexisting cervical intraepithelial neoplasia.

In cattle bovine papillomavirus has been associated with the development of alimentary warts. There is strong evidence of interplay between bovine papillomavirus and carcinogens in malignant transformation but that persistence of the bovine papillomavirus genome is not necessary for progression to or maintenance of the malignant state.¹⁷ It seems likely that the colposcopically abnormal tissue is in a site at which the virus is acting in concert with local cofactors of potentially equal importance, which may be viruses such as herpes simplex virus¹⁸ or human cytomegalovirus¹⁹ or indeed other chemical or biological cocarcinogens, to produce dysplastic changes.

The finding that in some cases the site on the cervix of maximum viral genome copy number is not the site of colposcopic abnormality may suggest that more virus DNA may be present in differentiating cells in which HPV replicates. Completion of the viral replication cycle as assessed by evidence of papillomavirus capsid antigen expression (table I) in our study suggests that mature virions are more often found in relatively poorly differentiating tissue of cervical intraepithelial neoplasia (six cases; 22%) than in well differentiated normal epithelium (three cases; 11%). Expression of the early region of the viral genome may also be increased in the abnormal tissue compared with the normal and stimulate other cellular genes—for example, cellular oncogenes.²⁰

The high rates of DNA detection in colposcopically normal areas of the transformation zone raise questions regarding patient management. It is standard practice when treating patients with local ablative measures such as carbon dioxide laser therapy to destroy the whole transformation zone. HPV DNA in adjacent, untreated normal tissue, however, may predispose to reinfection.⁶ The sexual nature of virus transmission implies that patients with cervical wart infection will also harbour HPV DNA in the vagina and vulva, which are lined with mature keratinised squamous epithelium. After ablation of the cervical transformation zone it therefore seems inevitable that residual HPV DNA will be left outside the treatment area. Additionally, the male factor,²¹ makes it highly likely that the cervix will become reinfected on subsequent sexual contact. Residual HPV infection in vaginal and vulval tissue may result in the failure of ablative treatment to the transformation zone. We believe that there is no case for intervention in the absence of dysplasia, as it may prove to be totally inefficient in preventing cervical cancer.

HPV-16 and HPV-18 were first detected in cervical carcinomas^{5,22} and it has been assumed that their detection is relevant to prognosis⁴ compared with that of HPV-6 and HPV-11. Reportedly in Glasgow HPV-16 and HPV-18 are the HPV subtypes predominantly detected in lesions of cervical intraepithelial neoplasia.²³ The results from our main and separate studies confirm these findings and also the strong association of HPV-16 and HPV-18 with cancer,⁷ these being the only subtypes detected in cases in the west of Scotland; thus "evidence of viral infection" as found histologically or cytologically is probably HPV-16 or HPV-18. If we accept that HPV-16 and 18 are prognostic indicators in cervical intraepithelial neoplasia⁴ then patients in the west of Scotland who harbour HPV DNA in their cervical tissue are at particularly high risk of progression to invasive disease. Data on the prevalence of grade 3 cervical intraepithelial neoplasia in the region are incomplete owing to lack of notification; nevertheless, the incidence of cervical cancer

FIG 2—Autoradiograph of cervical intraepithelial neoplasia DNA specimen digested with BamHI (track a). Sample digested with PstI, SstI, and HindIII in track b is compared with plasmid similarly digested in track c. Six submolar bands in track b (s) indicate that HPV DNA with cervical intraepithelial neoplasia is inserted into three different sites in host chromosome. Molecular weight markers in kb are to left of tracks.

TABLE II—Numbers (percentages) of HPV positive lesions related to histopathology

	HPV-6, 11	HPV-16	HPV-18
Normal (n=15)	0	9/15 (60)	0
Virus only (n=15)	0	6/15 (40)	0
Cervical intraepithelial neoplasia grade 1 (n=9)	0	5/9 (56)	1/9 (11)
Cervical intraepithelial neoplasia grade 2 (n=10)	0	6/10 (60)	1/10 (10)
Cervical intraepithelial neoplasia grade 3 (n=5)	0	4/5 (80)	2/5 (40)

in the west of Scotland defined by the age standardised rate against a world standard population was 9/100 000 women in the period 1975-7. This compares favourably with an incidence of 10·6/100 000 women in Oxford and 10·6/100 000 white women in New York State during the same period (Cancer Surveillance Unit, west of Scotland). In our area cure rates for cervical intraepithelial neoplasia exceed 95%. In our main study HPV-16 and HPV-18 were found in most grade 3 lesions (table II); nevertheless, two thirds of the HPV-16 positive biopsy specimens came from histologically normal tissue or mild lesions (viral atypia alone and grade 1 cervical intraepithelial neoplasia). Probably, therefore, the type of HPV infection may be subject to geographical variation rather than to the degree of histological abnormality or its potential for progression to metastatic disease. This is substantiated by our separate study of HPV subtypes, in which HPV-16 was detected in 90 (58%) of abnormal specimens.

Integration of HPV DNA in lesions of cervical intraepithelial neoplasia has been described^{23,24} and was found in this study. Integration of viral DNA may occur in the malignant state¹⁶ but the prognostic relevance of its detection in cervical intraepithelial neoplasia remains to be defined.

HPV infection of the uterine cervix is a common event, and despite the impressive body of data the association between HPV and malignant change remains to be firmly established. Screening for wart virus infection by hybridisation to cervical scrapes has been suggested²⁵ but would be expensive in terms of manpower and resources. Its value will be limited by the increasing evidence of viral DNA in histologically normal tissue detected by hybridisation studies and additionally by the difficulty in interpreting dot (slot) blot hybridisation at low copy numbers.²⁶ Furthermore, the high correlation between established methods of diagnosis (cytology and histology) and DNA-DNA hybridisation for detecting viral infection question the value of this additional test.

The deficiencies of national cytological screening for cervical intraepithelial neoplasia—an entity generally accepted as a precancerous condition—are well known.²⁷ Well coordinated and intensive cytological screening programmes have been successful in reducing the incidence of cervical cancer.²⁸ A recent prospective study in Australia of patients with cytological evidence of HPV infection showed that the presence of HPV on the cervix was associated with a greatly increased risk of future development of carcinoma in situ. This was especially so in women under 25, whose risk increased by a factor of 38·7 compared with women with no cytological evidence of HPV infection.²⁹ Thus established cytological screening with colposcopic back up based on histological diagnosis may be made more efficient and can identify high risk groups for intensive surveillance further to improve cost effectiveness.

In conclusion we believe that screening the population for HPV infection using the present probes and subsequent ablation will not lead to the eradication of malignant genital disease. This is substantiated by our data, which show that HPV-16 remains in tissue after successful treatment for cervical intraepithelial neoplasia²⁷ and that HPV-16 in tissue adjacent to excised malignant lesions does not suggest a prognosis of recurrence or death.³⁰ Nevertheless, the high incidence of HPV-16 DNA in normal tissue of patients with evidence of malignancy or premalignancy suggests the need for caution and close follow up of all patients treated for histologically abnormal lesions.

We thank the histopathologists at the Royal Infirmary, Glasgow, for the

histological data; Alison Chudleigh for excellent technical work; Professor J W F Jarrett and Mr Brian O'Neill for help in setting up the antigen studies; and Professor Harald zur Hausen and Dr L Gissmann for the HPV containing plasmids. JBM was supported by a Wellcome research training fellowship in surgery, LJC by a research grant from the Greater Glasgow Health Board, and KF by a Medical Research Council studentship for training in research techniques. We thank Professor J H Subak-Sharpe, Professor C W Whitfield, and Professor Sir Malcolm Macnaughton for supporting this study.

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(Accepted 7 December 1987)